The effect of paternal dietary folic acid on male fertility and embryo development in Balb/c mice

By

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Abstract

In 1998, Canada fortified white flour with folic acid targeting women of childbearing age to reduce the incidence of neural tube defects. Resultantly, FA intake in men increased. This study investigated the effect of paternal FA intake on male fertility, embryo development, and methylation dependent gene expression in unexposed descendants of Balb/c mice. Folate deficiency resulted in a higher number of embryos with congenital anomalies and developmental delay, and a lower prevalence of ossification in bones of the skull. Placenta size was higher with higher FA intake. BiSulfite Amplicon Sequencing was used to characterize methylation changes in heritable epigenetically regulated genes for which hepatic expression was dependent on grand-paternal FA exposure during early development or post-weaning. CpG islands in promoters of three genes had subtle diet-induced methylation changes. This study highlights the importance of adequate paternal FA intake, emphasizing that fortification can directly impact embryo development and affect future generations.

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List of Abbreviations

AdoHcy: adenosylhomocysteine

AdoMet: adenosylmethionine

AICART: aminoimidazolecarboxamide ribonucleotide transformylase

ALH: amplitude of lateral head displacement

BSAS: bisulfite amplicon sequencing

CACC: Canadian Council on Animal Care

CASA: computer-assisted sperm analysis

CNS: central nervous system

CL: corpus luteum

CRL: crown rump length

DHF: dihydrofolate

DHFR: dihydrofolate reductase

DMR: differentially methylated region

DNA: deoxyribonucleic acid

DNMT: DNA methyltransferase

dsDNA: double stranded DNA

dTMP: deoxythymidine monophosphate

dUMP: deoxyuridine monophosphate

ED: early development

EtOH: ethanol

FA: folic acid

FIGLU: formiminoglutamic acid

FTHFS: 10-formyltetrahydrofolate synthetase

GART: glycinamide ribonucleotide transformylase

GD: gestational day

Hcy: homocysteine

IAP: intracisternal A particle

ICR: imprinting control region

IVOS: integrated visual optical system

KOH: potassium hydroxide

miRNA: micro ribonucleic acid

MTHFC: methenyltetrahydrofolate cyclohydrolase

MTHFD: methylenetetrahydrofolate dehydrogenase

MTHFD1: C1-tetrahydrofolate synthase

MTHFR: methylenetetrahydrofolate reductase

MTR: methionine synthase

NGS: next generation sequencing

NTD: neural tube defect

***p*ABA:** para-amino-benzoic acid

PCD: post-coital day

PCR: polymerase chain reaction

PGC: primordial germ cell

PND: post-natal day

PW: post-weaning

RBC: red blood cell

RRBS: reduced representation bisulfite sequencing

SSC: spermatogonial stem cell

SEM: standard error of the mean

SHMT: serine hydroxymthethyltransferase

SNP: single nucleotide polymorphism

STR: straightness

SUMO: small ubiquitin-like molecules

THF: tetrahydrofolate

TYMS: thymidylate synthase

VAP: average path velocity

VCL: track velocity/ curvilinear path

VSL: straight line velocity

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Chapter 1: Introduction

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Chapter 2: General Overview of the Methods

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Chapter 3: Paternal folic acid intake in Balb/c mice affects embryo development.

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Chapter 5: Discussion

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Chapter 1:

Introduction

1 Chapter 1: Introduction

1.1 Folate-mediated one carbon metabolism

Folate is an essential water-soluble B vitamin naturally occurring in various food groups, primarily leafy green vegetables, beans, and lentils. Natural folate exists as reduced tetrahydrofolate (THF) derivatives, made up of a pteridine ring, a para-amino-benzoic acid (*p*ABA), and a (poly)glutamate group [1,2]. FA is found in dietary supplements and food fortification, and exists in the oxidized synthetic form, containing a pteridine ring, a *p*ABA, and a monoglutamate moiety [2,3]. Folate and FA are biotransformed in the intestinal mucosa and liver, respectively, where they are reduced to 5-methyl-THF, the major circulating form of folate in the human body [4]. While it is necessary for folates to be monoglutamated by gamma-glutamyl hydrolase at the intestinal apical brush border membrane in order to be absorbed, these molecules must be subsequently polyglutamated within the cell [4]. Polyglutamation of folates by foly-poly-gluatamate-synthase allows them to become effective substrates for folate dependent enzymes in one-carbon metabolism [1,4] THF, in its various reduced states, plays an integral role in one-carbon metabolism, where one-carbon units are activated for nucleotide and methionine synthesis [1,5].

One-carbon metabolism in eukaryotic cells occurs, in the cytosol, the mitochondria, and the nucleus [3,5]. Cytoplasmic folate-mediated one-carbon metabolism is a network of pathways in which folate catalyzes *de novo* purine synthesis, as well as methionine synthesis via the methylation of homocysteine in the cytoplasm [5] (Figure 1.1). *De novo* thymidylate synthesis is mainly activated in the nucleus during S phase at the replication fork [6,7].

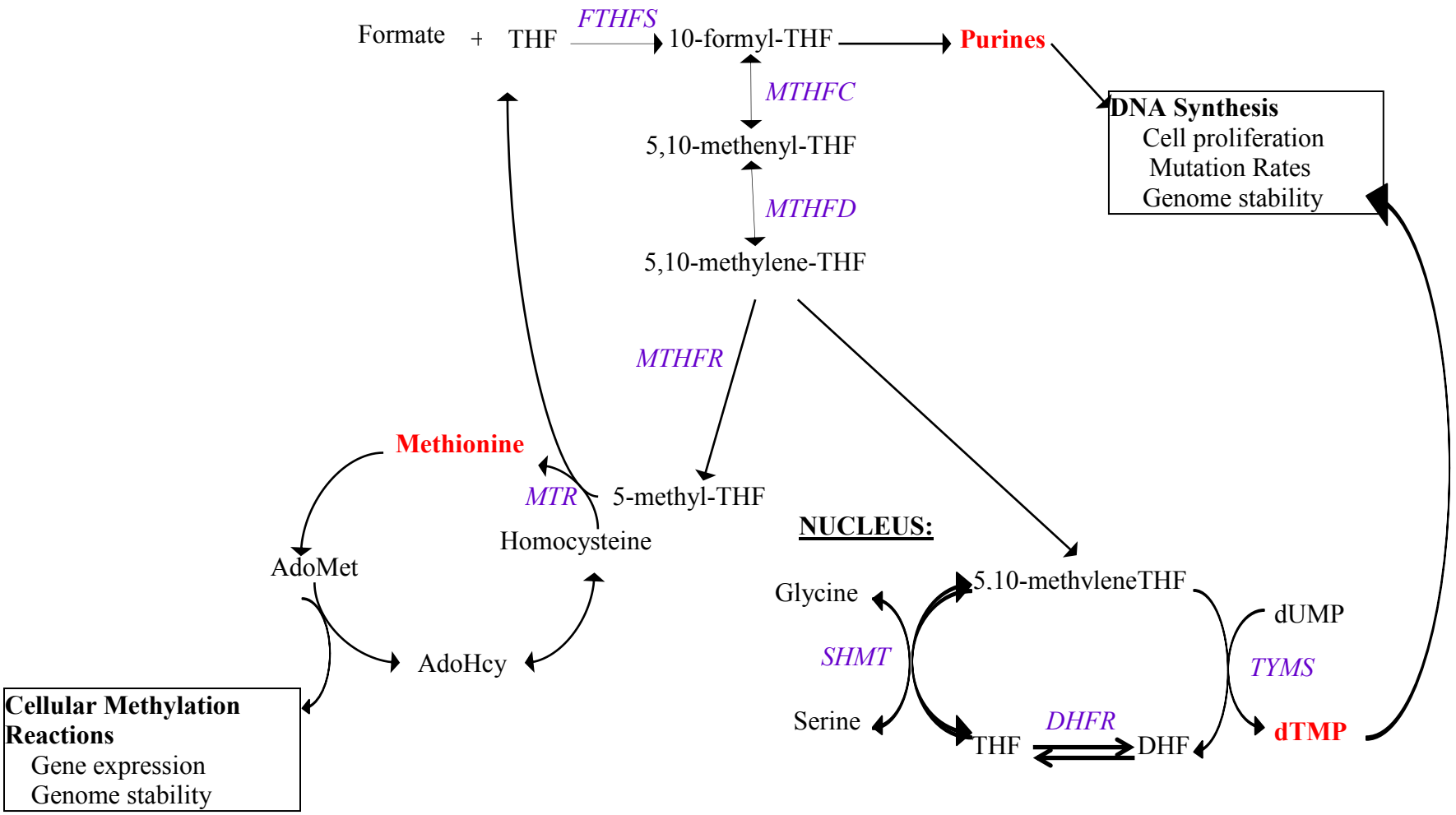

Figure 1.1: Cytoplasmic Folate-Mediated One Carbon Metabolism. AdoHcy, adenosylhomocysteine; AdoMet, adenosylmethionine; DHF, dihydrofolate; DHFR, DHF reductase; FTHFS, 10-formylTHF synthetase; MTHFC, methenylTHF cyclohydrolase; MTHFD, methyleneTHF dehydrogenase; MTHFR, methyleneTHF reductase; MTR, methionine synthase; SHMT, serine hydroxymthethyltransferase; THF, tetrahydrofolate; TYMS, thymidylate synthase**.** Enzymes are indicated in purple, and products of one-carbon metabolism are indicated in red bold.

In the mitochondria, amino acids are catabolized to produce formate, which enters the cytosol for subsequent metabolism [1]. Glycine is converted to serine via serine hydroxymethyltransferase (SHMT2), a vitamin B6-dependent enzyme that also catalyzes the conversion of THF to 5,10-methylene-THF [5]. 5,10-methylene-THF undergoes sequential oxidation of THF to formate, which upon entering the cytosol, condenses with tetrahydrofolate (THF) to form 10-formyl-THF by the 10-formyl-THF-synthetase (FTHFS) activity of C1-THF synthase (MTHFD1) [5]. C1-THF synthase, encoded by *MTHFD1* has three different enzymatic activities including the FTHFS activity found on the C-terminal domain [5]. The other two enzymatic activities include methenyl-THF-cyclohydrolase (MTHFC) and methylene-THF-dehydrogenase (MTHFD), and are found on the N-terminal domain of C1-THF synthase, and work sequentially to reduce the formyl entity of 10-formyl-THF to 5,10-methylene-THF (Figure 1.1) [5,8]. Depending on nucleotide requirements and purine and thymidylate availability from salvage pathways, 10-formyl-THF can either be used for *de novo* purine synthesis, be sequentially dehydrated and reduced to 5,10-methylene-THF for use in *de novo* thymidylate synthesis, or further reduced to 5-methyl-THF (Figure 1.1) [5].

If purines are limited, *de novo* purine biosynthesis is activated, whereby a complex of enzymes associated with purine biosynthesis, also known as the “purinisome”, assemble [9]. 10-formyl-THF is partitioned toward purine synthesis through its donation of carbons 2 and 8 to the purine ring, by the activities of aminoimidazolecarboxamide ribonucleotide transformylase (AICART) and glycinamide ribonucleotide transformylase (GART), respectively [5]. Alternatively, if the cell does not require the *de novo* synthesis of purines, 5,10-methylene-THF can either be irreversibly reduced to 5-methyl-THF by 5,10-methylene-THF-reductase (MTHFR)

and used for methionine synthesis, or be used as a cofactor in the synthesis of thymidylate (dTMP) [5].

5-methyl-THF is a one-carbon donor for the synthesis of methionine, a reaction catalyzed by methionine synthase (MTR), encoded by the gene *MTR* [5]. MTR is a Vitamin B12-dependent enzyme that remethylates homocysteine to form methionine using the methyl group of 5-methyl-THF [3]. Through the action of S-adenosylmethionine synthetase, an adenosine group from ATP is transferred to methionine, forming S-adenosylmethionine (AdoMet), which is the major methyl donor in cellular methylation reactions [3]. Removal of the methyl donor from AdoMet by methyltransferases produces S-adenosylhomocysteine (AdoHcy), which is then catabolized to homocysteine (Hcy) and adenosine (Ado) by AdoHcy hydrolase [3]. Hcy is remethylated once again using a methyl group from 5-methyl-THF and the cycle for methionine synthesis repeats, creating a consistent source of methyl groups for methylation reactions [3].

In contrast to *de novo* purine synthesis and methionine synthesis, *de novo* dTMP synthesis is localized to the nucleus, where DNA replication takes place during the S phase of the cell cycle [6,7]. When dTMP synthesis is required, the necessary enzymes such as SHMT1, thymidylate synthase (TYMS), and dihydrofolate reductase (DHFR) are sumoylated [5,7]. Small ubiquitin-like molecules (SUMO) are proteins that facilitate their nuclear import. Within the nucleus, thymidylate synthase (TYMS) mediates the transfer of a methyl group from 5,10-methylene-THF to deoxyuridine monophosphate (dUMP), creating dTMP and DHF [10]. This is followed by the NADPH-dependent conversion of DHF to THF by SUMO-DHFR and the conversion of THF and serine to 5,10-methylene-THF and glycine by SUMO-SHMT1, constituting a cycle that supports dTMP synthesis [5].

The products of folate-mediated one-carbon metabolism include purines and thymidylates, which are important in maintaining proper cell proliferation. Because nucleotide synthesis is required for cell proliferation, folate status is important in cells that are rapidly dividing and proliferating, such as cancer cells and cells involved in embryogenesis. Thus, aberrant folate metabolism can influence nucleotide synthesis and as such, impact cell proliferation and tissue growth.

Folate deficiency results in reduced nucleotide synthesis, causing a decrease in cell proliferation. Aberrant nucleotide synthesis results in increased mutation rates, as well as genome instability, caused DNA strand breakage and chromosome damage. One consequence of folate or Vitamin B12 deficiency, or a functional single nucleotide polymorphism (SNP) in the *MTR* gene, is decreased MTR activity and the resultant accumulation of 5-methyl-THF (Figure 1.1). In that case, cellular THF would decrease thereby resulting in depressed nucleotide biosynthesis [5]. The accumulation of 5-methyl-THF in this manner is known as the "methyl trap" since the function of MTHFR is irreversible *in vivo,* and only the MTR activity can regenerate THF, which is required for purine and dTMP synthesis [5,8].

Folate deficiency is also associated with increased micronucleus formation during cell division [5,8]. Micronuclei are small nuclei containing chromosomal acentric or centric fragments that had not moved to the polar ends of the cell during anaphase. The chromosomes develop a nuclear membrane and become micronuclei in the daughter cell. Micronuclei can be formed under conditions of impaired *de novo* thymidylate synthesis, an indicator of chromosome damage [5,8]. If *de novo* thymidylate synthesis is impaired, levels of deoxyuridine triphosphate (dUTPs) increase, resulting in increased incorporation of uracil into DNA [5]. As DNA repair mechanisms proceed to excise the misincorporated uracils via uracil-DNA glycosylase and

apyrimidinic endonuclease, single-strand breaks are created. Under conditions of chronic folate deficiency, double-strand breaks can occur if single strand breaks occur on opposing sides of the double-stranded DNA (dsDNA) [5].

Additionally, folate deficiency has been associated with global DNA hypomethylation [11]. DNA methylation is critical in the regulation of gene expression and in the maintenance of genome stability. The methylation status of gene-specific DNA can regulate gene repression/expression, interfere with the binding of transcription factors to DNA, and/or alter chromatin structure, affecting transcription [4]. In addition, global DNA hypomethylation can result in increased mutation rates or genome instability [5]. Since folate is required for the maintenance of cellular methylation potential, it can affect genome methylation status, gene expression, and genome stability.

1.2 The history of folic acid and neural tube defects

Folate deficiency has been associated with increased risk for neural tube defects (NTDs), including spina bifida, anencephaly, and encephalocele. NTDs are congenital birth defects involving the central nervous system (CNS), which includes the brain, spine, or spinal cord. In spina bifida, vertebrae do not develop fully, remaining open and in some cases, allowing a portion of the spinal cord to extend through the opening. In anencephaly and encephalocele, the neural tube that forms the brain fails to close.

Several observational studies led to the discovery of a relationship between maternal folate status and the occurrence of NTDs. Bryan Hibbard first suggested such a relationship in 1964, following his investigation into the folate status of 1,484 pregnant patients in Liverpool. He found that there was a higher rate of congenital abnormalities in newborns of folate-deficient mothers (3.0%) than in control mothers (1.6%) [12]. As a result, Hibbard accurately surmised

that abnormal formiminoglutamic acid (FIGLU) excretion in the urine (an indicator of folate deficiency) is related to various pregnancy complications, including congenital defects. In 1965, Hibbard and Smithells suggested that folate deficiency was specifically related to CNS malformations in developing embryos [13], and in 1976, Smithells et al. had observed that maternal vitamin deficiencies were associated with higher incidences of NTDs in humans [14].

Following these observational studies, Smithells et al. conducted an intervention study in Leeds, during which a multivitamin supplement containing 0.36 mg/day FA was given to women who had already given birth to an infant afflicted by an NTD [15]. From the group of supplemented mothers, 1 of 178 infants had an NTD (0.6%), whereas 13 of 260 infants from the non-supplemented mothers had an NTD (5.0%) [15]. In order to investigate further the effect of FA on NTD risk, the Medical Research Council launched a large-scale randomized trial [16]. Their main goal was to determine whether multivitamin supplementation either with or without FA would prevent the recurrence of NTDs in a high-risk population (those who have a history of infants born with NTDs). Starting in 1991, the MRC trial entailed giving women either a periconceptional supplement containing 4.0 mg/day FA, a multivitamin (containing Vitamins A, D, B1, B2, B6, C, and nicotinamide), both, or neither [16]. Results of this trial indicated that periconceptional supplementation with FA had a 72% protective effect against NTD incidences. Moreover, a large-scale primary prevention trial conducted in 1984 in Hungary in women who had no history of NTDs established that periconceptional use of 0.8 mg/day of FA prevented the first occurrence of NTDs [17]. They found that with supplement use, 0 of 2394 women gave birth to an infant with an NTD, whereas without supplement use, 6 of 2310 women gave birth to an infant with an NTD [17].

In light of these trials and increased public health promotion of periconceptional FA supplementation, Health Canada among others recommended that women eat more folate-rich foods and take a daily multivitamin supplement containing FA. The recommended daily intake of FA, the synthetic form of folate, is 0.4 mg for women who plan to become pregnant [18]. Additionally, women with an increased risk of delivering a baby with an NTD, such as women with a family history of NTDs, or women using antifolates, should be taking 4 – 5 mg FA/day.

Despite increased public health promotion, not all women use supplements with FA as recommended. Thus, to ensure women's adequate intake of FA and to decrease the incidence of NTDs, Canada and the US made the fortification of white flour with FA mandatory in 1998 [19]. Mandatory FA fortification in Canada has reduced the prevalence of folate deficiency from 30% to less than 1% in the general population [20,21]. There has been a 45% reduction in the incidence of NTDs [22], and 78% of women of child-bearing age had an RBC folate concentration of greater than 906 nmol/L, the concentration above which is associated with protection from NTDs [21]. In fact, it was also found that, amongst women who were not consuming a supplement with FA, 76.4% have an RBC folate concentration of greater than 906 nmol/L [23].

1.3 Folic acid and male fertility

While it is generally accepted that folate status is associated with female fertility, there is a growing body of evidence that it can also have important effects on male fertility. The target population of mandatory fortification was women of childbearing age. However, since white flour and "enriched" grain products are a staple food consumed by the general population, non-target groups, including men and boys, also demonstrate increased FA intake and higher folate status [21].

Men may benefit from increased FA intake because folate deficiency has been associated with male infertility. Male infertility is defined as the inability of a male to induce a pregnancy in a female. Male infertility underlies approximaely 40% of infertility cases in couples and is typically caused by semen deficiencies. Low sperm number is known as oligozoospermia, and is characterized by a decreased amount of spermatozoa in the semen; azoospermia is characterized by the absence of spermatozoa in the semen. Non-obstructive azoospermia is a lack of spermatozoa in the semen due to abnormal sperm production, whereas obstructive azoospermia is a lack of spermatozoa in the semen due to a physical obstruction. Teratospermia and asthenozoospermia are characterized by increased sperm morphology abnormalities and reduced sperm motility, respectively. Aspermia is when there is a complete lack of semen, whereas hypospermia is there is a lower amount of seminal volume. Some cases of male infertility are also considered to be idiopathic male infertilities, where the underlying pathogenesis is unknown. Approximately 30% of male infertilities are considered cases of idiopathic oligozoospermia, or unexplained sperm deficiencies [24].

Folate deficiency has been associated with decreased sperm counts, increased sperm DNA damage, and increased frequency of aneuploid sperm in both sub-fertile and fertile men [25–27]. The association of folate deficiency with decreased sperm counts and increased aneuploidy sperm were established in healthy North American men, both pre-fortification and post-fortification, respectively [25,27].Increased sperm DNA damage was associated with low seminal plasma folate in Dutch men undergoing *in vitro* fertilization in the Netherlands, where FA fortification is not implemented [26]. A therapeutic trial in the 1990s showed the treatment of idiopathic male infertility in men with the administration of 15 mg/day of 5-formyl-THF, which resulted in increased spermatozoa number and motility [28]. Another trial in the

Netherlands consisted of supplementation of sub-fertile men with both 5 mg/day FA and 66 mg/day zinc, resulting in an increase in sperm numbers [29]. Of note, only those men who were homozygous for the major allele (CC) of the *MTHFR* gene variant (677C>T) displayed a significant improvement in sperm numbers [30].

MTHFR, which irreversibly reduces 5,10-methylene-THF to 5-methyl-THF, is critical in the maintenance of methylation capacity. Impaired MTHFR activity results in decreased levels of 5-methyl-THF, decreased Hcy re-methylation, and consequently, decreased methionine synthesis, ultimately decreasing the availability of methyl groups for methylation reactions. The *MTHFR* gene has a common SNP, 677C>T (rs1801133) that leads to the substitution of Alanine by Valine at amino acid number 222. This change in the amino acid code results in a thermolabile protein, with lowered enzymatic activity [31]. A decrease in enzymatic function by approximately 35% and 70% occurs when the *MTHFR* gene is in the heterozygote (677CT) or homozygote state (677TT), respectively [32]. In patients with low folate status, this SNP was found to be associated with mild hyperhomocysteinemia [33], a condition characterized by elevated Hcy levels and a biomarker of folate deficiency. Homozygosity (TT) for the *MTHFR* SNP is also associated with lower folate status [34].

Various epidemiological studies have been conducted on the relation between the *MTHFR* 677C>T SNP and male infertility. An association between the two was first reported in 2001 by Bezold et al., who found that 18.8% of subfertile men were homozygous for the *MTHFR* 677TT mutation, compared to 9.5% of fertile men [35]. Subsequent studies were conducted regarding the association between this SNP and male infertility in various populations (Caucasian, Asian, African, and South American populations) with mixed results. Some studies showed a positive association, while others did not observe a significant relation between the two

parameters [36,37]. Some studies noted differences in the association of the *MTHFR* genotypes between different ethnicities. For example, two meta-analyses showed the association of this SNP with male infertility in Asian groups, but not in Caucasians [36,37]. A recent meta-analysis by Gong et al. examined 26 case-control studies, encompassing a total of 5,575 cases and 5,447 controls, and found that there was an association between heterozygosity and homozygosity for the *MTHFR* 677C>T variant and male infertility, specifically azoospermia and oligoasthenoteratozoospermia (decreased sperm count, decreased motility, and increased morphological abnormalities) [38]. In addition to irreversibly reducing 5,10-methylene-THF to 5-methylTHF, MTHFR has been associated with spermatogenesis [39].

1.4 Spermatogenesis

Spermatogenesis consists of a multistep process by which a diploid spermatogonium, the primordial germ cell, replicates and differentiates into mature spermatozoa [40]. The two main stages of spermatogenesis include spermatocytogenesis, and spermiogenesis. In spermatocytogenesis, a diploid spermatogonium either replicates itself to produce more spermatogonium (Type A spermatogonium) or undergoes mitotic division (Type B spermatogonium) to produce diploid primary spermatocytes, which will then undergo meiosis I to produce haploid secondary spermatocytes. Secondary spermatocytes divide via meiosis II to produce haploid spermatids. In spermiogenesis, mature spermatozoa are produced. Sperm maturation is characterized by the formation of a tail from one of the centrioles of the spermatids, the removal of the cytoplasm, and the formation of an acrosome, all of which occur in the presence of Sertoli cells in the seminiferous tubule [41]. Once mature, the spermatozoa will undergo spermiation, allowing them to be released from the Sertoli cells into the lumen of

seminiferous tubules. While the sperm are mature, they lack motility and are incapable of fertilization until they are transported to the epididymis of the testes.

In post-meiotic spermatids, chromatin remodeling occurs. In almost all cells, histones are required to condense DNA in the nucleus by wrapping DNA tightly around them 1.6 times. Known as the building blocks of a structure called the nucleosome, there are five subtypes of histones: H1, H2A, H2B, H3, H4 [42]. However, the majority of DNA in a mature sperm is condensed around protamines. A histone to protamine exchange takes place during spermiogenesis, at which time histones are first acetylated and then replaced by sperm-specific transition proteins [43,44]. The transition proteins are subsequently removed during the final stage of spermiogenesis and replaced by protamines [43,44]. Protamines are arginine-rich nuclear proteins critical for sperm head condensation and DNA stabilization. The positively charged arginine residues allow the protamines to bind to the negatively charged phosphate in DNA, leading to the formation of a toroid [45]. The tightly packaged structure of the protamine toroid allows for increased protection of the sperm chromatin, rendering it more stable and less prone to modifications than histones. Protamination is incomplete however, with approximately 5 – 10% of histones retained in humans [46], and somewhat less in mice.

1.5 Folate metabolism and spermatogenesis

Folate dependent nucleotide synthesis and methionine synthesis play a vital role in spermatogenesis. Altering either of these processes can affect germ cell differentiation and sperm production, which could lead to male infertility.

During the early stages of spermatogenesis, germ cells are successively undergoing mitosis, which requires efficient nucleotide synthesis. Thus, folate metabolism is important for germ cells and spermatogonia as they are constantly replicating, dividing, and differentiating. If,

for example, the MTHFR enzyme were to be compromised, levels of 5-methyl-THF would decrease, thereby potentially reducing purine and thymidylate synthesis, possibly resulting in a downstream reduction in sperm production. Impairment of *de novo* thymidylate synthesis can cause increased uracil misincorporation, dsDNA breaks, and consequently increased chromosome damage in sperm. Consistent uracil misincorporation due to impaired thymidylate synthesis can lead to an accumulation of uracil during ongoing cell proliferation of progenitor cells, leading to an increase in DNA strand breakages and genomic instability [47].

Methionine synthesis is also important in the maintenance of proper methylation capacity for both DNA and chromatin in spermatogenesis. Aberrant AdoMet synthesis as a result of aberrant folate metabolism can affect the maintenance of methylation of sperm DNA, thereby affecting sperm differentiation. In fact, altered sperm DNA methylation has been shown to affect the differentiation of spermatogonia into spermatocytes in neonatal mice exposed to a hypomethylating agent [48]. Aberrant methylation status as a result of folate deficiency can also affect chromatin packaging and lead to chromosome instability, affecting sperm production. A study by Young et al. (2008) has shown that low folate intake can cause sperm aneuploidy in healthy non-smoking men [27]. Another study showed that mice exposed to a folate antagonist had decreased sperm counts and increased sperm DNA damage [49].

Our previous research showed that FA deficiency in male mice resulted in decreased sperm numbers, increased germline chromatin damage, and increased sperm DNA mutation frequency [50]. Germline chromatin damage was measured using a DNA fragmentation index (DFI) and sperm mutation frequency was assessed as tandem repeat mutation frequencies. These results were similar to those of an observational study by Wallock et al. (2001), who also showed that low folate concentration in the seminal plasma was associated with low sperm density and

low sperm count in men [25]. It was also found that subfertile men consuming a healthy diet high in folates had a lower DFI than in those consuming a diet heavy in carbohydrates [51].

It has been suggested that the *MTHFR* gene plays an important role in spermatogenesis. In male mice, *Mthfr* is more active in the adult testes than in other organs [39]. Knockout of the *Mthfr* gene in male mice leads to decreased sperm counts [52], as well as impaired spermatogenesis and infertility [53]. In men, hypermethylation of the *MTHFR* gene promoter region in both testes and sperm has also been associated with non-obstructive azoospermia and idiopathic male infertility [54,55]. Consequently, impaired MTHFR function and altered MTHFR levels may result in impaired spermatogenesis and male infertility, likely due to its key role in folate metabolism. Together, the data indicate that MTHFR function is important in spermatogenesis and male fertility.

1.6 Supplemental folic acid intake

In addition to folate deficiency, there may also be unforeseen effects associated with FA intake above that recommended for men. This is important in the Canadian context where data from the Canadian Health Measures Survey indicate that 63.5% of the Canadian population has a RBC folate status indicative of folate intakes above the tolerable upper intake level (UL) for FA (1 mg) [21]. The UL is defined as "the highest level of daily nutrient intake that is likely to pose no risk of adverse health effects to almost all individuals in the general population." [56]. Individuals may achieve FA intake above the UL by consuming the RDA (0.4 mg/day FA) and an FA-containing multivitamin (can contain up to 1 mg/day FA) [21,57]. Regarding the male population, 65-73% of boys and men have a folate status indicative of FA intakes above the UL [21].

Over-supplementation of FA may result in unmetabolized FA in circulation. FA is absorbed by the intestine. However, metabolism of FA in the intestine is limited and it is shuttled through to the liver via the hepatic portal vein. In the liver, FA is sequentially reduced by dihydrofolate (DHF) reductase to DHF and THF. However, DHFR activity has been shown to be slow and inefficient at the conversion of large amounts of FA to THF, and thus supplementation with FA leads to the presence of unmetabolized FA in circulation [58]. In fact, there have been several studies showing that prevalence of unmetabolized FA found in circulation from exposure to high levels of FA nears 100% in fortified regions [59–61].

Unmetabolized FA remaining in circulation can be absorbed directly by the tissues and be metabolized to cellular DHF. Increased FA would result in an increase of DHF, which is also a competitive inhibitor of DHFR and could prevent the rate of reduction of DHF to THF [47]. Consequently, there would be an accumulation of DHF, which may pose a risk given that DHF is an inhibitor of both MTHFR and *do novo* thymidylate synthesis. Inhibition of MTHFR can result in decreased methionine synthesis and a decrease in 5-methyl-THF production, leading to decreased remethylation of homocysteine, as well as decreased purine and thymidylate synthesis. In turn, methionine synthesis will be reduced, and methylation capacity will be impaired, allowing for changes in gene expression or genomic instability. Additionally, DHF has been shown to inhibit TYMS, thereby also decreasing synthesis of dTMP [62]. Impaired nucleotide synthesis may subsequently cause an increase in mutation rates, genome instability, and reduced cell proliferation. Maintaining proper cell proliferation is critical for cells involved in embryogenesis, as they are rapidly dividing and proliferating.

1.7 Paternal FA intake and pregnancy outcomes

A previous animal study done in our lab has shown that a FA defined diet, including either a deficient and supplemented diet, affected male fertility rates (Table 1.1) [MacFarlane et al., unpublished]. Measured endpoints included litter sizes and rates, resorption numbers and rates, implantation sites, and fertilization events. Male mice consuming a FA deficient diet had lower fertility rates, where the number of litters produced decreased by a third compared to the control diet. Deficient male mice did not differ significantly from control mice in litter size or number of implantation sites, but the deficient mice showed a significant increase in the number of embryo resorptions compared to the control mice. An implantation site indicates that a blastocyst successfully implanted into the uterine wall at embryonic day 4.5 (E4.5), four days post-fertilization.

Table 1.1: Fertility outcomes from previous animal study. Male mice were weaned to a FA deficient (0 mg/kg), control (2 mg/kg), or supplemented (6 mg/kg) diet for 14 weeks. Values in a row that do not share a letter are significantly different (MacFarlane et al., unpublished data).

	Diet (mg FA/kg diet)			***P***
	Deficient (0) n=12	Control (2) n=11	Supplemented (6) n=12	
Litters Produced (% of matings)	58.3[a]	90.9[b]	66.7[a]	<0.0001[1]
Litter Size (no./female)	5.6 ± 2.0	4.9 ± 2.0	5.0 ± 3.1	ns[2]
Implantation sites (no./female)	8.6 ± 2.2[a]	8.5 ± 3.4[a]	12.6 ± 3.3[b]	0.003[2]
Resorptions (no. resorptions/female)	5.8 ± 3.8[a,c]	3.6 ± 5.0[a]	8.6 ± 6.3[b,c]	0.03[2]
Resorption rate (% embryos resorbed/female)	64.9 ± 33.0[a]	34.7 ± 0.58[b]	62.7 ± 0.47[a]	0.06[2]

[1] Chi square analysis
[2] One-way ANOVA Tukey's HSD post-hoc analysis

Of interest, negative effects on fertility rates were also observed in male mice consuming a FA supplemented diet with three times the recommended intake despite having no effects on sperm counts, sperm mutation frequency or sperm chromatin damage [50]. The supplemented male mice demonstrated a 30% reduction in the number of litters born, similar to the reduction in litters born to male mice fed a FA deficient diet (Table 1.1) [MacFarlane et al, unpublished data]. The reduction in litter rates occurred despite an increase in the number of uterine implantation sites. Furthermore, supplemental FA intake in male mice resulted in an increase in the number of resorptions and resorption rates, compared to those fed the control diet. This suggests that while there was an increase in the number of fertilization events and implantation sites, there was abnormal embryo development and death resulting in fewer litters born. The reduced pups, coupled with no change in implantation sites, suggest a reduction in embryonic viability at the post-implantation stage of embryonic development. Therefore, in the absence of DNA damage or increased mutation frequency, FA supplementation of fathers may lead to abnormal embryo development and death. This is similar to what Pickell et al. observed; maternal FA supplementation (twenty times higher than the recommended daily intake) was associated with embryonic delay and embryonic heart defects in mice [63].

Changes in gene expression may be one mechanism that underlies the reduction in embryo viability. Since folate is required for the maintenance of cellular methylation potential, and consequently, can affect genome methylation status and gene expression, altering paternal FA intake may alter methylation status of sperm DNA, which may be stably transmitted to the offspring. These changes in methylation profiles of the sperm, if stable, may also result in trans-generational and heritable epigenetic modifications in the descendants of the exposed males.

1.8 Epigenetic regulation during spermatogenesis

Epigenetics is defined as changes in gene expression without alteration of the DNA sequence [64]. Mechanisms of epigenetic regulation of gene expression include DNA methylation, histone modifications (i.e. acetylation, methylation, phosphorylation, ubiquitinylation), and miRNAs.

DNA methylation is the most studied form of epigenetic modification and results from the covalent addition of a methyl group to a cytosine nucleotide, leading to the formation of a methyl-cytosine. DNA methylation occurs at CpG dinucleotide sequences, within CpG-rich regions known as CpG islands, which can be found in the 5' promoter region of genes. Cytosine methylation is mediated by DNA methyltransferases (DNMTs), including DNMT3A, DNMT3B, and DNMT1, all of which are expressed throughout spermatogenesis [65]. DNMT3L, a DNMT-like protein has been shown to regulate DNMT3A and DNMT3B. Both DNMT3A and DNMT3B activate *de novo* methylation [66,67], whereas DNMT1 is involved in the maintenance of DNA methylation. DNA methylation is mitotically stable allowing for consistent regulation of DNA sequences between mother and daughter cells in a tissue, or potentially trans-generationally among organisms if they occur in the germline.

Once an oocyte is fertilized by a sperm, two separate rounds of demethylation take place that can potentially alter gene expression within the developing embryo. The first round of demethylation in mice occurs between E0.5 and E3.5 days post-fertilization, and results in the erasure of gametic epigenetic marks [68]. Paternal and maternal DNA undergo both active and passive demethylation [69,70], with the exception of certain imprinted genes and intracisternal A particles (IAPs) [68,71,72]. Imprinted genes are observed in clusters and form imprinted domains, which are regulated by imprinting control regions (ICR). ICRs are characterized by

differentially methylated regions (DMRs), in which only the maternally or paternally inherited allele is methylated and expression from that allele is repressed. Since DMRs and IAPs (endogenous retrotransposons) are able to escape the first round of demethylation, they provide a mechanism by which methylation patterns can be trans-generationally inherited.

Once the epiblast is formed, post-implantation global *de novo* DNA remethylation occurs, Between E10.5 and E13.5, primordial germ cells (PGCs) will undergo their own round of demethylation, which results in erasure of their genome, including imprinted genes, ensuring totipotency in the offspring [73]. However, complete erasure of all epigenetic information is not guaranteed. At the point of the lowest levels of methylation, approximately 5% of the genome still possesses methylated DNA, and characterization of such areas has shown that the majority are related to brain and growth-related functions [74,75]. Environmentally acquired information, such as those from dietary changes, can affect expression of these areas, providing a mechanism by which changes can be transferred trans-generationally [76,77], inducing phenotypic changes in the offspring [74]. *De novo* methylation is maintained throughout spermatogenesis and as such, information may be potentially inherited during each cell division.

Additional mechanisms by which paternal transmission of epigenetic information can occur include histone modifications and miRNAs. During the histone to protamine exchange process during spermatogenesis, approximately 10% of the original histone content remains intact in human sperm nuclei [78], whereas in mice, it is thought to be approximately 1% [79]. Studies by Hammoud et al. have shown that in humans, the retained histones are those related to genes of embryo development, as well as transcription factors, imprinted genes, and miRNAs [46,80]. Furthermore, histone replacement by protamination is a process facilitated by histone acetylation during late spermatogenesis. Epigenetic alterations of the acetylation patterns of

histone can affect the protamination process during spermatogenesis. Recently, miRNAs have also been found to be regulators of DNA methylation [81], where certain miRNAs are predicted to be down-regulators of DNMT3A, a stimulator of *de novo* methylation [82,83]. Consequently, changes in miRNA can affect other epigenetic markers such as DNA and possibly histone methylation.

Our lab, in collaboration with the lab of Dr. Carole Yauk, performed a microarray analysis on liver tissue from the F3 descendants of male mice exposed to FA deficient, control, or supplemented diets, in order to determine whether paternal FA exposure resulted in heritable changes in gene expression. Indeed, grandpaternal diet dependent gene expression changes in the liver tissue of the F3 descendants of viable male offspring fed a FA deficient or supplemented diet were observed. Genes identified by microarray analysis were differentially expressed depending on grandpaternal FA exposure (Table 1.2). It is of interest to examine the mechanism underlying these changes in gene expression in the offspring/descendants of male mice. We hypothesize that the mechanism underlying differences in gene expression in the offspring and descendants of males fed different FA diets is altered DNA methylation.

Table 1.2: Hepatic gene expression, as measured by quantitative RT-PCR in F3 male descendants of male mice fed FA defined diets. Grandfathers were fed a FA deficient (0 mg/kg), control (2 mg/kg), or supplemented (6 mg/kg) diet in either early development or post-weaning. Gene expression changes are relative to the FA control diet, as determined by qPCR. Genes in red are candidate genes chosen for further methylation analyses based on the presence of CpG islands in their promoter regions.

	Gene	Up/Down Regulation compared to Control			
		Early Development		Post-weaning	
		Deficient	Supplemented	Deficient	Supplemented
Choline Pathway	Mgll	0.80	0.99	0.87	0.99
	Chpt1	0.99	1.10	0.91	1.22*
	Chka	2.09*	1.43	1.75	1.49
	Chdh	1.07*	1.13*	0.99	1.08*
	Maob	1.05	1.05	0.99	1.15
NTD - associated Genes	Cited2	1.17*	1.05	1.26*	1.21*
	Mapk8	1.09*	1.03	1.09*	1.11*
	Pdgfc	1.27*	1.11	1.27*	1.16
	Mthfr	1.64	1.37	1.46	1.58
	Gcm1	1.23	1.14	1.19	1.21
Others	Acnat2	0.56*	0.66	0.53*	0.82
	Cish	0.16	0.53	0.55	0.29
	Cyp4a10	0.46	0.56	0.51	0.53
	Cyp4a14	0.28	0.22*	0.40	0.25*

* Indicates a significant change in gene expression relative to the control group. All others were identified in the microarray but were not significant by qPCR.

1.9 Hypothesis

Paternal FA intake will affect male fertility and embryo development. Folate deficiency will have a negative effect on parameters of male fertility and embryo development. Folate supplementation will not have a negative effect on male fertility parameters, but will negatively impact embryonic development. Changes in sperm DNA methylation in males fed different FA diets will alter gene expression in the offspring in the absence of overt changes to sperm number or quality, which results in altered embryo viability.

1.10 Objectives

1. Determine the effect of paternal FA intake on parameters of male fertility (Chapter 3).
2. Determine the effect of paternal FA intake on embryo development (Chapter 3).
3. Determine the effect of paternal FA intake on methylation dependent gene expression in viable descendants (Chapter 4).

Chapters Overview

This thesis is organized by chapters according to the methods used and objectives for the study. Chapter 2 is written as an overview of the various methods used in this study and their associated endpoints. Chapter 3 addresses the first two objectives and Chapter 4 addresses the third objective. Chapters 3 and 4 are written as stand-alone publishable units. As such, there may be redundant parts between the two. A discussion consisting of a summary of the results, implications of the study to human health, future directions, and a conclusion is provided in Chapter 5.

Chapter 2:
General Overview of the Methods

2 Chapter 2: General Overview of the Methods

The study designs and methods described in this thesis are quite varied. As such, I have included an overview of the two animal study designs as they relate to the study goals. Also, an overview of a selection of the methods and endpoints analyzed are also presented.

2.1 Animal Study Models

To characterize the effect of paternal FA intake on male fertility and embryo development, we designed an animal study where male Balb/c mice were fed deficient (0 mg/kg), control (2 mg/kg), and supplemented (6 mg/kg) FA diets, which have been used extensively in our lab [47,50,84,85] (Figure 2.1). The FA diets are physiologically relevant. The control diet (2 mg/kg) is recommended by the American Institute of Nutrition as the adequate dietary FA intake for rodents [86], and as such is equivalent to 0.4 mg per day the Recommended Daily Allowance for human adults. The deficient diet (0 mg/kg) represents an inadequate dietary FA intake. Mice fed the folate deficient diet have lower tissue folates but do not become completely folate depleted, as folate can be synthesized by intestinal bacteria [87]. Therefore, only a mild folate deficiency was induced over the study period, as has been observed in previous studies, where no overt effects on mice body weight and activity were observed due to decreased folate intake (Honours thesis, Danielle Leblanc, 2015). The supplemented diet (6 mg/kg) is threefold the control diet. The supplemented diet was designed to approximate the combined intake of FA from fortification (~0.15-0.2 mg) and over-the-counter supplements (1 mg) available on the Canadian market.

The animal study was conducted using the Balb/c inbred mouse strain for various reasons. First, this study was developed based on results from a previous study in our lab, where negative male fertility effects were observed in response to paternal FA diet in Balb/c mice

(MacFarlane et al, unpublished). We therefore designed a similar animal study using the same mouse strain and diets in order to confirm and expand on those findings. An inbred mouse strain, in comparison to an outbred mouse strain, was used for reproducibility since mice of an inbred strain are isogenic, or genetically identical. The isogenicity of an inbred strain allows us to characterize epigenetic features, such as methylation, in the absence of any genetic effects on the epigenome.

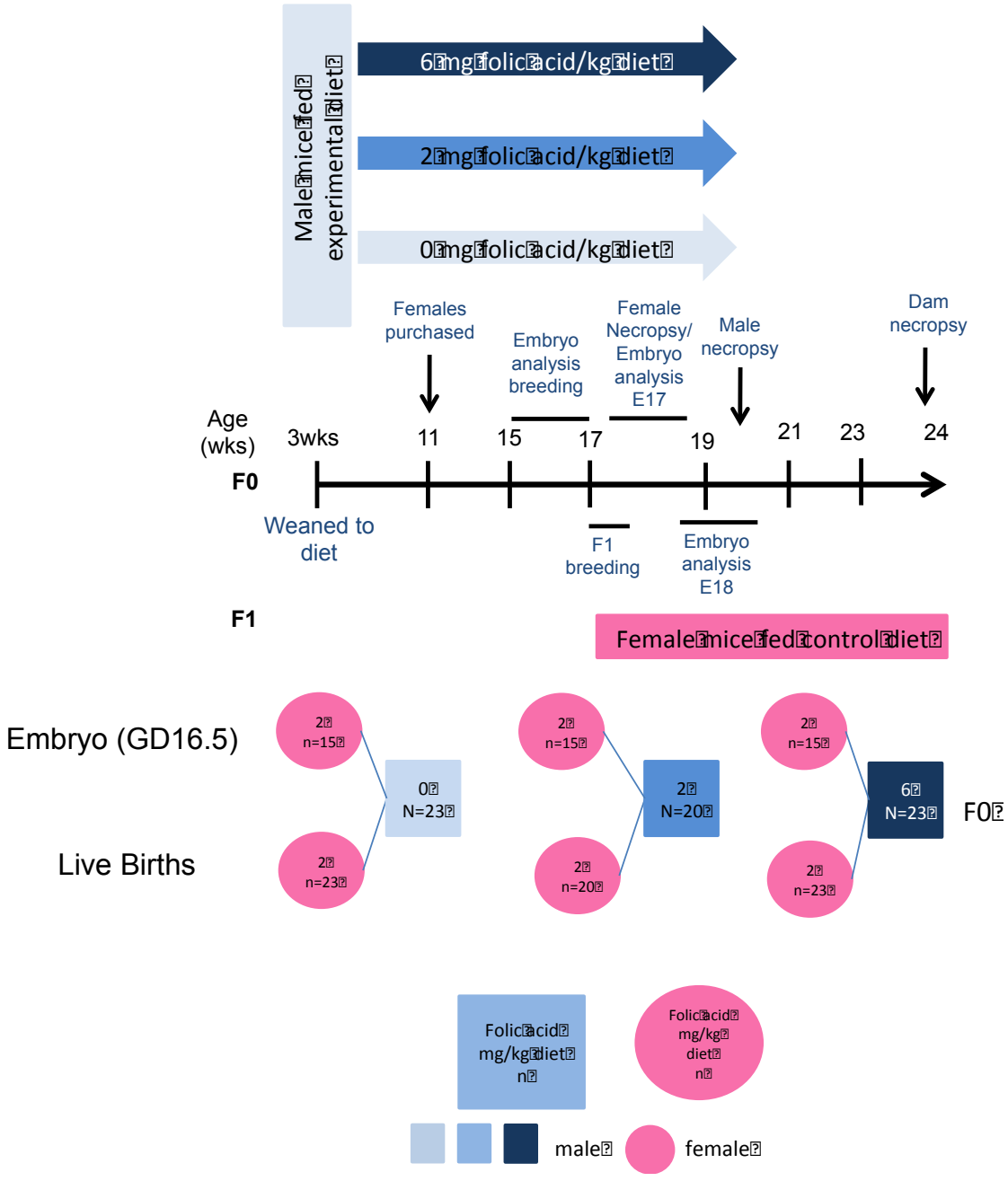

Figure 2.1 Breeding scheme and timeline of animal study for fertility and embryo analysis. Male F0 Balb/c mice were fed a FA deficient (0 mg/kg), control (2 mg/kg), or supplemented (6 mg/kg) diet from weaning for 15 weeks and bred with female Balb/c mice fed the control diet. One set of F0 females (n = 45) were killed at gestational day 16.5 (GD16.5) to examine fertility and embryo development endpoints. The second set of F0 females (n = 66) were allowed to deliver F1 pups to examine fertility and live pup viability endpoints. 0, deficient diet (0 mg/kg); 2, control diet (2 mg/kg); 6, supplemented diet (6 mg/kg); Male mice are represented by blue squares; females by pink circles.

Male Balb/c mice (aged 3 weeks) were weaned to their respective diets for 15 weeks before being bred with female Balb/c mice that had been fed the control diet for 4 weeks to ensure that they had reached tissue folate homeostasis (Figure 2.1). Male mice were fed their respective diets for 15 weeks to ensure exposure of the paternal sperm to the diet. In germ cell development in mice, a complete cycle of spermatogenesis lasts 49 days [88]. Spermatogenesis in male mice begins at 3 weeks of age and it takes a spermatogonium approximately 7 weeks to mature into a spermatozoon and be released into the lumen of the testes [89,90]. Prior to spermatogenesis, spermatogonial stem cells (SSCs) will also undergo stem cell divisions (Type A spermatogonium) [88]. Spermatogonium present in the testes that do not self-renew will undergo mitotic divisions to produce Type B spermatogonium, at which point spermatogenesis will begin. It takes 8 – 9 days to produce a Type B spermatogonium from a spermatogonial stem cell, and 49 days for Type B spermatogonia to divide and produce mature spermatozoa. Thus, a total of 15 weeks on diet means that the male offspring were exposed to their respective diets during 7 SSC divisions, 2 mitotic divisions, and 2 meiotic divisions to produce mater spermatozoa.

After 15 weeks on their respective diets, male mice were bred with two sets of female mice fed the control diet. The first females were used for the embryo analysis and were sacrificed at gestational day 16.5 (GD16.5), where GD0.5 represented the morning a positive mating plug was observed. Since we assumed that embryos were being lost, but did not know at which time point, we chose GD16.5 as a benchmarker to determine if embryos were being lost before or after that particular time point. The second females used to examine the impact of paternal FA intake on live births.

2.2 Male Fertility Outcomes

As a means of assessing male fertility in the F0 generation, we measured testes weight, conducted sperm analyses, and analyzed pregnancy outcomes.

2.2.1 Testes Weight

Testes weight correlates with total sperm count in mice [91–93]. It has been shown that species with larger testes had a higher daily production rate of sperm, as well as a larger sperm reserve and a larger number of sperm per ejaculate [92]. Therefore, testes weight can be a marker of fertility, as sperm number is an indicator of male fertility [91–93]. Testes weight can vary among mouse strains. A study by Le Roy et al. measured paired testes weight in various mice strains and found that a Balb/c strain had a mean paired testes weight of 0.200 ± 0.010 (mg), resulting in an individual testicle weight of approximately 0.100 mg [94], as shown in additional studies [95,96]. Additionally, it was important to calculate the testes to body weight ratio since testes size is dependent on body weight; testes to body weight ratios in mice have been shown to be approximately 0.38 ± 0.02 (%) [97].

2.2.2 Sperm Analysis

Sperm counts and motility were evaluated using the integrated visual optical system (IVOS) for sperm analysis, which is a computer-assisted sperm analysis (CASA) instrument. IVOS is able to track sperm motion by creating a centroid at the sperm head of a spermatozoon and reconstructing its trajectory. Parameters of sperm analyses assessed include the percentage of motile sperm cells, average path velocity (VAP, distance traveled along a smoothed average path divided by the elapsed time), straight line velocity (VSL, straight-line distance of space gain of the sperm head divided by the elapsed time), track velocity (VCL, distance traveled by the sperm head between adjacent points divided by the elapsed time), and straightness (STR, ratio of

VSL to VAP; a higher value is indicative of a straighter trajectory) [98]. An important parameter is the amplitude of lateral head displacement (ALH; μm), which is representative of the amplitude of the beating flagellum (Figure 2.2). The amplitude of this beating wave determines the degree to which the sperm thrusts forward against a surface, such as the mucus of the cervix, which is the first surface the sperm head will encounter post-ejaculation. Consequently, larger amplitudes would propel the sperm further than smaller ones, and with a greater force across such a surface [55, 56].

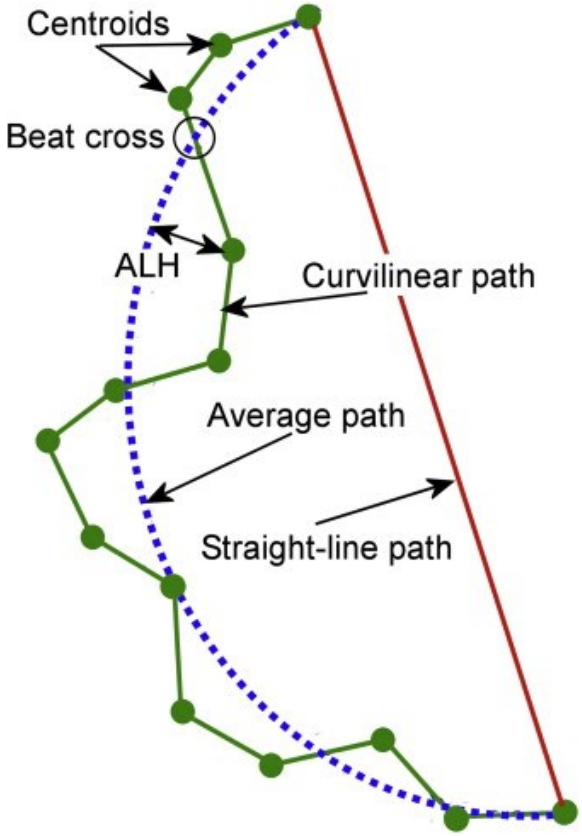

Figure 2.2 Schematic of sperm velocity paths including average path (VAP), curvilinear path (VCL), and straight-line path (VSL). Reprinted from "Computer-assisted sperm analysis (CASA): Capabilities and potential developments," by Amman RP and Waberski D, 2014, Theriogenology, 81(1):5-17. Reprinted with permission from RightsLink ©2015.

A kinematic definition has been established for spermatozoa in humans showing ideal mucus penetrating characteristics include a VAP of greater than 25 μm/s, a STR of greater than 0.8, and an ALH of greater than 2.5 μm, where all three characteristics must be met [101]. Therefore, sperm that do not meet these criteria may be indicative of poor fertility. In mice, an established definition for progressive sperm is a VAP of greater than 50 μm/s and a STR of greater than 0.5 [102].

2.2.3 Analysis of Pregnancy Outcomes

Number of uterine implantation sites in females sacrificed at GD16.5 was evaluated and litter size and rates established. Corpus luteum:litter size ratios were also evaluated.

An implantation site occurs when a blastocyst has successfully implanted into the uterine wall at four days post-fertilization. Implantation of the blastocyst is an integral process for a successful pregnancy, as it is the beginning of the formation of the placenta and will facilitate the transfer of nutrients from the mother, allowing for embryo growth and development. The corpus luteum (CL) is an endocrine gland in the ovary that forms post-ovulation and secretes progesterone [103]. The number of CL is representative of the number of eggs that ovulated [104,105]. Resorptions occur post-implantation due to an impairment of embryo viability and embryo death, whereas a pre-implantation loss is indicative of a lack of fertilization or embryo loss prior to implantation of the blastocyst [106]. The number of implantation sites per female was determined by adding the number of viable embryos and resorptions. The number of fertilization events per female was determined by calculating the ratio of implantations to CL. Resorptions were determined visually at necropsy and the rate of resorption was determined by calculating the number of resorptions per implantation per female.

2.3 Multi-generational Study

In order to determine whether an environmental exposure results in a heritable, not just trans-generational, effect, the effect must be observed in the first unexposed generation. The exposed generations in the context of the paternal line would include the animal and its gametes (in the case of pregnant females, the mother, the embryo and its gametes), and would only provide evidence for the direct effects of the environmental factor. To identify heritable effects through the paternal line, the study design must therefore include a third generation. Therefore, to determine the heritable transgenerational effect of paternal FA intake on altered gene expression and methylation capacity, a multigenerational study was conducted where male mice were fed a folic acid defined diet (Deficient, Control, and Supplemented), either in early development (*in utero* and during lactation) or post-weaning (Figure 2.3). Their offspring and future descendants were all fed the control diet. In collaboration with the lab of Carole Yauk, microarray analysis was used to identify gene expression changes in the liver tissues of F3 descendants of these male mice.

The F3 generation was the first generation that was unexposed to the FA-defined diet (Figure 2.3). Effects in the F2 generation would not be considered heritable because they would have been the exposed as germ cells. Therefore, the F3 generation is the first unexposed generation and the observed gene expression changes can be considered trans-generationally heritable.

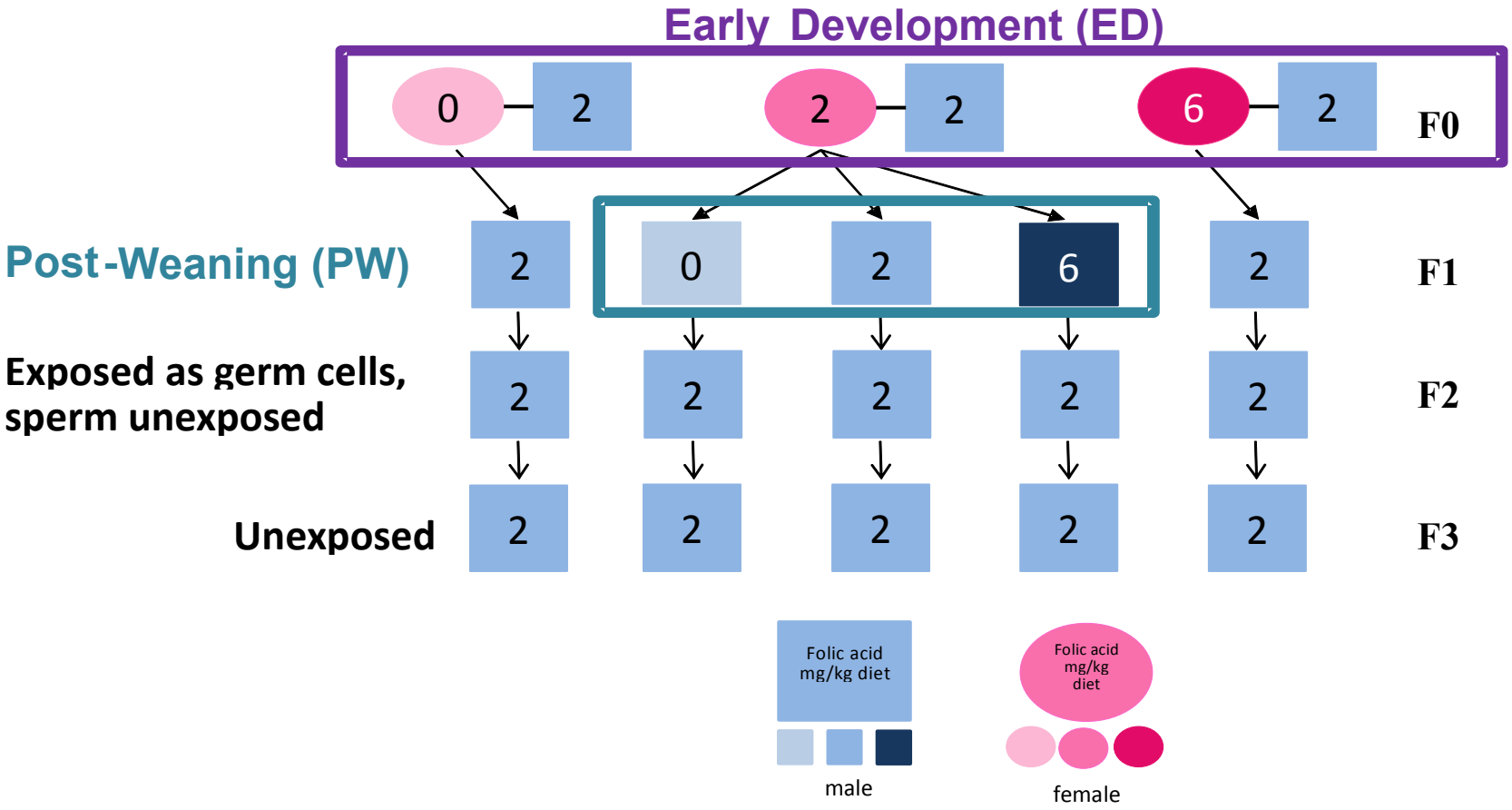

Figure 2.3 Breeding scheme of multigenerational animal model [MacFarlane et al., unpublished]. F0 female Balb/c mice a FA deficient (0 mg/kg), control (2 mg/kg), or supplemented (6 mg/kg) diet from weaning for 15 weeks and bred with male Balb/c mice fed the FA control diet. At 3 weeks of age, F1 male mice born from the FA control maternal diet group were weaned to a FA deficient (0 mg/kg), control (2 mg/kg), or supplemented (6 mg/kg) diet for 15 weeks and bred with female Balb/c mice fed the FA control diet. F1 male mice born from the FA deficient and supplemented maternal diet groups were weaned to a FA control diet for 15 weeks. F1 male mice were sacrificed at 18 weeks of age. Subsequent F2 and F3 generations were fed a FA control diet. 0, deficient diet (0 mg/kg); 2, control diet (2 mg/kg); 6, supplemented diet (6 mg/kg); Male mice are represented by blue squares; females by pink circles.

2.3.1 BiSulfite Amplicon Sequencing

We hypothesized that the biological mechanism behind the change in diet-dependent gene expression was DNA methylation changes. To test this, we used bisulfite-treated DNA (BS-DNA) to characterize the DNA methylation status of the genes that had diet-dependent gene expression changes.

Bisulfite sequencing of DNA was first introduced by Frommer et al. in 1992 as a novel method in the characterization of DNA methylation. Since then, it has become the "gold-standard technology for detection of DNA methylation" [107]. In 2013, a method known as BiSulfite Amplicon Sequencing (BSAS) was developed by Masser et al., where a specific CpG island can be analyzed in a rapid and highly quantitative manner using next generation sequencing [108]. BSAS begins with the bisulfite conversion of DNA, at which point unmethylated cytosines are converted to uracils and 5-methylcytosines remain unchanged. PCR amplification using primers specific to the region of interest is subsequently carried out. PCR primers can be created using MethPrimer [109], which designs primers based on potential CpG island identification for methylation analyses. Once the region of interest is amplified, next generation sequencing is used to quantitatively assess the methylation status of specific CG di-nucleotides within the CpG island of interest.

While the BSAS method is fairly novel, it has been used before to quantify CpG methylation levels in the Rhodopsin promoter in RNA and DNA extracted from mouse retina and cerebellum [108,110], as well as to characterize the methylation status of a promoter CpG island involved in Alzheimer's disease [111].

One limitation of the BSAS is the lack of differentiation between identification of methylated cytosine and hemi-methylated cytosines. Hemi-methylated DNA is when only one of

two complementary DNA strands is methylated; or in the case of a hemi-methylated cytosine, a single cytosine is methylated on one of the two complementary strands. Another limitation is PCR bias, which is when the region of interest is amplified more frequently in one of the two complementary DNA strands than the other. However, this bias can be mitigated by using MethPrimer to design primers following the recommended guidelines for amplification of bisulfite converted DNA. For this study, primers were designed to anneal to sequences within the two hundred base pairs 5′ or 3′ of the identified CpG island sequence to ensure optimal primer design around the CpG island and not within it. If the primers were to be designed within the CpG island, the binding capacity of the primer to the DNA sequence would be decreased due to variable cytosine/methylcytosine content in the CpG island [109].

Another commonly used method for methylation analyses is Reduced-representation bisulfite sequencing (RRBS). RRBS uses the methylation insensitive MspI restriction enzyme, which cleaves DNA sequences at 3'CCGG5', thereby targeting CG dinucleotides. Once the DNA is digested, a sequencing library is prepared using the fragmented pieces, the fragments are bisulfite sequenced, and NGS is used to sequence the fragments. While RRBS reduces the amount of sequences that have to be sequenced, thereby reducing the cost and time, there are disadvantages. One such disadvantage is that only 85% of CpG islands and 60% of promoters are detected. Furthermore, only 12% of CG dinucleotides are identified [112]. In our study, we were targeting CpG islands in promoters of genes, and using RRBS may have limited the detection of methylation changes in these particular regions.

2.3.2 Next-Generation Sequencing

Next-generation sequencing (NGS) is a fairly recent method of DNA sequencing that has become the preferred method in comparison to first-generation sequencing methods (Sanger

sequencing). NGS, also known as massively parallel sequencing, is a high-throughput method of sequencing that potentially allows for the sequencing of an entire genome in one day [113]. The most widely used next-generation sequencing methods include pyrosequencing (454 Roche) and sequence-by-synthesis (Illumina Miseq) technologies. In this study, we used the Illumina MiSeq system. Whereas both the pyrosequencing and Illumina systems yield quantitative data of methylation frequencies at individual consecutive CpG sites, the Illumina Miseq system was found to have higher throughput of data per run. Performance of these two systems was compared by sequencing an *Escherichia coli* isolate [114]. Furthermore, the Illumina system had higher quality reads compared to the Roche system, as shown by the presence of substitutions and indels within the sequenced data. In comparison to the Roche system, the Illumina system had a lower rate of substitution at 0.1 substitutions per base pair, as well as no noticeable indels [114]. One area in which the Roche system excelled was in its read lengths, which averaged at approximately 600 base pairs (bps). However, the lower error rate in substitutions and indels produced by the Illumina system, as well as its higher throughput capabilities made it the more appropriate system to use within the scope of this study [114].

In light of this evidence, the BSAS method with the Illumina Miseq system for NGS was deemed the most appropriate for investigating the objectives outlined in this project.

Chapter 3:

Paternal FA intake in Balb/c mice affects embryo development.

3 Chapter 3: Paternal FA intake in Balb/c mice affects embryo development.

3.1 Abstract

In 1998, Canada fortified white flour with FA to reduce the incidence of NTDs in women of childbearing age. FA fortification of a staple food increased FA intake in the general population, such that there is essentially no folate deficiency. However, 73% and 65% of men and boys, respectively, have a folate status indicative of possible FA intake above the recommended Upper Tolerable intake level. Folate deficiency has been associated with decreased sperm counts and increased sperm DNA damage, but previous data in male mice suggested that FA supplementation may reduce the number of viable embryos. To investigate the effect of paternal FA intake on male fertility and embryo development, we fed male Balb/c mice a FA deficient (0 mg/kg), control (2 mg/kg), or supplemented (6 mg/kg) diet from weaning for 15 weeks, and bred them with FA control-fed female Balb/c mice. Male fertility outcomes such as sperm analyses and pregnancy outcomes were assessed. Embryo development outcomes assessed included viability parameters, placental size and diameter, presence of congenital birth defects and developmental delay, and skeletal ossification in GD16.5 embryos. Sperm velocity was higher in male mice fed the FA deficient diet than in those fed the FA control and FA supplemented diets, but all were still within the normal range. Paternal FA intake was directly proportional to placenta size. Fifty percent of litters from deficient fathers had embryos with congenital anomalies, including omphalocele and gastroschisis, or delayed development, compared to 10% of litters from control fed fathers. Prevalence of ossification was reduced from 100% in the frontal, parietal, and interparietal bones of embryos from fathers fed the FA-control diet to 90, 92, and 92%, respectively in the three bones of embryos from fathers fed the FA-deficient diet. Folate deficiency was associated with higher numbers of embryos affected by congenital anomalies or developmental delay, and by reduced ossification in the skull bones.

Lack of diet dependent embryo size differences among viable embryos suggested that viable embryos not affected by congenital anomalies or developmental delay grew normally. This study emphasizes the critical role of FA in male reproductive health and fitness.

3.2 Introduction

Folate is an essential water-soluble B vitamin required for *de novo* purine, thymidylate, and methionine synthesis [5]. Folate deficiency results in reduced nucleotide synthesis, causing decreased cell proliferation [5]. Impaired nucleotide synthesis can also cause increased mutation rates, as well as genome instability, including DNA double strand breaks and chromosome damage. Additionally, folate deficiency has been associated with aberrant DNA methylation because methionine is used to synthesize AdoMet, the major cellular methyl donor [11].

Since nucleotide synthesis is required for cell proliferation, rapidly dividing cells are folate dependent. Folate status is therefore important for periods of rapid growth, such as in cancer and embryogenesis. As such, maintenance of folate metabolism is critical in pregnancy for fetal development and folate deficiency has been associated with increased risk for neural tube defects (NTDs), such as spina bifida, in which the neural tube fails to close [12].

As a result of increased public health awareness of the importance of periconceptional FA supplementation, Health Canada recommends that women of childbearing age eat folate-rich foods and take a daily multi-vitamin supplement containing FA. Women who could become pregnant are advised to take 0.4 mg of supplemental FA per day, and those with an increased risk of delivering an NTD affected baby are advised to take 4 – 5 mg FA per day [10]. Unfortunately, a large proportion of women do not take a FA-containing supplement as recommended. As such, Canada made the fortification of white flour with FA mandatory in 1998 [11]. Mandatory FA fortification has reduced the prevalence of folate deficiency to less than 1% in the general population and importantly, reduced the incidence of NTDs by 45% [12, 13]. The target population of mandatory fortification is women of childbearing age. However, since white flour and “enriched” grain products are a staple food consumed by the general population, non-target

groups including men and boys also demonstrate increased FA intake. Men may benefit from increased FA intake because folate deficiency has been associated with male sub-fertility, including decreased sperm counts, increased sperm DNA damage, and increased frequency of aneuploid sperm [25–27]. During the early stages of spermatogenesis, germ cells are successively undergoing mitotic and meiotic divisions, both of which require efficient nucleotide synthesis and the maintenance of methylation profiles. Thus, folate metabolism is important for male germ cells as they are constantly replicating, dividing, and differentiating.

We previously found that FA deficiency in mice resulted in decreased sperm numbers, increased germline chromatin damage, and increased sperm DNA mutation frequency [50]. In 2001, Wallock et al. found that low seminal plasma folate concentrations were associated with low sperm density and low sperm count in men [25]. Low seminal plasma folate was also associated with increased sperm DNA damage and with sperm aneuploidy in healthy men [26,27]. Administration of folinic acid (5-formylTHF) to sub-fertile men in a therapeutic trial in the 1990s resulted in increased sperm numbers and motility [28]. In a randomized trial, Wong et al. showed that FA and zinc sulfate supplementation in men with subfertility resulted in a 74% increase in their sperm concentration [29]. Together, the data support a role for folate in male fertility.

While potential benefits for men may be intuitive, there may also be unanticipated risks due to over-consumption of FA. Data from the Canadian Health Measures Survey indicate that 65-73% of boys and men have a folate status indicative of possible FA intakes above the Upper Tolerable intake level (UL) [21]. Furthermore, adverse effects of FA fortification have been hypothesized, such as the masking of Vitamin B12 deficiency, as well as an increased risk for the recurrence of colorectal cancer [115].

We previously observed a negative effect of FA deficiency on fertility rates in male Balb/c mice (Table 1.1) [MacFarlane et al., unpublished data]. Male mice fed a FA deficient diet had ~33% fewer litters compared to males fed the control diet. However, no significant differences in litter size or number of implantation sites were observed. There was a significant increase in the number of resorptions in female mice bred to male mice fed the FA deficient diet, compared to those fed the FA control mice.

Of note, male Balb/c mice fed a FA supplemented diet also demonstrated a significant 30% reduction in the number of litters born [MacFarlane et al., unpublished data]. Females mated with supplemented male mice also demonstrated an increase in the number of uterine implantation sites, but many or all of the embryos in many litters were resorbed. Interestingly, these differences in fertility occurred despite having normal sperm numbers, mutation frequency and chromosome fragmentation index [47]. The data indicated that while there was an increase in the number of fertilization events, the embryos were not viable, suggesting a reduction in embryonic viability at the post-implantation stage due to paternal FA intake.

3.3 Methods

3.3.1 Animal Study Model

All mice were cared for in accordance with the Guidelines of the Canadian Council on Animal Care, described in the CACC Guide to the Care and Use of Experimental Animals and the study was approved by the Health Canada Animal Care Committee [116]. Mice were housed in plastic, HEPA-filtered cages and maintained at 22 ± 2°C and a room humidity of a minimum of 40% and a maximum of 60%, with a 12-h-light/-dark cycle.

Diets: Male mice (F0) were fed one of the following FA-defined diets containing three different levels of AIN-93G FA [86]: Deficient (0 mg/kg), Control (2 mg/kg), and Supplemented (6 mg/kg) (Figure 2.1). Male mice were weaned at 3 weeks of age to their diets and fed for 15 weeks (Figure 2.1), allowing for the completion of two full rounds of spermatogenesis. They were bred sequentially to two sets of female mice (F0) that had been fed the control diet for four weeks.

The first females were used to examine implantation, resorptions, and embryo development at gestational day 16.5 (GD16.5). The second set of F0 females were allowed to deliver F1 pups to examine endpoints such as implantation sites, resorption rates, litter size and rates, pup weights, sex ratios, etc.

Sixty-six male Balb/c mice (3 weeks old) were purchased from Charles River Laboratories (Saint Constant, QC). They were weaned to one of three FA-defined diets: Deficient, AIN-93G 0 mg/kg FA (n = 23), Control, AIN-93G 2 mg/kg FA (n = 20), or Supplemented, AIN-93G 6 mg/kg FA (n = 24) [86]. One hundred and eleven female Balb/c mice (6 weeks old) were purchased from Charles River Laboratories and fed the control AIN-93G 2mg/kg FA diet for four weeks until breeding. Male mice were pair-housed until breeding at ~15

weeks of age, at which point they were single housed for breeding over a two week period. At 15 weeks, a single female was placed with a single male overnight for the embryo analysis at GD16.5. In the morning after breeding (PCD 0.5), vaginal plugs were assessed and the females were weighed and placed back in their original cage. This was repeated with the same male mouse until a plug was observed the next morning, up to a maximum of three attempts. At 16 weeks, a single male was placed with a second single female overnight to produce the F1 generation. A weight gain of approximately two grams in the plug positive females, taken ten days later, was indicative of a successful pregnancy. Throughout pregnancy, females were fed the control AIN-93G 2 mg/kg FA diet. At 16.5 days post-coitum (PCD), the first set of pregnant females (n=45) were killed under isoflurane for embryo analyses. The second set of pregnant females (n=90) remained on the control AIN-93G 2 mg/kg FA diet throughout pregnancy and lactation to produce the F1 generation. The F0 males were killed under isoflurane post-breeding at 21 weeks of age.

3.3.2 Parameters of Male Fertility and Embryo Development

As a means of assessing male fertility in the F0 generation, we measured testes weight, conducted sperm analyses, and analyzed pregnancy outcomes.

3.3.2.1 Sperm Motility

The left and right cauda were dissected from F0 male mice, at which point, the left cauda was sent for sperm motility analyses and the right cauda was frozen. Sperm motility and morphology on the left cauda were analyzed immediately post-dissection by Francesco Marchetti using the integrated visual optical system (IVOS) motility analyzer (Hamilton-Thorne Research, Inc.). The control droplet had an equivalent volume of DMSO. An aliquot was transferred into each of two compartments on a glass cannula slide for computer-assisted sperm analysis (CASA)

using the IVOS. Sperm velocity was assessed by the percentage of motile sperm cells, smoothed path velocity (VAP), straight line velocity (VSL), track velocity (VCL), and straightness [98]. Sperm counts, as well as elongation and size (μm^2), were also evaluated using the IVOS system, as was the amplitude of lateral head displacement (ALH; μm).

3.3.2.2 GD16.5 Pregnancy Outcomes

The number of uterine implantation sites, CL, live embryos and resorptions in female mice killed at GD16.5 was evaluated. Litter size (number of embryos per female), pregnancy rates (percent of pregnancies per diet), and CL:litter size ratios were calculated, as well as resorptions and resorption rates. Resorptions were identified visually at necropsy.

Embryo weights and crown-rump length (CRL) were measured as an indicator of growth and developmental/gestational age [117]. The crown-rump length was determined by measuring the distance from the top of the head to the bottom of the rump [117]. Placental weight and diameter for each GD16.6 embryo was also measured.

3.3.2.3 Live Birth Pregnancy Outcomes

Pregnancy rate, litter size at post-natal day (PND) 0, number of live pups at PND 2 (neonatal survival), and number of dead pups at PND 2 were evaluated. Weight and crown-rump length (CRL) of live pups was measured on PND 0 as an indicator of development. The crown-rump length was determined by measuring the distance from the top of the head to the bottom of the rump.

3.3.2.4 Congenital Birth Defects

GD16.5 embryos were examined visually to determine the presence of major congenital birth defects and developmental delay. We examined the embryos for the presence of neural tube

defects such as spina bifida and anencephaly/exencephaly. Developmental delay were determined visually and confirmed by determining whether embryo weight or crown-rump length were 2 standard deviations below the mean of the control group. Additionally, the mouth was examined for deformities such as cleft palate with and without cleft lip, which is an opening in the mouth palate or lip. The limbs, digits, and tail were analyzed for any deformities or missing structures.

3.3.2.5 Sex ratios

Embryo tails were snipped for sex genotyping at the time of necropsy at GD16.5. Sex genotyping was carried out by Nathalie Behan, following the protocol by Clapcote and Roder [118]. Tail DNA was extracted the High Pure PCR Template Preparation Kit (Roche Applied Science, 2012). PCR was carried out using the 5PRIME 2.5X Master Mix, 10 μM forward and reverse primer, and 200 ng DNA, up to a total volume of 25 μL with DNase free water. The forward (5′-CTGAAGCTTTTGGCTTTGAG-3′) and reverse (5′-CCACTGCCAAATTCTTTGG-3′) primers target the sex-specific gene *Jarid1c* in both the X and Y chromosome, as they are homologous [118]. Amplified product was analyzed on a 2% agarose gel, where two bands (331 and 302bp) were indicative of a male embryo and one band (331bp) was indicative of a female embryo.

3.3.2.6 Skeletal Staining

GD16.5 embryos were fixed in 80% ethanol until skeletal staining. On day one of skeletal staining, the embryos were immersed in dH_20 for 24 hours. The skin and viscera were removed and the embryos were fixed in 95% ethanol for another 24 hours. The embryos were then stained with Alcian blue solution (Alcian blue, 95% Ethanol, and Glacial acetic acid), which stains the cartilage, for 24 hours. They were then washed in 95% ethanol for 24 hours and transferred to a

2% KOH solution for 24 hours, during which time the muscles were dissolved. The embryos were stained in 1% KOH containing 0.015% Alizarin red S solution (for the calcified bones) for 48 hours. Embryos were washed in a 0.5% KOH solution for 24 hours. The skeleton was immersed for 24 hours in a solution of two parts 70% EtOH, two parts glycerin, and one part benzyl alcohol. The final stained skeleton was stored in a 1:1 solution of glycerol and 70% EtOH. While troubleshooting this method, an embryo was stained without removing the skin and viscera and the stain was unsuccessful. As such, this serves as a control that staining of parts of the embryo other than cartilage and bone did not occur.

Post-staining, the skeletons were examined using the SMZ 1500 Zoom Stereomicroscope. The ratio of ossification in the mandible, as well as ossified parts of the humerus, radius, ulna, femur, tibia, and fibula were measured using an eyepiece micrometer (Figure 3.1). The ratio of ossification in the mandible was measured by calculating the ratio of ossified portion to that of the un-ossified portion. Pictures were taken using the Nikon D5000 SLR camera. The presence or absence of skull, vertebral, and pelvic bones were recorded. Prevalence of ossification was determined by calculating the number of ossified bones present divided by the total number of events for each bone for each diet group.

Figure 3.1: Skeletal analyses were carried out on GD16.5 embryos stained with Alizarin Red and Alcian Blue. Ossification ratio in the (A) mandible and long bones such as the (B) tibia, (C) fibula, (D) femur, (E) radius, (F) ulna, and (G) humerus, were measured. Ossification ratio in the mandible was measured by calculating the ratio of the length of the ossified portion to the total length of the mandible. Length of ossification was measured in the long bones.

3.3.3 Statistical Analyses

Differences in means among diet groups for body weight, sperm analyses, fertility outcomes, embryo and live pup viability parameters, placental weight and diameter, and ossification measurements were tested for significance using a one-way ANOVA. These analyses were done in Sigmaplot for Windows, version 12.5 (Systat Software, Inc.). Chi-square analyses were performed on pregnancy rates, sex ratios, and prevalence of congenital anomalies and developmental delay, and ossified bones. Data are presented as mean ± standard error of the mean (SEM) or percentage. Differences among the diet groups for ossification prevalence were done using a Fisher's exact test in R.

3.4 Results

3.4.1 Male Body Weights

Mice fed the FA supplemented diet had a smaller overall weight gain (10.2 ± 0.5 g) from age 4 weeks to 20 weeks than those fed the FA control diet (11.9 ± 0.3 g) ($p = 0.01$) (Table 3.1). There was no effect of FA deficiency on body weight compared to mice fed the control diet. Testes weight in male mice fed the FA supplemented diet was 3.8 g less than those in male mice fed the FA control diet. However, testes to body weight ratio was not different among the diet groups.

Table 3.1: F0 male body and testes weights. Body weights were measured at 4 and 20 weeks of age and overall weight gain from 4 to 20 weeks of age in F0 male mice fed a FA deficient (0 mg/kg), control (2 mg/kg), or supplemented (6 mg/kg) diet. Testes weight was measured at 20 weeks and ratio of testes to body weight was calculated. Values within a column that do not share a letter are statistically different. All values are expressed as mean ± SEM.

			Body Weight (g)				Testes Weight (mg) (20 Weeks)		Testes Weight/Body Weight (20 Weeks) x 1000	
			Age		Weight gain over 16 weeks					
			4 Weeks	20 Weeks						
Diet	mg/kg FA	n	Mean ± SEM	Mean ± SEM	Mean ± SEM	***P***[1]	Mean ± SEM	***P***[1]	Mean ± SEM	***P***[1]
Deficient	0	23	17.4 ± 0.3	28.6 ± 0.4	11.2 ± 0.4[a]		95.1 ± 0.3[a]		3.3 ± 0.01	
Control	2	20	17.3 ± 0.2	29.3 ± 0.4	11.9 ± 0.3[a]	< 0.05	96.3 ± 1.0[a]	0.05	3.3 ± 0.04	ns
Supplemented	6	24	17.6 ± 0.4	27.8 ± 0.4	10.2 ± 0.5[b]		92.1 ± 1.3[b]		3.3 ± 0.07	

[1] One-way ANOVA Tukey's HSD post-hoc analysis

3.4.2 Sperm Analyses

Sperm from F0 male mice fed the FA deficient had a 6.8% faster VAP ($p = 0.0161$), a 7.8% faster VSL ($p = 0.03$), and a 6.3% faster VCL ($p = 0.009$) than sperm in male mice fed the FA control diet (Table 3.2). Sperm from males fed the FA supplemented diet did not differ from the control group. No differences were observed in cauda sperm numbers, STR, ALH, linearity, elongation, or size among the diet groups.

Table 3.2: Outcomes of sperm motility assay. Sperm motility was measured in sperm cauda of male mice fed a FA deficient (0 mg/kg), control (2 mg/kg), or supplemented (6 mg/kg) diet. Values within a row that do not share a letter are statistically different. All values are expressed as mean ± SEM.

	Diet (mg FA/kg diet)			***P*** [1]
	Deficient (0) n=23	Control (2) n=20	Supplemented (6) n=24	
Smoothed path velocity (VAP), µm/s	128.8[a]	120.6[b]	123.4[a,b]	0.016
Straight line velocity (VSL), µm/s	102.2[a]	94.8[b]	97.7[a,b]	0.03
VSL/VAP (straightness)	0.8	0.8	0.8	ns
Track velocity (VCL), µm/s	239.0[a]	224.9[b]	228.8[b]	0.009
Amplitude of lateral head displacement, µm	11.4	11.1	11.0	ns
Beat cross frequency, Hz	40.0	39.7	39.7	ns
Straightness	73.3	72.1	72.7	ns
Linearity (VSL/VCL)	41.8	41.2	41.7	ns
Elongation (head shape)	47.2	49.1	47.6	ns
Size, μm^2	40.4	38.4	39.5	ns
Motile (%)	49.9	50.8	51.4	ns

[1]One-way ANOVA Holm-Sidak for multiple comparisons

3.4.3 Pregnancy Outcomes in GD16.5 Embryos

No differences were observed in pregnancy rates, litter size, CL numbers, resorption numbers and rates, uterine implantations, and fertilization events among the diet groups, suggesting that diet did not have an effect on these aspects of male fertility (Table 3.3). Sex ratios were significantly different in the FA deficient and supplemented groups compared to the control group ($p < 0.001$), such that the deficient and supplemented groups had a higher ratio of male to female embryos per litter.

There were no diet-dependent differences observed in embryo weight or crown-rump length of GD16.5 embryos. However, increasing FA intake was associated with increasing placental weight and diameter ($p < 0.05$) (Table 3.4).

Table 3.3: Fertility outcomes in GD16.5 embryos. Embryos are from female mice bred with male mice fed a FA deficient (0 mg/kg), control (2 mg/kg), or supplemented (6 mg/kg) diet for 20 weeks. Values within a row that do not share a letter are statistically different. All values are expressed as mean ± SEM.

	Diet (mg FA/kg diet)			*P*
	Deficient (0) n=8	Control (2) n=9	Supplemented (6) n=8	
Pregnancies (%/diet group)	53.3	60	53.3	ns [1]
Litter Size (no./female)	5.67 ± 0.73	5.00 ± 0.60	5.00 ± 0.54	ns[2]
Corpus Luteum (CL) (no. CL/female)	8.11 ± 1.12	9.40 ± 0.56	9.13 ± 0.61	ns[2]
Resorptions (no. resorptions/female)	1.67 ± 0.24	2.60 ± 0.43	2.75 ± 0.84	ns[2]
Implants (no. resorptions + embryos)	7.33 ± 0.82	7.60 ± 0.58	7.75 ± 0.82	ns[2]
Resorption rate (% embryos resorbed/Implant)	29.98 ± 9.06	34.58 ± 5.36	32.82 ± 6.91	ns[2]
Fertilization events (Implants/CL)	0.91 ± 0.05	0.81 ± 0.04	0.84 ± 0.05	ns[2]
Sex Ratios (mean % male:female / litter)	49:51[a]	30:70[b]	45:55[a]	< 0.001[1]

[1] Chi square analysis

[2] One-way ANOVA Tukey's HSD post-hoc analysis

Table 3.4: GD16.5 embryo viability parameters. Embryos were from female mice bred with male mice fed a FA deficient (0 mg/kg), control (2 mg/kg), or supplemented (6 mg/kg) diet. All values are expressed as mean ± SEM.

	Diet (mg FA/kg diet)			***P***[1]
	Deficient (0) n=8	Control (2) n=9	Supplemented (6) n=8	
Embryo Weight (mg)	446.7 ± 29.2	478.7 ± 34.4	464.9 ± 4.7	ns
Crown-Rump Length (mm)	14.4 ± 0.41	15.0 ± 0.42	14.9 ± 0.08	ns
Placenta Weight (mg)	107.7 ± 2.1[a]	110.6 ± 4.7[a,b]	121.9 ± 3.5[b]	<0.05
Placenta Diameter (mm)	7.7 ± 0.09[a]	7.9 ± 0.10[a,b]	8.1 ± 0.05[b]	<0.05

[1] One-way ANOVA Tukey's HSD post-hoc analysis

3.4.3.1 Pregnancy Outcomes in Live Pups

No differences were observed in pregnancy rates, litter size at PND 0, or number of live and dead pups at 48 hours in pups (Table 3.5).

There were no diet-dependent differences observed in live pup PND 0 weight or crown-rump length (Table 3.6).

Table 3.5: Fertility outcomes in live pups. Pups were from female mice bred male mice fed a FA deficient (0 mg/kg), control (2 mg/kg), or supplemented (6 mg/kg) diet for 20 weeks. All values are expressed as mean ± SEM.

	Diet (mg FA/kg diet)			*P*
	Deficient (0) n=15	Control (2) n=13	Supplemented (6) n=15	
Pregnancies (%/diet)	65.2	65	65.2	ns[1]
Litter Size (mean no./female)	3.93 ± 0.36	4.62 ± 0.46	4.67 ± 0.58	ns[2]
Live pups at 48 hrs (mean no./female)	3.73 ± 0.37	4.39 ± 0.50	3.73 ± 0.53	ns[2]
Dead pups at 48 hrs (mean no./female)	0.20 ± 0.11	0.23 ± 0.12	0.93 ± 0.38	ns[2]

[1] Chi square analysis

[2] One-way ANOVA Tukey's HSD post-hoc analysis

Table 3.6: Live pup viability parameters. Pups were from female mice bred with male mice fed a FA deficient (0 mg/kg), control (2 mg/kg), or supplemented (6 mg/kg) diet. All values are expressed as mean ± SEM.

	Diet (mg FA/kg diet)			***P***[1]
	Deficient (0) n=15	Control (2) n=13	Supplemented (6) n=15	
Pup Weight (g)	1.47 ± 0.05	1.43 ± 0.03	1.40 ± 0.05	ns
Crown-Rump Length (mm)	24.76 ± 0.45	24.56 ± 0.19	24.25 ± 0.25	ns

[1] One-way ANOVA Tukey's HSD post-hoc analysis

3.4.3.2 Congenital Birth Defects

There was a five-fold higher percentage of litters affected by congenital birth defects and developmental delay in the FA deficient group (50%), compared to the FA control group (10%; $p < 0.001$) (Table 3.7). The percentage of litters affected by congenital anomalies and developmental delay was 2.5-fold higher in the FA supplemented group (25%) than in the FA control group (10%), but this was not significant.

FA deficient fathers had 27.5% embryos affected by congenital birth defects and developmental delay, compared to 10% of embryos in the FA control group ($p < 0.001$). The percentage of embryos with congenital anomalies and developmental delay in the FA supplemented group was 15% compared to the FA control group (10%) (Table 3.7).

The specific congenital anomalies observed included omphalocele and gastroschisis, rare abdominal abnormalities in which the abdominal contents develop outside of the body, either out of or in a peritoneal sac, respectively (Figure 3.2).

Table 3.7: Proportion of GD16.5 litters and embryos affected by birth defects and development delay. Embryos are from females bred with males fed a FA deficient (0 mg/kg), control (2 mg/kg), or supplemented (6 mg/kg) diet.

Diet	mg/kg FA	n	% **litters** affected by congenital birth defects & developmental delay	***P*** [1]	n	% **embryos** affected by congenital birth defects & developmental delay	***P*** [1]
Deficient	0	8	50	0.001	51	27.4	< 0.001
Control	2	10	10		50	10	
Supplemented	6	8	25	ns	40	15	ns

[1]Chi-square analysis

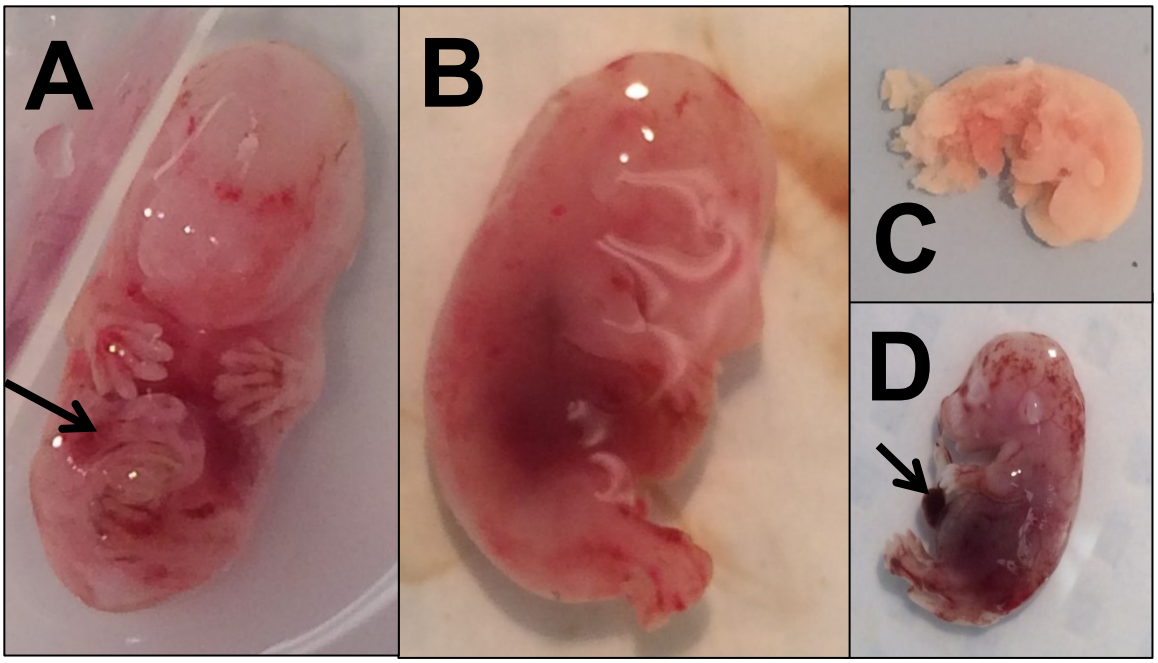

Figure 3.2: Congenital anomalies and developmental delay in GD16.5 embryos. Embryos were observed visually at time of necropsy and embryos were from female mice bred with male mice fed a FA deficient (0 mg/kg) diet. (A) omphalocele (B) developmental delay (C) late resorption (D) gastroschisis. Arrows in (A) and (D) point to the aforementioned anomalies.

3.4.4 Skeletal Analyses

Skeletal anomalies were characterized by abnormal craniofacial structure. FA deficient fathers had 9.8% embryos affected by congenital birth defects and developmental delay, compared to no affected embryos in the FA control and supplemented groups ($p = 0.01$) (Table 3.8).

There were no differences in the ratio of ossification in the mandible, or in the length of the ossified portions of the forelimb bones (humerus, radius, ulna) and the hindlimb bones (femur, tibia, fibula) among the embryos of male mice fed the FA deficient, control, and supplemented diets (Table 3.9). Where bones could not be quantitatively measured, presence or absence of ossification was recorded and percentage of embryos with ossification in each bone was calculated for each FA diet group.

Table 3.8. Proportion of GD16.5 litters and embryos affected by skeletal anomalies. Embryos are from females bred with males fed a folic acid FA deficient (0 mg/kg), control (2 mg/kg), or supplemented (6 mg/kg) diet.

Diet	mg/kg FA	n	% **litters** affected by skeletal anomalies	***P*** [1]	n	% **embryos** affected by skeletal anomalies	***P*** [1]
Deficient	0	8	25		51	9.8	
Control	2	10	0	ns	49	0	0.01
Supplemented	6	8	0		39	0	

[1]Fisher's exact test

Table 3.9: Ossification measurements in the mandible and long bones of GD16.5 embryos. Ossification ratio in the mandible was measured by calculating the ratio of the length of the ossified portion to the total length of the mandible. Length of ossification was measured in the long bones. Embryos are from females bred with male mice fed a FA deficient (0 mg/kg), control (2 mg/kg), or supplemented (6 mg/kg) diet. All values are presented as mean ± SEM.

			Diet (mg FA/kg diet)			***P***[1]
			Deficient (0) n = 47	Control (2) n = 47	Supplemented (6) n = 39	
Measurement of ossified bone (mm)	Mandible (Ossification Ratio)		0.83 ± 0.01	0.84 ± 0.01	0.84 ± 0.01	ns
	Forelimb	Humerus	1.19 ± 0.03	1.22 ± 0.04	1.22 ± 0.03	ns
		Radius	0.97 ± 0.03	1.00 ± 0.04	0.97 ± 0.03	ns
		Ulna	1.18 ± 0.04	1.21 ± 0.05	1.17 ± 0.03	ns
	Hindlimb	Femur	0.98 ± 0.03	1.02 ± 0.03	1.01 ± 0.02	ns
		Tibia	1.00 ± 0.03	1.05 ± 0.04	1.03 ± 0.02	ns
		Fibula	0.93 ± 0.03	0.97 ± 0.04	0.95 ± 0.02	ns

[1]One-way ANOVA Tukey's HSD post-hoc analysis

Significant differences between the diet groups were observed for the prevalence of ossification in the frontal ($p < 0.05$), parietal ($p < 0.05$), and interparietal ($p < 0.05$) bones of the skull (Table 3.10). Ossification of the frontal, parietal, and interparietal bones was present in all embryos of male mice fed the FA control or supplemented diets. However, in embryos of male mice fed the FA deficient diet, prevalence of the frontal, parietal, and interparietal bones was 90, 92, and 92%, respectively. Prevalence of ossification in the basiooccipital, supraoccipital, exooccipital, arcus post atlantis, ossification base of dens, premaxilla, tympanicum, temporal bones, and hyoid was not different among the diet groups. However, the prevalence of ossification in the arcus ant atlantis tended to be different among the diet groups ($p = 0.06$), such that 6.4% embryos of mice fed the FA control diet displayed ossification in this bone, whereas none did in embryos fed the FA deficient or supplemented diet (Table 3.10).

Table 3.10: Proportion of GD16.5 embryos with ossification in the skull bones. Embryos are from female mice bred with male mice fed a FA deficient (0 mg/kg), control (2 mg/kg), or supplemented (6 mg/kg) diet.

		Diet (mg FA/kg diet)			*P*[1]
		Deficient (0) n = 50	Control (2) n = 48	Supplemented (6) n = 39	
% Embryos with bones present	Frontal	90	100	100	< 0.05
	Parietal	92	100	100	< 0.05
	Interparietal	92	100	100	< 0.05
	Basiooccipital	0	0	0	ns
	Supraoccipital	0	6	3	ns
	Exooccipital	92	100	97	ns
	Arcus post atlantis	96	100	100	ns
	Arcus ant atlantis	0	6	0	ns
	Ossification base of dens	2	0	0	ns
	Premaxilla	90	98	97	ns
	Tympanicum	68	64	77	ns
	Temporal Bones	0	2	0	ns
	Hyoid	0	4	0	ns

[1]Fisher's exact test

No differences were observed in the prevalence of ossification of the clavicula, scapula, sternebrae, or ribs among the embryos of male mice fed the FA defined diets (Table 3.11). In the vertebrae, prevalence of ossification was not different among the diet groups in the cervical, thoracic, sacral, or cauda bones. However, there was a difference of ossification in the lumbar vertebrae bones ($P < 0.0001$), such that ossification was present in 78% of the embryos of male mice fed the FA deficient diet, compared to 100% of embryos of male mice fed the FA control or FA supplemented diets (Figure 3.3). No differences were observed in the prevalence of ossification of the ilium, ischium, or pubis among embryos of male mice fed the different FA defined diets (Table 3.11).

Table 3.11: Proportion of GD16.5 embryos with ossification in bones of the pectoral girdle, sternum, ribs, vertebrae, and pelvic girdle. Embryos are from female mice bred with male mice fed a FA deficient (0 mg/kg), control (2 mg/kg), or supplemented (6 mg/kg) diet.

			Diet (mg FA/kg diet)			
			Deficient (0) n = 47	Control (2) n = 48	Supplemented (6) n = 39	***P*** [1]
% Embryos with bones present	Pelvic Girdle	Clavicula	100	100	100	ns
		Scapula	100	100	100	ns
	Sternum	Sternebrae	0	4	0	ns
	Ribs	Ribs	100	100	100	ns
	Vertebrae & Tail	Cervical	98	100	100	ns
		Thoracic	96	100	100	ns
		Lumbar	79	100	100	< 0.0001
		Sacral	2	11	3	ns
		Cauda	0	0	0	ns
	Pelvic Girdle	Ilium	98	100	100	ns
		Ischium	67	65	84	ns
		Pubis	67	65	84	ns

[1]Fisher's exact test

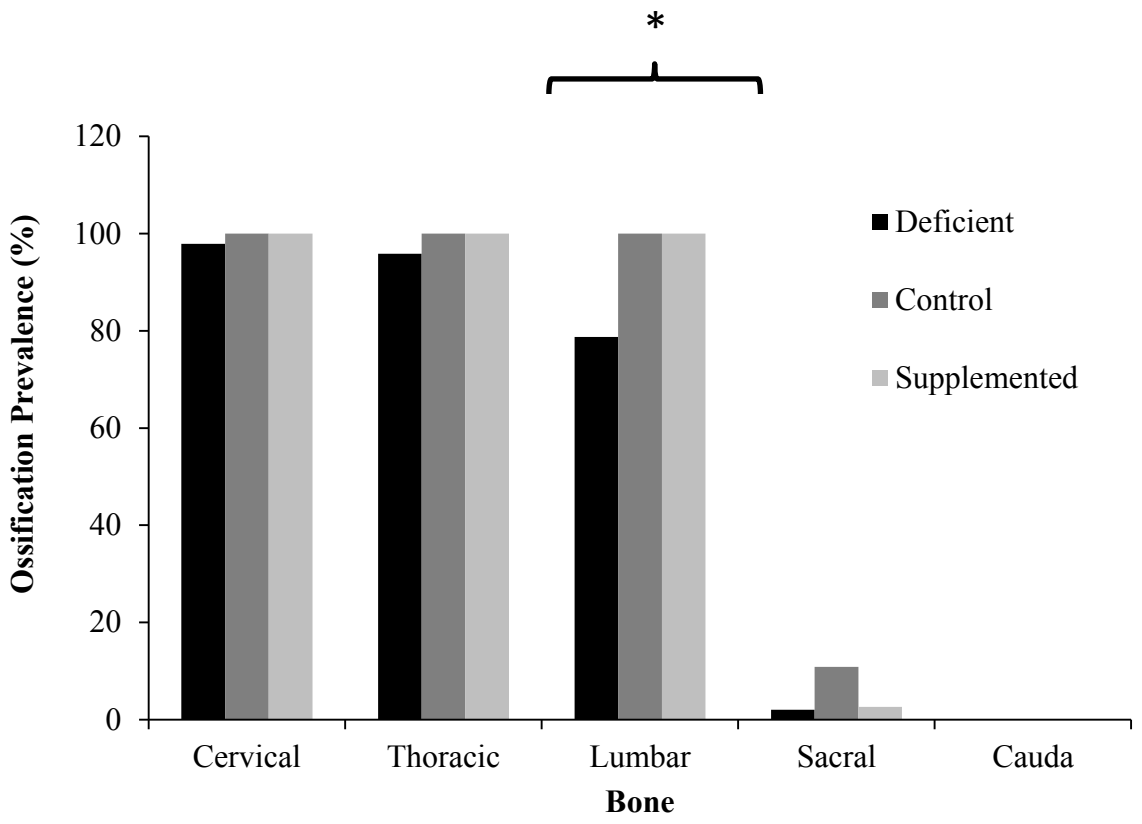

Figure 3.3: Proportion of GD16.5 embryos with ossification in the cervical, thoracic, lumbar, sacral, and cauda bones of the vertebrae and tail. Embryos are from female mice bred with male mice fed a FA deficient (0 mg/kg), control (2 mg/kg), or supplemented (6 mg/kg) diet. *$P < 0.0001$ as assessed by a Fisher's exact test.

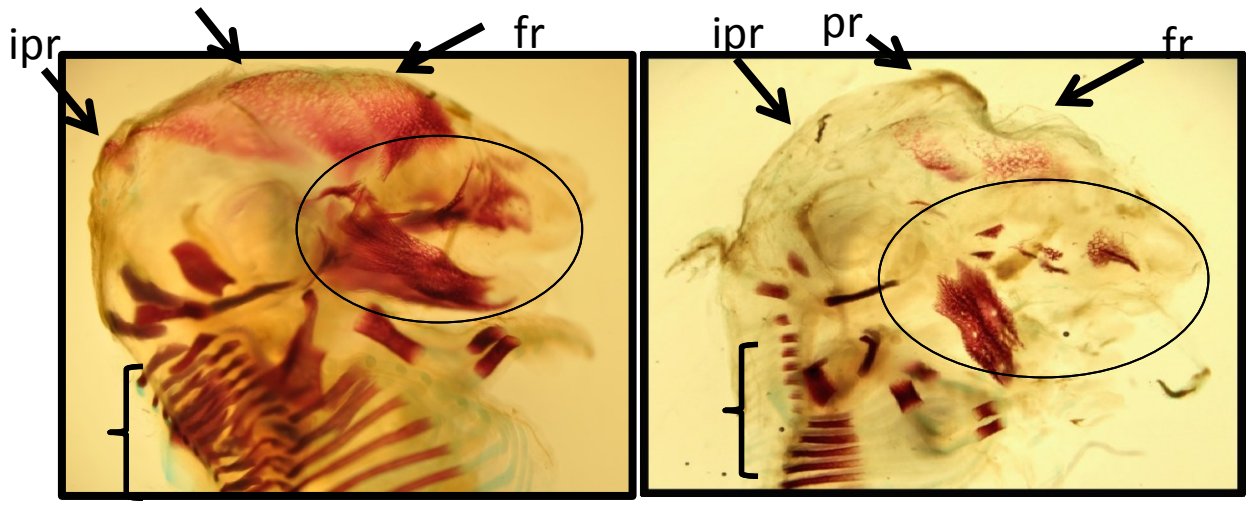

Figure 3.4: Ossification differences found in GD16.5 embryos of male mice fed a FA defined diet. (Right) Reduced ossification found in GD16.5 embryos of a female mouse bred with a male mouse fed a FA deficient (0 mg/kg) diet, in comparison to (left) GD16.5 embryo of a female mouse bred with a male mouse fed a FA control (2 mg/kg) diet. Some notable differences in ossification can be seen the reduced ossification in the vertebrae and skull, including the frontal (fr), parietal (pr), and interparietal (ipr) bones of the embryo on the right. Abnormal craniofacial structure (in circle) is also present in the skeleton depicted on the right.

3.5 Discussion

Results from a previous study indicated altered male fertility and possibly reduced embryo viability in male mice weaned to a FA deficient or supplemented diet [39; MacFarlane et al., unpublished] (Table 1.1). We sought to explore these findings by designing a similar study where male Balb/c mice were fed a FA defined diet (deficient, control, or supplemented) for 15 weeks. The control diet (2 mg/kg) approximates the recommended daily intake of FA for adults (0.4 mg/day), whereas the supplemented diet (6 mg/kg) approximates three times the recommended daily intake of FA for adults. The supplemented diet is relevant as an adult consuming fortified foods and over-the-counter supplements in Canada can reach this level of FA intake.

Similar to its benefits for female fertility, FA has also been associated with beneficial effects on male fertility. Successful treatment of male infertility was achieved in a therapeutic trial led by Bentivoglio et al. in 1993, during which 15 mg/day leucovorin (5-formylTHF) was administered to men over a three month period [18]. Administration of leucovorin resulted in improved sperm numbers and sperm motility [18]. Studies have also shown that low seminal plasma folate concentration was associated with low sperm density and low sperm count in men, as well as increased sperm DNA damage [15, 16]. Folate deficiency has also been associated with sperm aneuploidy in healthy men [17]. FA deficiency in male mice was previously associated with decreased sperm numbers, as well as increased sperm DNA damage and increased germline chromatin damage [39].

In contrast to these studies, however, we found no FA dependent differences in sperm numbers. There was a modest but significant increase in smoothed path (VAP), straight line (VSL), and track (VCL) velocity in sperm from male mice fed the FA deficient diet compared to

those fed the control diet. However, the ratio of the VSL to VAP velocities, which gives the straightness (STR) of the path of the sperm head was not different among the three diet groups, nor was amplitude of lateral head displacement (ALH). The STR measurement, along with the VAP and ALH are those that are used to predict its ability to fertilize an oocyte. The ALH measures the amplitude of the beating wave of the sperm head, determining the degree to which the sperm thrusts forward against a surface, such as the mucus of the cervix, the first surface the sperm head will encounter post-ejaculation. Consequently, larger amplitudes would propel the sperm further than smaller ones, and with a greater force across such a surface [55, 56]. A kinematic definition established for spermatozoa showing ideal mucus penetrating characteristics include a VAP of greater than 25 um/s, a straightness of greater than 0.8, and an ALH of greater than 2.5 um, where all three characteristics must be met [101]. Sperm velocity is indicative of how fast the sperm moves and while there was a difference in male mice fed the FA deficient diet compared to those fed the FA control diet, all VAP, ALH, and STR values for all diet groups meet the criteria for successful sperm penetration of cervical mucus [101]. Therefore, the observed differences in VAP and VSL may not have physiological consequences.

Confirming the observations on sperm counts and motility, fertility outcomes from male mice fed the FA deficient diet did not differ in pregnancy rates, litter size, CL numbers, resorption numbers and rates, implants, and fertilization events from those fed the FA control diet. This is in contrast to results of the previous study, where negative fertility outcomes, such as reduced pregnancy rates, resorption numbers, and resorption rates were observed in the FA deficient group, compared to the FA control group [MacFarlane et al., unpublished]. Furthermore, we did not find differences in fertility outcomes between male mice fed the FA supplemented diet compared to those fed the FA control diet. Interestingly, this is also in contrast

to the previous study, which found that FA supplementation had a negative effect on litter rates similar to that of FA deficiency. FA supplementation resulted in decreased litter rates by 30%, and significantly higher resorption numbers and rates compared to those found in male mice from the FA control group. These differences occurred despite there being an increase in implantation sites within the group of male mice fed the FA supplemented diet. One reason as to why our results did not replicate those of the previous study may be due to the decreased pregnancy rates observed in the male mice fed the FA control diet in our study, which was 60%. In comparison, we have previously observed litter rates of approximately 85 – 90% across multiple studies and multiple generations [MacFarlane et al., unpublished]. The reason for the reduction in pregnancy rate between the control groups in the two studies is unknown. However, we speculate that ongoing renovations at the time of breeding may have interfered with the hormone regulation and thus affected the pregnancy rates.

Our previous study suggested that embryo viability was affected by paternal FA intake. Similarly, a study by Lambrot et. al showed that lifelong folate deficiency in male mice, from conception throughout life, was associated with embryo congenital defects [119]. Conversely, while maternal FA supplementation has been associated with embryonic delay and embryonic heart defects in mice [44], the effect of paternal FA supplementation on embryo development had not previously been assessed. However, other studies have examined the effect of other nutrients in the paternal diet on offspring development. A chronic high-fat paternal diet in rats has been shown to produce female offspring with early-onset impaired insulin secretion and glucose intolerance [120]. A low-protein paternal diet in mice negatively impacted cardiovascular and metabolic homeostasis in offspring [121].

To elucidate the effect of paternal FA deficiency and supplementation on embryo development and viability, we analyzed GD16.5 embryos from male mice fed a FA defined diet (deficient, control, supplemented) for gross anatomical abnormalities, developmental delay, and altered skeletal development. We found a 5-fold increase in litters affected by congenital anomalies or developmental delay in the FA deficient group compared to the FA control group and a more modest 2.5 fold increase in litters affected by congenital anomalies and developmental delay in the FA supplemented group compared to the FA control group, although the latter was significant. We mostly observed abdominal wall malformations, such as omphalocele and gastroschisis, in addition to cases of developmental delay. Our data indicate that male folate deficiency is associated with increased risk for congenital anomalies and developmental delay in offspring. The effect of FA supplementation on embryo viability needs to be assessed further to determine whether it consistently increases risk for congenital anomalies in offspring.

These results are comparable to those found by Lambrot et al., who found that 27% of GD18.5 embryos from male mice fed a FA deficient diet throughout life (from conception throughout life), at GD18.5, had gross anatomical abnormalities compared to 3% of embryos from FA sufficient (2 mg/kg) fathers [119]. They observed craniofacial, spinal, limb, and dorsal malformations, as well as craniofacial abnormalities such as hydrocephalus, reduced ossification in the skull, and delays in digit development. In our study, we found that 9.8% of FA deficient embryos were affected by skeletal anomalies, in comparison to no embryos within the FA control and FA supplemented litters. Embryo skeletons that had prominent craniofacial abnormalities, as shown in Figure 3.4, were considered to have skeletal anomalies. We conducted quantitative skeletal analysis of the skulls, bodies, and limbs of the embryos, and

while we saw some cases of delayed limb ossification in the FA deficient group, quantitative measurements of forelimb and hind limb bones did not show significant differences among the three paternal diet groups.

We found that in the skulls of embryos from males fed the FA deficient diet, the prevalence of ossification in the frontal, parietal, and interparietal were less than that of the FA control diet. This is similar to what was found in a study by Lambrot et al., who observed that some embryos of male mice exposed to a FA deficient diet throughout life had reduced ossification in the skull [119]. In the vertebrae, we saw evidence for reduced ossification in the FA deficient embryos, as there was increasingly reduced ossification moving down from the cervical bones to the lumbar bones, compared to the full presence of ossification in the vertebrae of embryos from male mice fed the FA control and supplemented diets. Interestingly, when looking at the sacral bones, which are normally unossified at this stage, embryos of male mice fed FA control had a significantly higher prevalence of ossification than in embryos of male mice fed either the FA deficient or FA supplemented diets. Other differences were observed in ossification of the tympanicum, ischium, and pubis bones, which were significantly more ossified in the FA supplemented embryos than either the FA deficient or FA control embryos. Lambrot et al. also observed delayed ossification in the sternebrae of GD18.5 embryos from FA deficient fathers [119]. While we found that there was a trend for reduced ossification in the sternebrae in the FA deficient and FA supplemented groups compared to the FA control group, differences were not significant. Together the data indicate that overall, embryos from male mice fed the FA deficient diet had delayed skeletal development.

It is important to take note of key differences between this study and the study conducted by Lambrot et al. [119], which may account for the slight differences in results obtained between

the two studies. In the Lambrot study, male mice were exposed to their experimental diets from conception and throughout life, whereas in the current study, male mice were exposed to the FA experimental diets only during the post-weaning period. Additionally, embryos in the Lambrot study were analyzed at GD18.5, whereas embryos in this study were analyzed at GD16.5.

Two possible mechanisms by which paternal diet, specifically paternal FA intake, might affect offspring health and development include genotoxicity or altered methylation capacity. Due to its role in *de novo* nucleotide synthesis, altered folate metabolism can cause DNA damage and genome instability. Folate deficiency can result in impaired purine synthesis, which can be genotoxic, as well as the misincorporation of uracil into DNA if *de novo* thymidylate synthesis is impaired, which can lead to DNA strand breaks, and thus chromosome instability [2]. A recent study by MacFarlane et al. [122], showed that folate deficiency is genotoxic in red blood cells, and Swayne et al. demonstrated that folate deficiency causes male germline chromatin damage and higher sperm mutation rates [39]. However, the absolute frequency of sperm mutation is low and to observe an effect of folate deficiency on a mutation-dependent phenotype would require the examination of hundreds, if not thousands, of mice. In contrast, the congenital anomalies and developmental delay that we observed was highly penetrant affecting 50% of litters and over 25% of embryos derived from folate deficient fathers. Therefore, we believe that the more plausible mechanism by which paternal diet affects offspring health is via dysregulated methylation of the sperm methylome.

Folate is important in the formation of AdoMet and thus the maintenance of cellular methylation capacity. Two separate waves of demethylation and remethylation occur as part of the epigenetic programming process, through which gene expression and methylation changes can occur. During the first wave, genome-wide DNA methylation is removed post-fertilization

when both maternal and paternal DNA undergo active and passive demethylation, respectively, in order to erase gametic epigenetic marks [68–70]. The first wave of demethylation, however, does not include erasure of gametic epigenetic marks of imprinted genes [68,71,72]. The second wave of demethylation occurs in the epiblast, post-implantation. During this wave, a subset of cells that will give rise to PGCs will undergo demethylation, at which point epigenetic marks, including those on imprinted genes, are erased in order to ensure totipotency in the offspring [73,123]. Recently, however, the belief that genomic patterns are being completely erased is being challenged, as evidence of genes escaping epigenetic reprogramming in the PGCs is being presented [80]. A study by Hammoud et al., showed that humans are able to retain epigenetic information related to genes involved in embryo development from one generation to the next [80]. Other studies have shown that altered paternal FA intake changed the sperm epigenome and reduced hepatic DNA methylation in offspring, where genes that have been implicated in development were differentially methylated between FA deficient and FA control male mice [11,119]. Our data also indicate that FA-dependent changes in the sperm methylome can be retained in the developing embryo. As such, this epigenetic inheritance could be a mechanism by which paternal diet is affecting the sperm methylome, and consequently, offspring development and health. Further investigation into this mechanism will be discussed in Chapter 4, where trans-generational methylation status is analyzed in F3 offspring of male mice fed a FA defined diet.

The placenta is a key organ required for the development of the embryo. It is required for the transfer of nutrients and genetic information between the fetus and the mother. We found that with increasing paternal FA intake, placental size increased, as measured by placental weight and diameter. *In vivo* quantification of placental diameter predicted a diameter of 7.8 mm for a

placenta of GD16.5 [117], which is consistent with our measurements, where the mean placenta diameters were 7.7 mm and 7.9 mm within the paternal FA deficient and FA control groups, respectively. The paternal FA supplemented group, however, had a mean placenta diameter of 8.1 mm. While a larger placenta may be considered beneficial for more efficient transfer of nutrients between the fetus and the mother, there may also be some unforeseen risks associated with a larger placenta. A long-term Norwegian population study found that a larger placenta was associated with increased risk for cardiovascular disease mortality in the child [124]. Another study in Finland found that a larger placenta was associated with decreased mental health, specifically with increased attention deficit hyperactivity disorder, in boys, ages 8 – 16 years old [125].

Whereas maternal contribution to placental development post-implantation is known, it is important to acknowledge that placental development is also dependent, in part, on the epigenetic information passed on by the father [126]. Many imprinted genes that are paternally expressed in the placenta act as embryo growth promoters. One such example is insulin growth-like factor 2 (*Igf2*), which leads to abnormal growth in mice when inappropriately expressed [127]. A recent study has shown that paternal FA deficiency resulted in reduced placental folate content, suggesting that paternal FA intake is important in the regulation of placental folate metabolism [128]. Altered gene expression and DNA methylation was also detected in the placenta of embryos from FA deficient sires, although no overlap of genes was detected between the two [119]. Together, this evidence highlights the importance of paternal FA intake in placental development and regulation, as well as its effect on offspring via both maternal and paternal contributions and supports our data, which shows that placental size and diameter increased with increasing paternal FA intake.

Overall, we found that altered FA intake had no effect on many male fertility endpoints. One exception was sperm motility, although all diet groups had sperm parameters within the normal range. Embryo congenital anomalies and development were found to be dependent on paternal FA intake, where male folate deficiency was associated with an increased number of embryos affected by congenital anomalies, developmental delay, and reduced skeletal ossification. No diet dependent embryo size differences were observed, suggesting that viable embryos not affected by a congenital anomaly grew normally. Increasing FA intake also resulted in increased placental size and diameter. Together the data suggest that FA intake is an important factor for appropriate placenta and embryo growth and development. We propose that changes in the sperm methylome rather than increased genotoxicity is the likely biological mechanism underlying abnormal embryonic and placenta development. This study highlights the importance of FA intake for male reproductive health and reproductive fitness.

Chapter 4:

Investigating FA diet-induced methylation dependent transgenerational gene expression changes through the male line

4 Chapter 4: Investigating FA diet-induced methylation dependent transgenerational gene expression changes through the male line

4.1 Abstract

Folate is a water soluble B vitamin required for the synthesis of *de novo* methionine, which is converted to S-adenosylmethionine (AdoMet), the major methyl donor in methylation reactions. Altering paternal FA intake may alter methylation status of sperm DNA, which may be stably transmitted to the offspring. Paternal transmission of epigenetic information can occur via DNA methylation, histone modifications, or methylation of miRNAs. Altered hepatic gene expression profiles were identified by microarray analysis in the F3 descendants of male mice exposed to FA deficient, sufficient, or supplemented diets either in early development (*in utero* and during lactation) or post-weaning. A pathway analysis identified genes with differential expression belonging to the choline metabolism pathway and those associated with neural tube defects. In this study, we identified candidate CpG islands in the promoter regions of the candidate genes *Chdh, Chka, Cish, Mapk8, Mthfr,* and *Pdgfc* that had differential hepatic gene expression, and used BiSulfite Amplicon Sequencing to assess the methylation status of their respective CpG islands. We found no differential methylation among the diet groups in the sequenced CpG promoter regions of *Mthfr, Pdgfc,* and *Cish.* In the *Chka* gene, the grandpaternal FA deficient diet in early development resulted in increased overall methylation compared to the FA control diet, suggesting that DNA methylation of this region may be regulating gene expression changes and mediating its inheritance to future generations. This study emphasizes the need to examine how paternal FA diet induced changes are being inherited and affecting future generations.

4.2 Introduction

Folate is an essential water-soluble B vitamin required for *de novo* nucleotide and methionine synthesis. In folate metabolism, Hcy is remethylated to form methionine, using the methyl group of 5-methyl-THF. Methionine is then converted to AdoMet, which is the major methyl donor in cellular methylation reactions. DNA methylation of both DNA and histones is important for the regulation of gene expression and the maintenance of genome stability. Methylation status of DNA can regulate gene expression, as it can interfere with the binding of transcription factors to DNA, and/or alter chromatin structure, affecting transcription [4]. Global DNA hypomethylation can result in increased mutation rates and genomic instability, through chromosomal instability or aneuploidy [5]. Since folate is essential in the maintenance of cellular methylation potential, consequently, it can affect genome methylation status, gene expression, and genome stability.

Altering paternal FA intake could alter methylation status of sperm DNA, which may be stably transmitted to the offspring. Proper methylation of both DNA and histones is critical in spermatogenesis. Aberrant folate metabolism can result in altered methionine synthesis, which can in turn affect cellular methylation capacity, the methylation profile of sperm chromatin, and consequently, sperm differentiation. Altered sperm DNA methylation has been shown to affect the differentiation of spermatogonia into spermatocytes in neonatal mice exposed to a hypomethylating agent [48]. Hypomethylated sperm DNA may also affect the embryo that is derived from the affected sperm. Changes in methylation profiles of the sperm may result in trans-generational and heritable epigenetic modifications in the descendants of the exposed males, as DNA methylation can be mitotically and meiotically stable, allowing for the

transmission of DNA methylation patterns among dividing cells of a tissue or potentially trans-generationally among organisms through the germline.

The effect of altered MTHFR expression has also highlighted the importance of methylation capacity on spermatogenesis and male fertility. Impaired MTHFR expression may result in impaired spermatogenesis and male infertility, likely due to its role in folate metabolism. The *MTHFR* gene irreversibly reduces 5,10-methylene-THF to 5-methyl-THF, which is used in the re-methylation of homocysteine. As a result, *MTHFR* is important for methionine synthesis and the maintenance of methylation capacity. Knockout of the *Mthfr* gene in male mice has been shown to cause decreased sperm counts [52], as well as impaired spermatogenesis and infertility [53]. Hypermethylation of the *MTHFR* gene promoter region in both testes and sperm has been associated with non-obstructive azoospermia and idiopathic male infertility [54,55].

Epigenetics is the study of changes in gene expression in the absence of changes to the DNA sequence [64]. Paternal transmission of epigenetic information can occur via DNA methylation, histone modifications, or methylation of miRNAs. Although two separate rounds of DNA demethylation and remethylation occur during development that can potentially alter gene expression within the male germline, environmentally acquired information, such as from dietary changes, may still be transmitted trans-generationally [76,77]. Studies have recently shown the presence of zygotic resistance to epigenetic reprogramming at loci other than imprinted genes, in contrast to what had been previously thought [129–131].

Histone modifications may also be a mechanism by which epigenetic information is transmitted. When histones are replaced by protamines during spermatogenesis, approximately 1% and 10% of the original histone content remain intact in mice and human sperm nuclei,

respectively [78,79]. Studies by Hammoud et al have shown that in humans, the retained histones are those related to genes of embryo development, as well as transcription factors, imprinted genes, and miRNAs [46,80]. Similarly, in response to folate deficiency, differential DNA methylation was found in genes related to placenta and early embryonic development [119]. Recently, miRNAs have also been found to be regulators of DNA methylation [81], where certain miRNAs are predicted to be down-regulators of DNMT3A, a stimulator of *de novo* methylation [82,83]. Consequently, changes in miRNA can affect epigenetic markers such as DNA and histone methylation, which can be trans-generationally inherited.

In chapter 3, we demonstrated that paternal FA intake had an impact on embryo viability, where FA deficiency resulted in 5-fold more embryos affected by congenital anomalies and developmental delay compared to the FA control group. In collaboration with the lab of Dr. Carole Yauk, our lab has also observed altered hepatic gene expression profiles by microarray analysis in the F3 descendants of male mice exposed to FA deficient, sufficient, or supplemented diets (Table 1.2) either in early development (*in utero* and during lactation) or post-weaning (Figure 2.3). It is of interest to examine the mechanism underlying these changes in gene expression to determine their relationship to altered phenotypes in the offspring/descendants of male mice fed defined FA diets, including embryo development and viability. One proposed mechanism underlying these phenotypes is that (grand)paternal FA intake alters DNA methylation and results in heritable altered gene expression.

4.3 Methods

A bioinformatics approach was used to identify candidate genes with CpG islands and to determine the DNA methylation profiles of differentially expressed hepatic genes identified by microarray analysis of the F3 descendants of male mice fed a FA deficient (0 mg/kg FA), control (2 mg/kg FA), or supplemented diet (6 mg/kg FA) (Figure 2.3).

4.3.1 Previous Animal Study

A schematic of the following animal study is depicted in Figure 2.3.

1. The Post-Weaning group: F1 male mice, derived from female F0 mice fed a FA sufficient diet, were fed FA deficient (0 mg/kg FA), control (2 mg/kg FA) or supplemented (6 mg/kg FA) diets and bred to female mice fed a FA control diet (2 mg/kg FA) from 3 weeks for 15 weeks.
2. The Early Development group: F1 male mice, derived from F0 females fed a FA deficient (0 mg/kg FA), control (2 mg/kg FA) or supplemented (6 mg/kg FA) diet during pregnancy and lactation, were weaned to the FA control diet (2 mg/kg FA) from 3 weeks for 15 weeks.

All subsequent generations were fed the FA control diet. F3 male mice were necropsied and liver dissected at 8 weeks of age.

Microarray analysis of gene expression was performed on liver RNA from F3 male mice to identify candidate differentially expressed genes as a result of grandpaternal exposure to FA deficiency or supplementation. Pathway analyses were then conducted using Ingenuity Pathway Analysis to highlight which ones consisted of multiple genes affected by grandpaternal dietary FA intake.

4.3.2 Primer Design

For differentially expressed genes, the Epigenomics database of the National Center for Biotechnology Information (NCBI) was used to identify candidate CpG island sequences within gene promoters [132]. Identified CpG island sequences were taken from the Nucleotide database of NCBI in FASTA format and submitted to MethPrimer [109] to design bisulfite sequencing PCR primers for amplification. CpG islands in MethPrimer were identified based on a criteria set by Gardiner-Garden and Frommer (Minimum length = 200 bp; Observed/Expected ratio > 0.6; GC content > 50%) [133]. Two hundred base pairs before and after the identified CpG island sequences were included in the sequence input from NCBI to ensure primers were designed around the CpG island and not within it. If the primers were to be designed within the CpG island, the binding capacity of the primer to the DNA sequence would be decreased, since these primers may discriminate against methylated or unmethylated DNA [109]. In some instances, no CpG islands were predicted in MethPrimer, despite being identified in the NCBI Epigenomics database, at which point, they were excluded from primer design and further analysis.

4.3.3 DNA Extraction

Frozen liver tissue was cut into pieces that weighed between 10 – 30 mg (ideally 15 – 25 mg). 600 µL Buffer RLT (Qiagen), containing 1% β-mercaptoethanol, was added to the tissue and the sample was homogenized. DNA was extracted from liver tissue using the AllPrep DNA/RNA/Protein Mini Kit (Qiagen, Valencia, CA), following the protocol "Simultaneous Purification of Genomic DNA, Total RNA, and Total Protein from Animal and Human Tissues" (steps 1 – 5, 21 – 24) [134]. Extracted DNA was eluted in 40 µL Buffer EB and concentrations were measured using the Nanodrop ND1000 Spectrophotometer (Thermo Scientific, Waltham, MA). 100 µg of the extracted DNA was run on a 1.2% E-gel with SYBR Safe (Life

Technologies, Burlington, ON) to verify DNA quality before bisulfite treatment. Gel images were taken with the ChemiDoc MP Imaging System (BIORAD).

4.3.4 Bisulfite Conversion

Bisulfite conversion and clean-up of bisulfite treated DNA was carried out according to the protocol "Sodium Bisulfite Conversion of Unmethylated Cytosines in DNA" [135] and using the Epitect Bisulfite Kit (Qiagen). One μg of extracted DNA was bisulfite converted using the PTC-200 Peltier Thermal Cycler (MJ Research, St. Bruno, QC), in which DNA underwent alternating steps of denaturation and incubation (5 minutes at 95°C, 25 minutes at 60°C, 5 minutes at 95°C, 85 minutes at 60°C, 5 minutes at 95°C, and 175 minutes at 60°C; the reaction was then held 20°C until cleanup). Bisulfite-treated DNA was cleaned and eluted in 20 μL EB Buffer. Concentrations were measured using the Nanodrop ND1000 Spectrophotometer.

To verify the bisulfite treatment converted 100% of unmethylated cytosines to uracils, PCR amplification of the CpG island was performed, followed by cloning into a plasmid vector and sequencing. PCR amplification was carried out using primers designed with MethPrimer [109]. A CpG island was identified within the *Mthfr* gene and multiple primers were designed to amplify the 1132 base pair sequence containing this CpG island. Illustra puReTaq Ready-To-Go PCR Beads (GE Healthcare, Mississauga, ON) were used for PCR amplification of the CpG islands. As recommended by GE Healthcare [26], 50 ng of DNA and 10 pmol of each primer were mixed with PCR beads and water, up to a total volume of 25 μL. PCR beads were stable at room temperature and contained 50 mM KCl, 200 mM dNTPs in 10 mM Tris-HCl, ~ 2.5 units of PureTaq DNA Polymerase, and 1.5 mM $MgCl_2$. PCRs with temperature gradients were carried out to determine the optimal temperature for the annealing step of the reaction using the C1000 Thermal Cycler (BIORAD). The PCR products were run on a 1.2% E-gel with SYBR Safe (Life

Technologies) to verify the correct band sizes. Gel images were taken with the ChemiDoc MP System. Cloning of the PCR product into a plasmid vector was carried out using the TOPO TA Cloning Kit [136]. The clones were transformed in One Shot Mach1-T1 Competent Cells and positive transformants were selected and sent to Genome Quebec for sequencing to verify cytosine conversion.

4.3.5 Next Generation Sequencing

In the first step of sample preparation for Next Generation Sequencing (NGS), PCR was used to amplify the bisulfite-treated DNA using the primers (designed by MethPrimer and ordered from IDT) for the region of interest with overhang adaptors attached to them (Forward overhang: 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-*locus-specific sequenc*e; Reverse overhang: 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-*locus-specific sequence*). Locus-specific primer sequences used are depicted in Table 4.1. The primer overhang adaptors allowed for compatibility with the Illumina sequencing adaptors. Once amplified, the bands were separated by gel electrophoresis on a 2% agarose gel with SYBR Safe (Life Technologies). The band size of interest was then cut out and purified using the QIAquick Gel Extraction Kit (QIAGEN). Twenty-one animal samples for each of the 10 primer pairs were quantified and sent for Next Generation sequencing by Robyn Kenwell at the Bureau of Microbial Hazards (BMH) core sequencing facility at Health Canada.

Table 4.1: Locus-specific primer sequences used for PCR amplification of promoter CpG islands for the genes *Chka, Cish, Mthfr,* and *Pdgfc.* Primers were designed by MethPrimer.

Gene	Primer Name	Direction	Locus-Specific Primer Sequence
Chka	Chka1a-1	Forward	5' TTTTAGGGTTTGTTAAAGTTGTAGAAGTT 3'
		Reverse	5' ACACAACCCCTCCTAAACTCAA 3'
	Chka1b-1	Forward	5' GTTTGAGTTTAGGAGGGGTTGT 3'
		Reverse	5' ATACAAAACTTAATTTTCATACTAAACAAA 3'
	Chka1c-1	Forward	5' TTGTTTAGTATGAAAATTAAGTTTTGTAT 3'
		Reverse	5' TCCTTACACCACAAATAAACCCTAC 3'
	Chka1c-5	Forward	5' GTAGGGTTTATTTGTGGTGTAAGGA 3'
		Reverse	5' AATACTAAAAACAACATCCCTTTTT 3'
Cish	Cish1b-1	Forward	5' GAAATTTGTTAAAGGTGTTTTTTTT 3'
		Reverse	5' AAACCTATAATCTTCCATATCTCCC 3'
Mthfr	Mthfr1a-5	Forward	5' GGGGAAGTTGGATTATTAGTGATTTA 3'
		Reverse	5' CCCCAACCAAAATTCTATTCTATAAC 3'
	Mthfr1b-2	Forward	5' TAGAATAGAATTTTGGTTGGGGTA 3'
		Reverse	5' AAAAAAAATTAACCTAAATAATCC 3'
	Mthfr1c-3	Forward	5' GGATTATTTAGGTTAATTTTTTTT 3'
		Reverse	5' AATACCACCTCCCTAAAACTAAC 3'
	Mthfr1d-1	Forward	5' TATTTGGTTGGTTTTTTTTGAGATT 3'
		Reverse	5' CCTACCCACAAATCTAACTTAC 3'
Pdgfc	Pdgfc1a-1	Forward	5' GTTTAGGGGAAAGGAAGTTGG 3'
		Reverse	5' AAAAACCTAACTAACCTAAAAAATC 3'
	Pdgfc1b-1	Forward	5' TTGTTTTGTTTTAGGGTAGGTATT 3'
		Reverse	5' AAAATCAATAACAAAACTACTAAT 3'
	Pdgfc1b-2	Forward	5' TGGAGATATAGAAGAGGGTTTTAGGA 3'
		Reverse	5' CCAAAACAAATATCAACAAAAAAAA 3'
	Pdgfc1b-3	Forward	5' GTTTTGTTATTGATTTTAAGGTGTT 3'
		Reverse	5' TAAACCAATTTCCCAAAATTTAAAC 3'
	Pdgfc1c-1	Forward	5' TTTTTTTGTTGATATTTGTTTTGGT 3'
		Reverse	5' AATCACAATTAACTTTTCCAATCCTT 3'

NGS was used to sequence the bisulfite-treated DNA in order to characterize the methylation patterns of the differentially expressed genes from the F3 descendants of male mice fed deficient, sufficient, and supplemented diets. The protocol for preparation of bisulfite-treated DNA for sequencing was adapted from "16S Metagenomic Sequencing Library Preparation: Preparing 16S Ribosomal RNA Gene Amplicons for the Illumina MiSeq System" [137]. Index PCR was used to amplify the amplicon and attach an index primer and sequencing adaptor on each end of the amplicon. The library was normalized, pooled, denatured, and sequenced. NGS was carried out using the Illumina MiSeq System [137].

4.3.6 Analysis of NGS Results

NGS was used to quantitatively assess the degree to which each CpG island is methylated at the sequenced locations. Animals were grouped by diet (Early development: deficient (0 to 2 mg/kg FA), control (2 to 2 mg/kg FA), and supplemented (6 to 2 mg/kg FA); Post-weaning: deficient (2 to 0 mg/kg FA), control (2 to 2 mg/kg FA), and supplemented (2 to 6 mg/kg FA)). Overall average methylation of all animals in each diet group was determined. Raw reads obtained from NGS were trimmed for quality using Trimmomatic 0.32 [138]. A sliding window approach was applied with a window length of 4 and minimum quality of 20 was used to trim the raw reads. A sliding window approach is when the genome region of interest is split into windows and tests are performed in each window [139]. Reads with leading and trailing bases of quality less than 20 were removed and a minimum read length of 36 was required. Quality assessment was done using FastQC, both before and after trimming (Figure 4.1) [140]. Sequences were mapped using Bismark v0.14.3, using Bowtie2 to perform alignments [141]. One non-bisulfite mismatch was allowed per read and counts of methylated and non-methylated cytosines were extracted from the alignments using the Bismark methylation extractor.

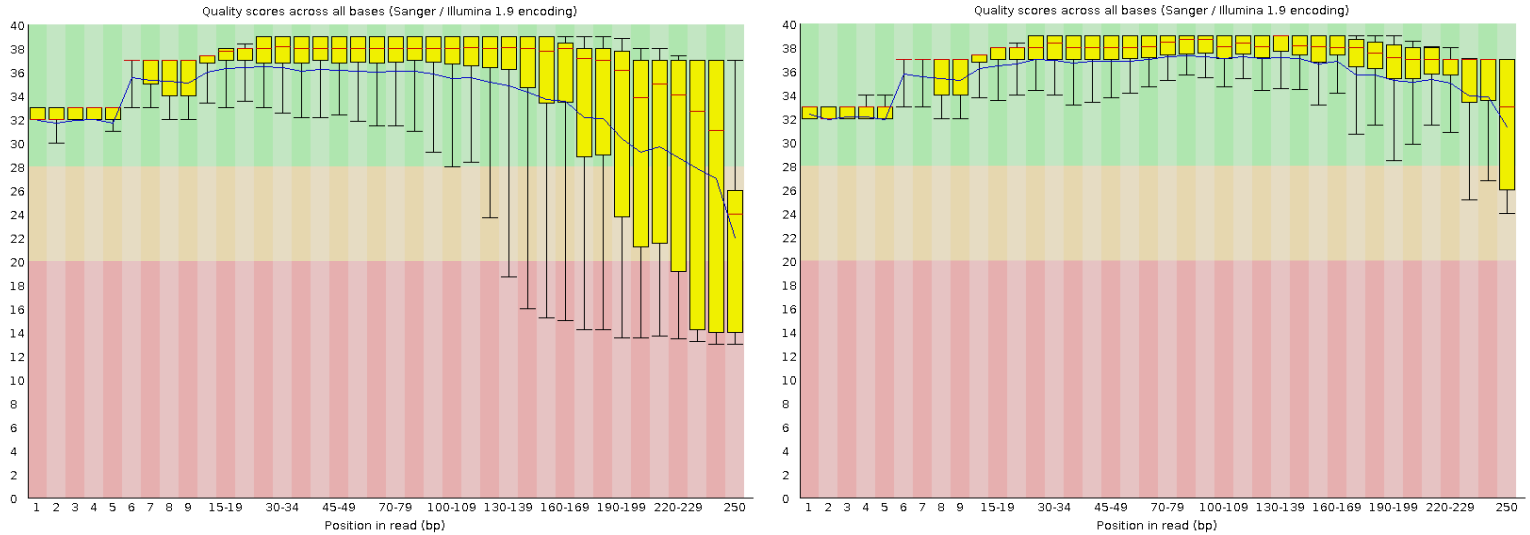

Figure 4.1: An example graph showing quality score across all bases of the forward read of one animal sample, both (left) pre- and (right) post-trimming by Trimmomatic 0.32. Quality assessments were calculated using FastQC.

4.3.7 Statistical Analyses

Statistical analysis on differential methylation at the gene level was done using generalized linear models in R, using the glm() function. For each region, the number of methylated cytosines was modeled as a function of treatment, CG site, and total number of reads. Diet groups were compared to the control diet group.

4.4 Results

4.4.1 CpG Island Identification

Candidate CpG islands, according to Gardiner-Garden and Frommer conditions were identified in 11 different genes, including *Chdh, Chka, Chpt1, Cish, Mapk8, Mg11, Mthfr, Pcyt1a, Pdgfc, Pld2, Sgms1*. However, of these 11 genes, *Chdh, Chka, Cish, Mapk8, Mthfr,* and *Pdgfc* were chosen for promoter CpG island methylation analyses due to their more prominent involvement in the choline pathway (*Chdh, Chka*) or their association with NTDs (*Mthfr, Pdgfc, Mapk8),* or because they had a greater degree of differential hepatic expression among the diet groups (*Cish)*. In total, 17 primer pairs were designed spanning the 6 genes' CpG islands. Nine of the 17 primer pairs successfully amplified their target sequences in 4 of the 6 genes (Table 4.2) (*Chka, Cish, Mthfr, Pdgfc*). Primer pairs (3 in total) designed for the *Chdh* (2 primer pairs) and *Mapk8* (1 primer pair) genes did not amplify their target sequence. The remaining primer pairs that did not successfully amplify their target sequence included 3 pairs for the *Chka* gene and 2 pairs for the *Pdgfc* gene. Primer pairs designed for the *Mthfr* (Figure 4.2) and *Cish* (Figure 4.3) CpG islands all successfully amplified the targeted regions, which were 932 and 365 bps, respectively. In the *Chka* gene, one of the four primer pairs designed for amplification of the promoter CpG island successfully amplified its target sequence (287 bp) (Figure 4.4), and in the *Pdgfc* gene, 3 primer pairs were a success, amplifying a total of 904 bps (Figure 4.5).

Table 4.2: Characteristics of CpG islands in candidate genes identified by microarray analysis. Chromosomal location, total and sequenced lengths of CpG islands, and total and completed number of primer pairs are presented.

Gene	Chromosome	Total length of CpG Island (bp)	Sequenced length of CpG island (bp)	Total # of Primer Pairs	# of Primer Pairs completed
5,10-methylenetetrahydrofolate Reductase (Mthfr**)**	4	916	916	4	4
Choline kinase alpha (Chka**)**	19	1311	287	4	1
Cytokine inducible SH2-containing protein (Cish**)**	9	365	365	1	1
Platelet-derived growth factor, C polypeptide (Pdgfc**)**	3	950	904	5	3

Figure 4.2: Visual schematic of the (a) epigenomic profile of *Mthfr* in the Epigenomics database of NCBI. Blue data tracks are presented, the peaks of which represent areas of enriched epigenetic features. (b) A snapshot of the *Mthfr* primers designed for BiSulfite Amplicon Sequencing by MethPrimer.

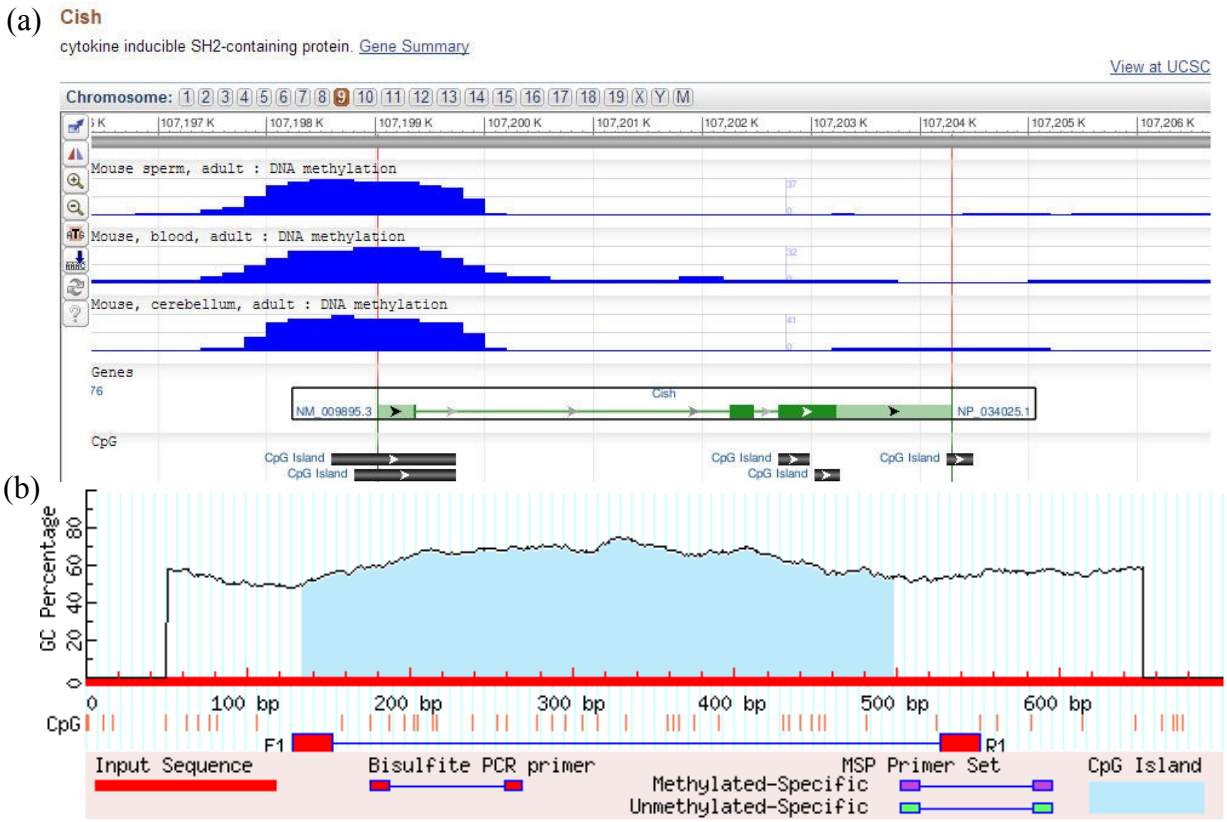

Figure 4.3: Visual schematic of the (a) epigenomic profile of *Cish* in the Epigenomics database of NCBI. Blue data tracks are presented, the peaks of which represent areas of enriched epigenetic features. (b) A snapshot of the *Cish* primers designed for BiSulfite Amplicon Sequencing by MethPrimer**.**

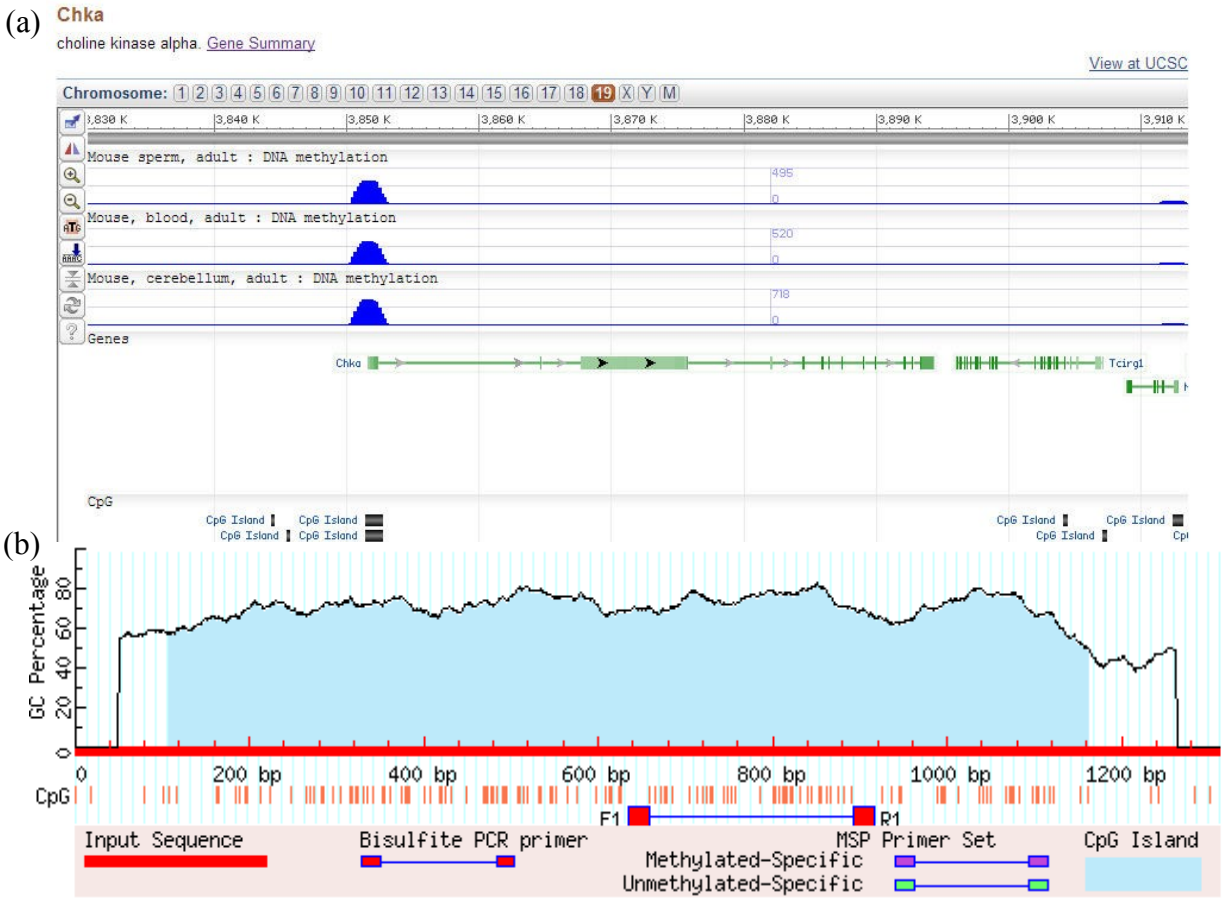

Figure 4.4: Visual schematic of the (a) epigenomic profile of *Chka* in the Epigenomics database of NCBI. Blue data tracks are presented, the peaks of which represent areas of enriched epigenetic features. (b) A snapshot of the *Chka* primers designed for BiSulfite Amplicon Sequencing by MethPrimer.

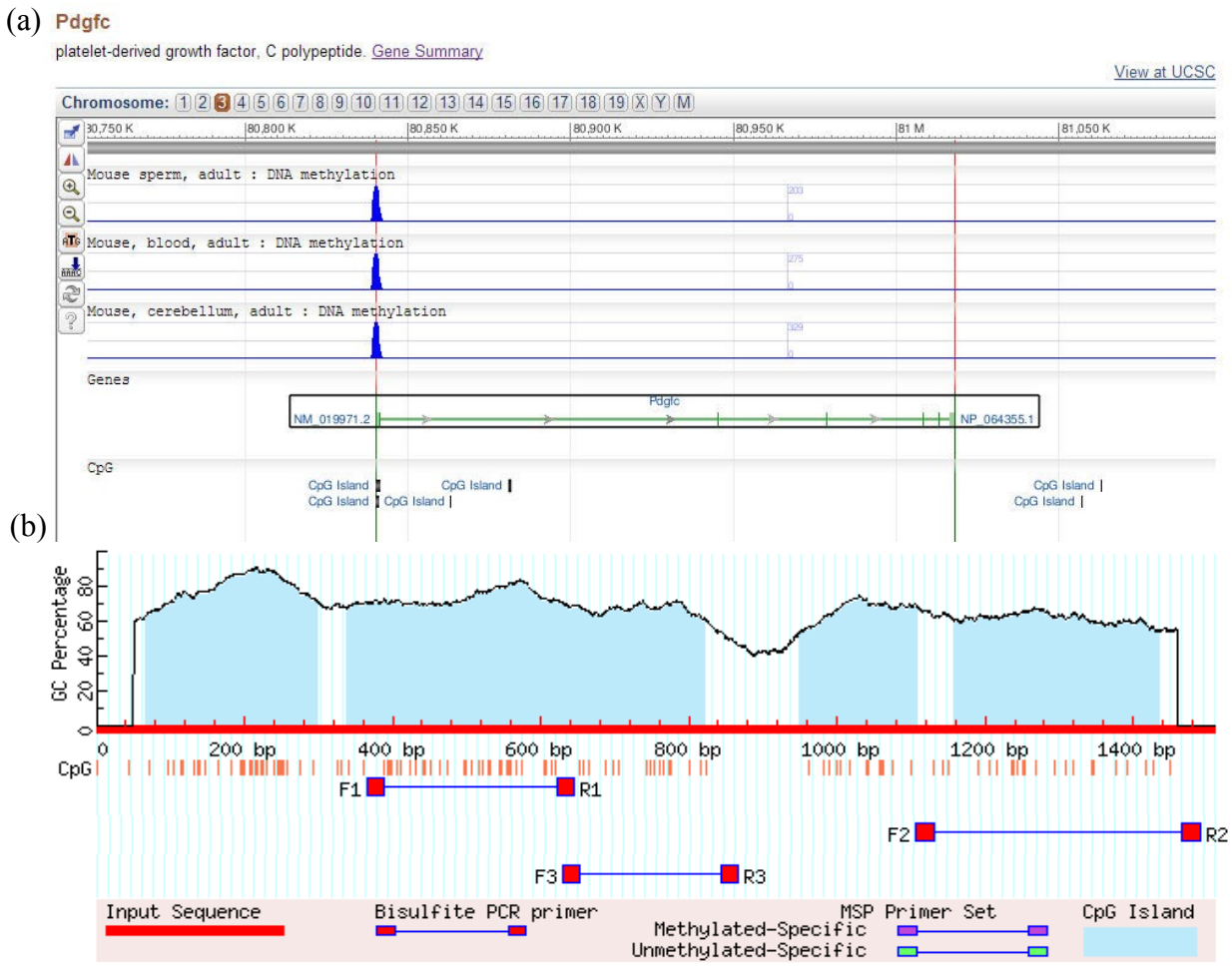

Figure 4.5: Visual schematic of the (a) epigenomic profile of *Pdgfc* in the Epigenomics database of NCBI. Blue data tracks are presented, the peaks of which represent areas of enriched epigenetic features. (b) A snapshot of the *Pdgfc* primers designed for BiSulfite Amplicon Sequencing by MethPrimer.

4.4.2 NGS Analyses

Choline Kinase Alpha (Chka)

Descendants of male mice fed the FA deficient diet post-weaning did not demonstrate significantly different methylation status of the promoter CpG island of the *Chka* gene, compared to that of male mice fed the FA control diet. However, descendants of male mice exposed to the supplemented diets both at early development and post-weaning, had a lower proportion of methylated CG sites ($p < 0.01$ & $p < 0.05$, respectively) across the sequenced portion of the *Chka* CpG island compared to the FA control group (Figure 4.6). In contrast, there was a higher proportion of methylated CG sites ($p < 0.01$) across the sequenced portion of the *Chka* CpG island compared to the FA control group in descendants of male mice exposed to the FA deficient diet in early development (Figure 4.6). Further analysis of the remaining un-sequenced portions of the promoter CpG island (1,024 bps) is required (Tables 4.1 and 4.2).

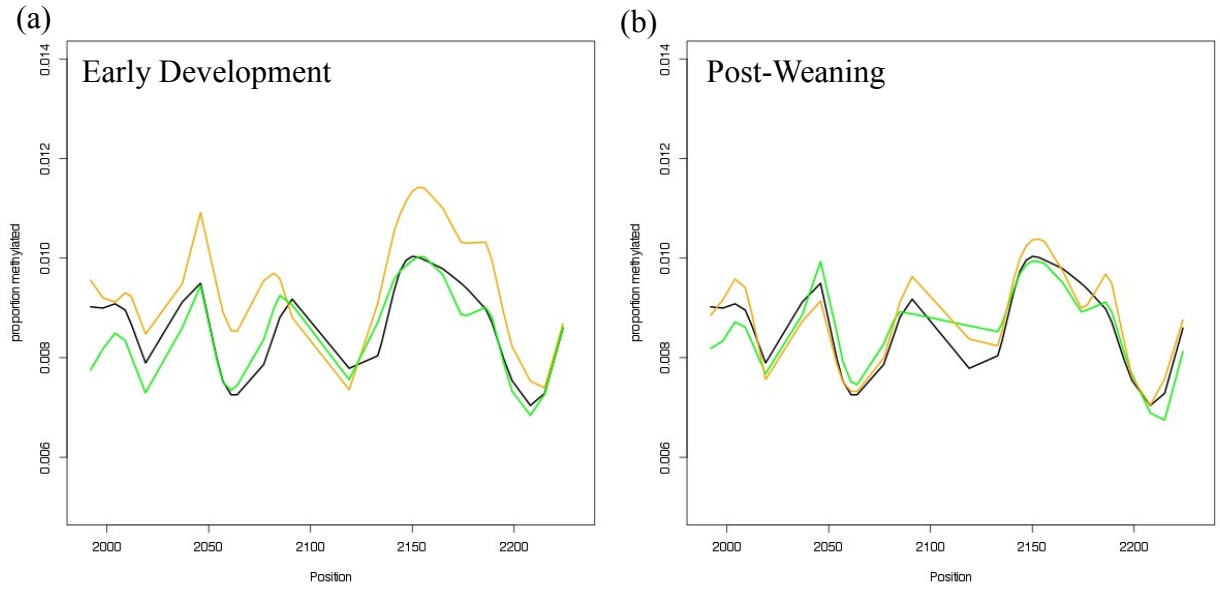

Figure 4.6: Hepatic methylation profile of a CpG island in the promoter region of the Choline kinase alpha (*Chka*) gene. Methylation profiles were characterized in F3 descendants of male mice fed a FA deficient (0 mg/kg FA), control (2 mg/kg FA), or supplemented (6 mg/kg FA) at (a) early development, *in utero* and during lactation or (b) at post-weaning from 3 to 18 weeks of age. Male mice exposed to a FA-defined diet post-weaning were exposed to a FA control maternal diet during early development. F2 and F3 males were fed a FA control diet. (a) black = 2 to 2, orange = 0 to 2, green = 6 to 2; (b) black = 2 to 2, orange = 2 to 0, green = 2 to 6. $P < 0.01$ for ED0 and ED6; $P < 0.05$ for PW6. All diet groups were compared to their respective FA control group.

Cytokine inducible SH2-containing factor (Cish)

Descendants of male mice fed the FA deficient diets had no significant differences in methylation in comparison to male mice fed the FA control diet within the promoter CpG island in *Cish* (Figure 4.7). Descendants of male mice fed the FA supplemented diet during early development also had no significant differences in methylation in comparison to male mice fed the FA control diet within the promoter CpG island in *Cish* (Figure 4.7). However, descendants of male mice fed the FA supplemented diet at post-weaning had an overall lower proportion of methylation ($p < 0.001$) at its promoter CpG island (Figure 4.7).

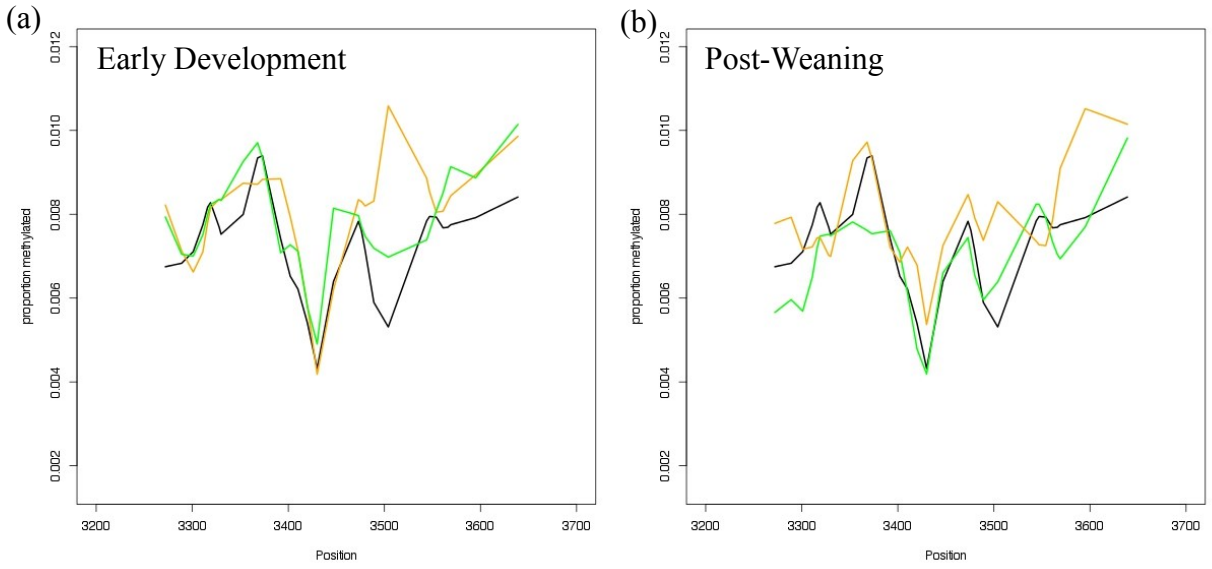

Figure 4.7: Hepatic methylation profile of a CpG island in the promoter region of the Cytokine inducible SH2-containing protein (*Cish*) gene. Methylation profiles were characterized in F3 descendants of male mice fed a FA deficient (0 mg/kg FA), control (2 mg/kg FA), or supplemented (6 mg/kg FA) at (a) early development, *in utero* and during lactation or (b) at post-weaning from 3 to 18 weeks of age. Male mice exposed to a FA-defined diet post-weaning were exposed to a FA control maternal diet during early development. F2 and F3 males were fed a FA control diet. (a) black = 2 to 2, orange = 0 to 2, green = 6 to 2; (b) black = 2 to 2, orange = 2 to 0, green = 2 to 6. $P < 0.001$ at PW6. All diet groups were compared to their respective FA control group.

Methylenetetrahydrofolate reductase (Mthfr)

Descendants of male mice fed the FA deficient and supplemented diets had no significant differences in methylation in comparison to male mice fed the FA control diet within the promoter CpG island in *Mthfr* (Figure 4.8).

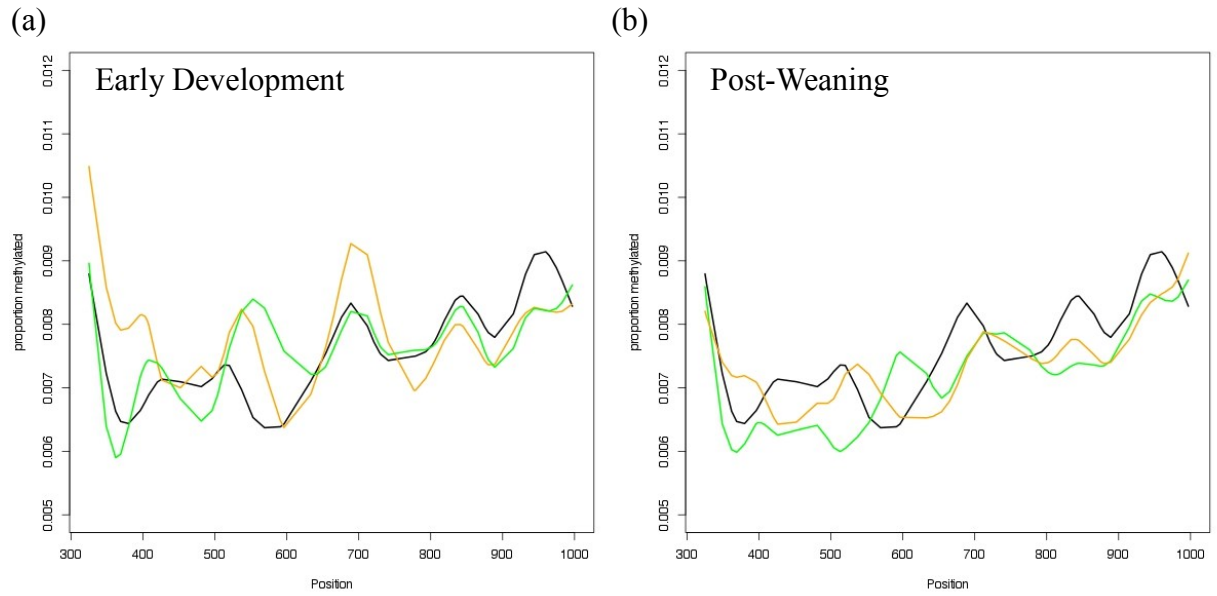

Figure 4.8: Hepatic methylation profile of a CpG island in the promoter region of the 5,10-methylenetetrahydrofolate reductase (*Mthfr*) gene. Methylation profiles were characterized in F3 descendants of male mice fed a FA deficient (0 mg/kg FA), control (2 mg/kg FA), or supplemented (6 mg/kg FA) at (a) early development, *in utero* and during lactation or (b) at post-weaning from 3 to 18 weeks of age. Male mice exposed to a FA-defined diet post-weaning were exposed to a FA control maternal diet during early development. F2 and F3 males were fed a FA control diet. (a) black = 2 to 2, orange = 0 to 2, green = 6 to 2; (b) black = 2 to 2, orange = 2 to 0, green = 2 to 6. All diet groups were compared to their respective FA control group.

Platelet derived growth factor C (Pdgfc)

Descendants of male mice fed the FA deficient diet at early development had no significant differences in methylation in comparison to male mice fed the FA control diet within the promoter CpG island in *Pdgfc* (Figure 4.9). Descendants of male mice exposed the FA deficient diet at post-weaning had a lower proportion of methylation ($p < 0.01$) in comparison to male mice fed the FA control diet (Figure 4.9). Post-weaning exposure of the grandfathers to both the FA deficient and supplemented diets resulted in a lower proportion ($p < 0.01$ for both diet exposures) of methylation in comparison to the FA control exposure at post-weaning (Figure 4.9).

Parts of the *Pdgfc* promoter CpG island (46 bps) region remain to be analyzed further (Tables 4.1 and 4.2).

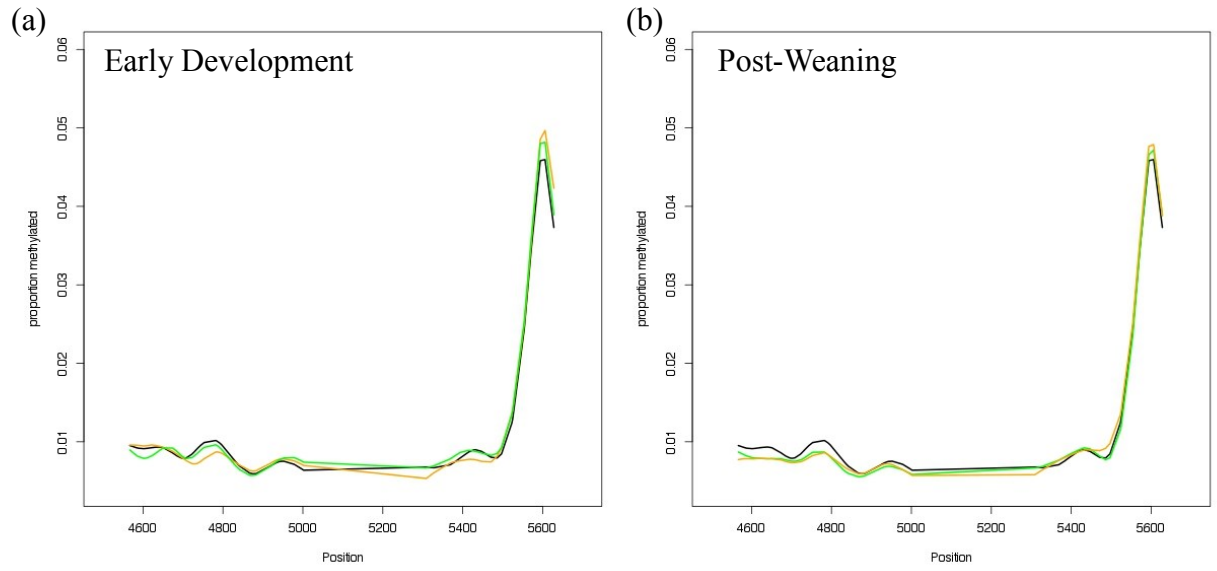

Figure 4.9: Hepatic methylation profile of a CpG island in the promoter region of the Platelet-derived growth factor, C polypeptide (*Pdgfc*) gene. Methylation profiles were characterized in F3 descendants of male mice fed a FA deficient (0 mg/kg FA), control (2 mg/kg FA), or supplemented (6 mg/kg FA) at (a) early development, *in utero* and during lactation or (b) at post-weaning from 3 to 18 weeks of age. Male mice exposed to a FA-defined diet post-weaning were exposed to a FA control maternal diet during early development. F2 and F3 males were fed a FA control diet. (a) black = 2 to 2, orange = 0 to 2, green = 6 to 2; (b) black = 2 to 2, orange = 2 to 0, green = 2 to 6. $P < 0.01$ for ED6; $P < 0.05$ for PW0 and PW6. All diet groups were compared to their respective FA control group.

4.5 Discussion

The objective of this study was to determine whether the effect of grand-paternal FA intake on F3 hepatic gene expression was due to altered DNA methylation. Male Balb/c mice were exposed to varying levels of FA (deficient, control, and supplemented) either in early development (*in utero* and during lactation) or post-weaning. In previous microarray analyses, it was hypothesized that grand-paternal FA intake had an effect on gene expression of unexposed offspring in the F3 generation via a trans-generational heritable effect through the male germline. Effects are considered trans-generationally heritable if they are passed down to the unexposed generations, as opposed to direct effects, which are based on direct exposure of the animal, including exposure of embryonic gametes. This multigenerational animal model allowed us to investigate the heritability of FA effects on gene expression and to determine whether it was due to methylation changes in the unexposed F3 generation.

Trans-generational epigenetics is the transfer of non-genetically induced changes from the parent to the offspring through the male or female germline; in some cases, these changes may be inherited by unexposed generations [142,143]. Trans-generational inheritance is only achieved when epigenetic effects are observed in the F3 generations, as they are the first unexposed generation (Figure 2.3). In this study, the F1 generation was exposed to the diet at post-weaning and the F2 generation was exposed as germ cells.

Examples of trans-generational inheritance of phenotypes are accumulating. In mice, a maternal high fat diet exposure from pre-conception to weaning resulted in increased body length and reduced insulin sensitivity in the F2 and F3 generations [144,145]. The most studied model of trans-generational inheritance is considered to be the agouti viable yellow mouse strain, in which coat color, adiposity, and methylation status are all transmitted through the female

germline [146]. Another study shows that paternal prediabetes resulted in the trans-generational inheritance of glucose intolerance and insulin resistance [147]. The mechanism by which male mice can confer heritable changes to their offspring is through their sperm, and epigenetics has been implicated as a major contributor to paternal transmission of diet-induced gene expression changes. Human archival data has shown an association between food availability in grandfathers during the pre-pubertal phase and increased risk for cardiovascular disease, diabetes, and mortality in grandsons [148]. Paternal transgenerational epigenetic inheritance has been shown in mice with variable methylation at the *Agouti* gene [149], as well as in rodents exposed to vinclozolin, an endocrine disruptor [150]. We hypothesized that epigenetics, and specifically DNA methylation, was the underlying mechanism behind the observed differential hepatic gene expression in our study.

Our approach was to investigate whether the differential expression of genes associated with grand-paternal FA intake was epigenetically regulated. DNA methylation is an epigenetic mechanism that regulates gene expression via the addition of a methyl group to cytosine. The dogma is that with increased DNA methylation, gene expression is reduced and vice versa. By identifying differentially expressed genes first, we could identify genes with candidate CpG islands and determine whether the expression differences were due to changes in DNA methylation.

Pathway analysis of the microarray data identified genes with differential expression involved in the choline pathway, and others associated with neural tube defects (NTDs). Choline, like folate, is a source of one-carbon units in the form of betaine for homocysteine re-methylation, which marks the point at which the folate and choline metabolic pathways intersect. Differentially expressed genes identified in the choline pathway included choline kinase alpha

(Chka), choline dehydrogenase *(Chdh)*, choline phosphotransferase *(Chpt1)*. Genes with differential expression that have been implicated in neural tube defects (NTDs) were differentially expressed in the descendants of male mice fed different FA diets. NTDs are congenital defects that occur when the neural tube fails to close during embryo development. Genes associated with NTD development that were shown to be differentially expressed included 5,10-methylenetetrahydrofolate *(Mthfr)*, platelet derived growth factor, C polypeoptide *(Pdgfc)*, Cbp/p300-interacting transactivator 2 *(Cited2)*, and mitogen-activated protein kinase 8 *(Mapk8)*. Other candidate genes of interest included the top differentially expressed genes, including acyl-coenzyme A amino acid N-acyltransferase 2 *(Acnat2)*, cytokine inducible SH2-containing protein *(Cish)*, and others. Of the identified genes with differential gene expression, the ones with candidate CpG islands included *Chdh, Chka, Chpt1, Cish, Mapk8, Mg11, Mthfr, Pcyt1a, Pdgfc, Pld2, Sgms1*.

Of the initial genes found to have differential expression, eleven genes had CpG islands in their promoter region. However, six of these were chosen for CpG promoter analyses and bisulfite sequencing due to their role in the choline pathway or NTDs, including *Chka, Chdh, Mapk8, Pdgfc, Mthfr*. *Cish* was chosen for further analyses as it was one of the genes that was differentially expressed the most. However, sequencing of *Mapk8* and *Chdh* has not yet been completed due to difficulties amplifying the CpG island in the promoter. Further experimentation needs to be conducted to characterize the methylation status of their promoter CpG islands.

Chka is involved in choline metabolism and was differentially expressed in the deficient diets both at early development and post-weaning. *Chka* encodes the initial enzyme of the CDP-choline pathway. The CHKA enzyme phosphorylates choline and plays an important role in choline metabolism. Microarray analysis showed that choline kinase alpha (*Chka)* gene

expression was significantly higher in descendants of mice exposed to a FA deficient diet by two-fold in the early development deficient (ED0) diet and by 66% in the post-weaning deficient (PW0) diet. For descendants of both the deficient and supplemented males, a greater effect was observed in F3 mice from grandparents exposed in early development compared to post-weaning, as well as a greater effect with FA supplementation than with FA deficiency. Our hepatic DNA methylation analyses show that in the *Chka* gene, there is no significant effect in the post-weaning deficient diet, but that there is overall decreased methylation in the post-weaning supplemented diet. However, the early development deficient diet had overall increased methylation in a part of its promoter CpG island, whereas the early development supplemented diet had overall decreased methylation (Figure 4.6). The rest of the promoter CpG island needs to be analyzed further.

The *Mthfr* gene encodes MTHFR, which is critical in the maintenance of methylation capacity, as it irreversibly reduces 5,10-methylene-THF to 5-methyl-THF. Impaired MTHFR activity results in decreased levels of 5-methyl-THF, decreased Hcy re-methylation, decreased methionine synthesis, and consequently decreased methylation capacity. In humans, a common single-nucleotide polymorphism (SNP), 677C>T (rs1801133), leads to a decrease in enzymatic function by approximately 35% and 70% when the *MTHFR* gene is in the heterozygote (677CT) or homozygote state (677TT), respectively [32]. This polymorphism has been associated with increased risk for NTDs [151,152]. *Mthfr* gene expression was higher in descendants of mice exposed to the FA deficient diet groups, compared to those exposed to the FA control diet, with an overall difference in expression of 54%. Likewise, the supplemented diet groups both at ED and PW also had increased expression of hepatic *Mthfr*. Our hepatic analyses showed no significant difference in methylation in descendants of male mice fed the deficient and

supplemented diets in comparison to those fed the FA control diet in the promoter CpG island in *Mthfr* (Figure 4.8). Other studies have also characterized the methylation status of *Mthfr*. The 5' untranslated region (UTR) of *Mthfr* was found to be hypermethylated in diffuse large B-cell lymphoma cells [153]. Hyper-methylation of the *Mthfr* promoter in both testes and lung tissues has been associated with male infertility and increased risk for lung cancer, respectively [54,154]. Similar to our findings, the methylation state of *Mthfr* was also found to be unchanged in response to folate status [154].

The *Pdgfc* gene is part of the PDGFR-alpha signaling pathway and plays a key role in palatogenesis, which is the development of the secondary palate in the mouth [155]. Knockout of *Pdgfc* in mice has been shown to be lethal in the perinatal period, due to respiratory difficulties attributed to cleft palate [156]. Cleft palate is a neural tube defect in which there is a split in the roof of the mouth. *Pdgfc* was differentially up-regulated in the deficient exposures at both early development and post-weaning. Our hepatic analyses show that the deficient exposure at post-weaning, but not at early development resulted in a lower proportion of methylated CG sites in the *Pdgfc* promoter. Additionally, exposure of the grandfather to the FA supplemented diet at both early development and post-weaning also resulted in a lower proportion of methylated CG sites in the promoter CpG island of *Pdgfc* (Figure 4.9).

While the *Cish* gene is not related to the choline pathway or associated with NTDs, our microarray analyses showed that it was significantly down-regulated in descendants of male mice fed either the FA deficient or FA supplemented diets, compared to those fed the FA-control diet. Therefore, it was of interest to determine whether the differential gene expression was associated with a difference in methylation status at its promoter CpG island. In a genome-wide methylome analysis of $CD4^+$ T cells, it was found that the *Cish* gene was differentially

methylated between memory and naïve T cells at its first intron and second exon, but not at its promoter region [157]. Similarly, our methylation analyses did not show any differences in promoter methylation of *Cish* among the three diet groups when the grandfathers were exposed to their diets at early development (Figure 4.7). In contrast to these results, however, we did find overall decreased methylation in grandsons of male mice exposed to the FA supplemented diet at post-weaning (Figure 4.7).

The observed methylation changes found in the gene promoters in this study are subtle and may raise speculation as to how they may affect gene expression and induce phenotypic effects. It is important to note however, that other studies have also observed subtle methylation changes in response to folate deficiency, with methylation changes ranging from approximately 4% to 10% [119]. Similarly, a study looking at methylation status differences in promoters of genes associated with schizophrenia and bipolar disorder found subtle methylation differences between patients affected by these disorders and patients unaffected by them. Schizophrenic and bipolar disorder patients had a methylation difference of about 8% and 6%, respectively, compared to those unaffected by the disorders [158].

Despite the overall subtle changes in methylation that were observed, this study highlights the potential for identifying gene expression changes in unexposed offspring that correlate with methylation changes. Another study that provides proof-of-principle for transgenerational epigenetic inheritance shows that undernutrition during prenatal life can potentially alter male germline development and epigenetic reprogramming, although the mice were weaned back to a normal diet postnatally [159].

There are a number of strengths and limitations of our analysis and study design. Our study design has the potential to determine at which life stages the sperm methylome may be

more susceptible to diet-induced epigenetic changes. We found two instances in which the proportion of methylation was generally proportional to changes in gene expression. The first instance is shown in descendants of male mice fed the FA deficient diet at post-weaning, in which the gene expression of *Pdgfc* was up-regulated. As expected, hepatic methylation analyses showed that the CpG promoter region sequenced had decreased methylation. Another instance can be seen in the *Chka* gene, where there was an increase in gene expression in F3 descendants of male mice exposed to the deficient diet at early development. Counterintuitively, there was also increase in methylation of the sequenced portion of its promoter. While these results are opposite of what may be expected (lower methylation is usually indicative of higher gene expression and vice versa), another study has also made similar observations. Renner et al. showed that methylation and gene expression of the *CDKNA* gene in soft-tissue sarcomas is positively correlated. Hypermethylation of the gene did not result in inactivation of the gene [160].

Another strength of this study lies in the use of next generation sequencing, specifically the Illumina MiSeq System, to quantitatively determine methylation frequencies at the candidate CpG islands. Compared to other methods, such as pyrosequencing, this system provides a greater throughput of data per run and higher quality reads, with a lower error rate per base pair [114]. Consequently, it was found to be the more appropriate method for investigating the objectives outlined in this project.

One limitation of this study lies in the fact that the BSAS approach was a more biased approach compared to others when assessing methylation status. A genome-wide approach in which the entire methylome is assessed may have produced more data enabling exploration across the genome. For example, we limited our search for CpG islands to the promoter regions,

meaning other regions could have been missed, such as within intragenic regions or in 3' untranslated regions. Intragenic CpG islands have been suggested to function as alternative transcription start sites, and altered methylation within these regions may consequently alter gene expression [161].

Another limitation was the assessment of one of many potential epigenetic modifications that may have been underlying the F3 hepatic gene expression changes observed by our microarray analyses. Such modifications include histone modifications (methylation, acetylation, phosphorylation, ubiquination) and miRNAs. Thus, in genes where no diet-dependent DNA methylation was observed, one of the other epigenetic mechanisms may have been heritably altering gene expression. Further analyses of other types of heritable epigenetic modifications will either confirm or contradict our current findings. Additionally, given the level of technical expertise required for the implementation of this approach, acquisition and setup of the technical assistance required was rather time-consuming.

In conclusion, this study provides us with proof-of-principle that trans-generational gene expression changes can be correlated with subtle changes DNA methylation and highlights the importance of assessing the impact of dietary changes in future generations.

Chapter 5:

Conclusion

5 Chapter 5: Discussion

5.1 Summary of Results

The purpose of this study was to investigate the effect of paternal dietary FA intake on parameters of male fertility, embryo development, and methylation dependent gene expression in viable offspring.

In chapter 3, we examined parameters of fertility and embryo development of offspring from male mice fed diets of varying FA levels from weaning to maturity. We found no significant differences in male fertility outcomes other than sperm motility, where sperm velocity was higher in male mice fed the folate deficient diet compared to those fed the control diet. However, sperm characteristics for all diet groups were within the normal range.

We showed that embryo congenital anomalies were dependent on folate status, such that male folate deficiency resulted in a higher number of embryos affected by gastroschisis, omphalocele, developmental delay, or skeletal anomalies. Embryos from male mice fed a folate deficient diet also had a lower prevalence of ossification in the frontal, parietal, and interparietal bones of the skull. We did not find any diet-dependent size differences among embryos. However, we did find that placental size was directly proportional to FA intake, such that increasing paternal FA intake resulted in larger placenta, as measured by diameter and weight. This suggests that viable embryos not affected by a congenital anomaly grew normally despite differences in placenta size, but that placenta size could have contributed to more subtle developmental effects, such as differences in bone ossification. Together, the data from this study show that altered FA intake does not negatively affect male fertility parameters, such as sperm number and fertilization events, but that it influences placenta and embryo growth and development.

In chapter 4, we hypothesized that differences in gene expression may underlie paternal FA-dependent phenotypes in his embryo/offspring and future descendants. To determine whether gene expression differences associated with grand-paternal FA intake were due to differences in CpG island methylation status, we identified candidate heritable, epigenetically regulated genes by characterizing their promoter CpG island densities. We then carried out bisulfite amplicon sequencing on liver tissue DNA from F3 descendants of male mice exposed to a FA defined diet either in early development or post-weaning. We identified diet-dependent methylation subtle differences in the sequenced region of the promoter CpG island of the *Chka, Cish,* and *Pdgfc* genes. This suggests that expression of these genes may be trans-generationally, epigenetically regulated under conditions of folate deficiency or supplementation. *Chka* and *Pdgfc* were the only genes for which there were gene expression and CpG island methylation differences. These findings do not exclude the possibility that other epigenetic mechanisms, such as histone modifications or miRNA expression, may underlie the F3 hepatic gene expression differences of the other candidate genes.

5.2 Implications in Human Health

In recent decades, the association between FA and NTDs has been at the forefront of public health awareness and policy. The aim of this study was to highlight the effect of FA intake on male fertility and embryo development parameters and determine whether transgenerational differential gene expression in descendants of male mice is epigenetically regulated.

Based on our results and other studies, we show that it is important that men of reproductive age, in addition to women of child-bearing age, consume a diet adequate in FA in order to prevent altered embryo development in their offspring. Our results suggest that while the fortification program has been a success in the prevention of NTDs, it may have also benefited

men in terms of their reproductive success. We did observe a higher number of congenital anomalies/developmental delays among embryos from male mice fed the supplemented diet; however, this was not significant. More studies will have to be conducted to further explore the degree to which FA in men may affect their reproductive and offspring health.

This study also highlights the potential for transgenerational, heritable effects of FA intake in men. We have clearly shown that direct exposure of males and their gametes can impact their immediate offspring. We have also shown that gene expression in unexposed descendants can also be dependent on grand-paternal FA intake and that they may be due, at least in part, to changes in CpG island methylation patterns. Interestingly, we found two instances in which gene expression and methylation changes, although subtle, were proportional. As such, it is imperative to examine future generations to determine whether there are heritable phenotypic effects due to epigenetic modifications in the ancestors, and whether time of paternal exposure exacerbates these effects. Our results suggest that there are heritable effects induced by altered paternal FA intake. Further studies may elucidate additional mechanisms behind these heritable phenotypes.

5.3 Future Directions

The results of this study have provided numerous potential avenues for future work. The animal model used for this study was designed similar to a previous one conducted in our lab, with the intention of reproducing previous findings. However, we did not see the same effects. It is possible that the reduced pregnancy rates in the FA control group, compared to those observed in the previous study, may have nullified any effect of FA deficiency or supplementation on fertility rates. Therefore, it would be of interest to repeat this experiment once again to confirm one way or another whether fertility rates are indeed affected by paternal FA intake.

Furthermore, characterization of promoter CpG island methylation in candidate genes identified by microarray analysis was not completed for all candidate genes. For example, we were not able to PCR amplify the promoter CpG islands of *Chdh* and *Mapk8*. It would be of interest to successfully amplify and sequence these regions to elucidate whether they are epigenetically regulated. Additionally, DNA methylation may not be the only epigenetic mechanism mediating heritable phenotypes. Thus, it would be beneficial to examine other potential mechanisms underlying these heritable phenotypes, such as histone modifications and miRNA expression.

5.4 Conclusions

In conclusion, results of this thesis highlight the importance of paternal dietary FA intake and its effects on male fertility and embryo development. Paternal dietary FA intake did not affect male fertility, but did have an effect on embryo development. Folate deficiency resulted in altered embryonic development as shown by the increased incidence of congenital anomalies and developmental delay, as well as reduced prevalence of ossification in embryonic skull bones. Embryos not affected by congenital anomalies or developmental delay grew normally. Finally, this thesis highlighted the importance of investigating the effect of (grand)paternal FA exposures on future generations, providing a proof-of-principle that altered gene expression in future generations have the potential to be epigenetically regulated through the male line and modifiable by diet.

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