The effect of dietary folic acid intake on somatic and germ cell mutations in the transgenic MutaMouse model

By

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Abstract

Folate is an essential water soluble B-vitamin required for DNA synthesis and methylation. Folate deficiency has been associated with increased risk for some cancers, male subfertility, and causes megaloblastic anemia and neural tube defects. We propose that the ability of folate deficiency to induce genetic mutations underlies, at least in part, its association with these conditions. *In vitro*, supplemental folic acid (FA) intake protects against chemical mutagens, however it remains unclear whether or not elevated FA intakes are detrimental or beneficial to DNA mutations *in vivo*. Using a transgenic mouse model (MutaMouse), we investigated the effects of FA deficient, control and supplemented diets on somatic and germline DNA mutation frequency and genome instability in male mice. Male mice were fed the diets for 20 weeks from weaning. Half of the mice from each diet group were injected with *N*-ethyl-*N*-nitrosourea (ENU) (a known mutagen) after 10 weeks on diet to evaluate a diet by environmental mutagen interaction. Inadequate FA intake induced micronuclei formation in red blood cells and DNA mutations in bone marrow. In contrast to previous studies, deficient FA intake had no effect on sperm counts or mutations in sperm DNA. Supplemental FA intake had no effect on somatic or germ cell mutations or the induction of mutations by ENU. This study highlights the importance of having an adequate FA intake to ensure DNA integrity and prevent chromosomal damage and also alleviates hypothetical concerns related to high intakes of FA in the Canadian population.

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Chapter 1: Introduction

Chapter 1: Introduction

1.1 Overview of folate mediated one-carbon metabolism

Folate is an essential, water-soluble B-vitamin present in cells as a family of enzyme cofactors responsible for chemically activating one-carbons [1]. Natural sources of folate include dark green vegetables, tropical fruits and legumes. Folic acid (FA), the synthetic and more stable form of folate, is added to fortified foods such as enriched white flour or pasta, and to vitamin supplements [1] . In populations with mandatory FA fortification, FA is the major form of folate consumed.

Folate, also termed pteroylglutamate, consists of a pteridine moiety attached via a methylene group to p-aminobenzoylglutamate (Figure 1-1) [1]. It can be degraded by irreversible oxidative cleavage releasing pteridine and *p*-aminobenzoylglutamate. Folate from natural food sources is typically in the form of 5-methyl tetrahydrofolate (5-methyl-THF) with a variable length polyglutamate tail ranging from 2 to 9 residues in length depending on the food. However, only monoglutamate forms of folate are transported effectively into intestinal cells; therefore the polyglutamate tail must be cleaved by γ-glutamyl hydrolase, which is located in the intestinal brush border, to allow for folate uptake. Intestinal absorption of folate is achieved by a proton-coupled folate transporter [2]. Following transportation into cells, the polyglutamate chain is restored via folylpolyglutamate synthetase; this ensures that folate will not be transported back out of the cell since folates with longer polyglutamate tails have higher affinity for folate-dependent enzymes [1]. Intracellularly, 5-methyl-THF is converted to THF by methionine synthase (MS) which transfers a methyl group to homocysteine to form methionine (Figure 1-2). THF is the bioactive form of folate.

FA, also termed pteroylglutamic acid, is the oxidized form of folate, and contains only a single glutamate. FA has a higher bioavailability than 5-methyl-THF and can be readily taken up by intestinal cells. However, FA is not bioactive and must be sequentially reduced to dihydrofolate (DHF) and then THF by dihydrofolate reductase (DHFR) (Figure 1-2) [1,3]. The bioavailability of folate from natural sources is approximately 50-60% of that of FA [4]. 5-methyl-THF is converted more rapidly to THF than FA as reduction of FA to DHF is a slow, rate-limiting step. Once FA is reduced to DHF, the reduction to THF is rapid [5].

THF, the active, carbon carrying form of folate is a cofactor for the three main biosynthetic pathways of one-carbon metabolism: *de novo* synthesis of purines; *de novo* thymidylate (dTMP) biosynthesis; and, the remethylation of homocysteine to methionine [6].

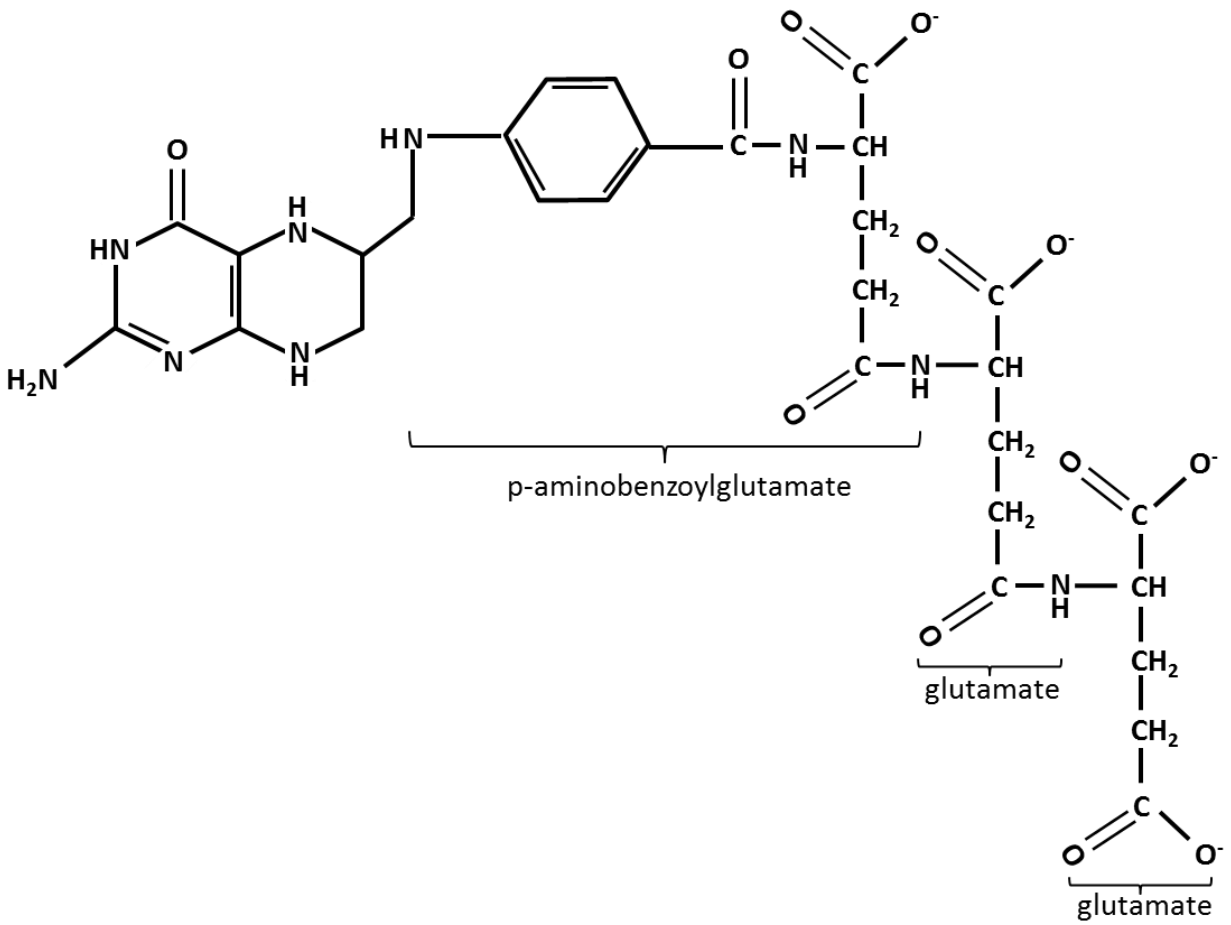

Figure 1-1 Tetrahydrofolate triglutamate structure.

1.2 Folate mediated one-carbon metabolism

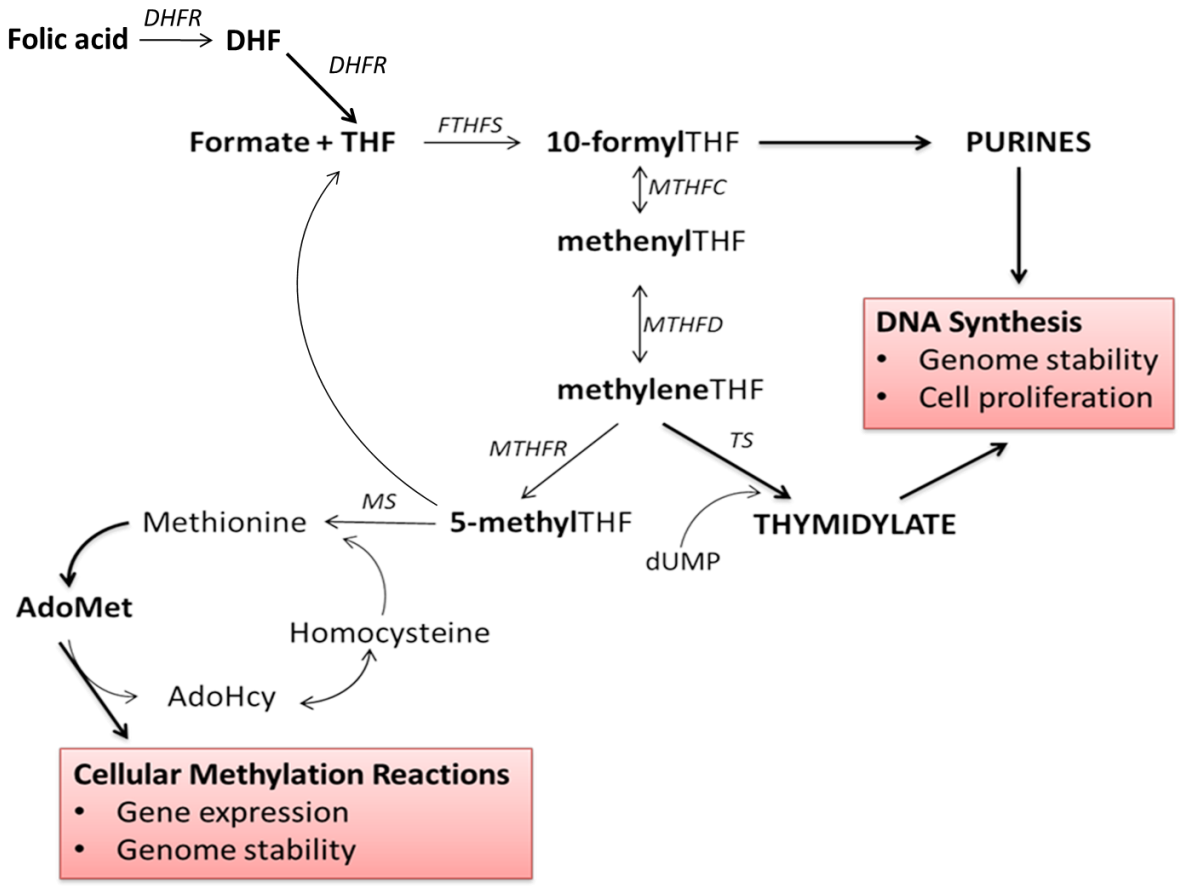

Figure 1-2 Folate mediated one-carbon metabolism. DHF, dihydrofolate; THF, tetrahydrofolate; DHFR, dihydrofolate reductase; FTHFS, formyl tetrahydrofolate synthetase; MTHFC, methenyltetrahydrofolate cyclohydrolase; MTFHD, methylenetetrahydrofolate dehydrogenase; MTHFR, methylenetetrahydrofolate reductase; MS, methionine synthase; AdoMet, S-adenosylmethionine; AdoHcy, S-adenosylhomocysteine; TS, thymidylate synthase; dUMP, deoxyuridine monophosphate.

Folate metabolism is compartmentalized in the cell in the mitochondria, cytoplasm and nucleus, each with specific metabolic endpoints [1]. One-carbon metabolism in the cytoplasm generates purines and methionine. One-carbon metabolism in the mitochondria has three primary functions: the production of one-carbon units in the form of formate to be used in cytoplasmic one-carbon metabolism; to produce glycine; and, the synthesis of formylmethionyl-tRNA used in protein synthesis [7]. One carbon metabolism in the nucleus generates dTMP and purines.

A series of sequential reductive reactions take place in the cytoplasm. Initially, mitochondria-derived formate donates a formyl group to THF, catalyzed by 10-formyl-THF synthetase, to form 10-formyl-THF in an ATP-dependent reaction (Figure 1-2). 10-formyl-THF, required for *de novo* purine biosynthesis provides carbons for positions 2 and 8 in the purine ring. The purinosome, a multi-enzyme complex made up of the purine synthesis enzymes, is responsible for *de novo* purine biosynthesis in the cell [8]. When purines recycled from the purine salvage pathway cannot meet the purine requirements of the cell, components of the purinosome, and consequently *de novo* purine synthesis, are upregulated [9,10]. Methylene-THF synthetase (MTHFS) localizes with the purinosome and enhances its efficiency by delivering 10-formyl-THF directly to the complex [10].

Alternatively, 10-formyl-THF can be reduced to 5, 10 methenyl-THF by methenyl cyclohydrolase and further reduced to 5, 10 methylene-THF in a NADPH dependent reaction catalyzed by methylene-THF dehydrogenase 1 (MTFHD1). 5, 10 methylene-THF is required in the nucleus for *de novo* production of dTMP through the methylation of deoxyuridine monophosphate (dUMP) to dTMP (Figure 1-2). Thymidylate synthase (TS) catalyzes the transfer of a methylene group from 5, 10 methylene-THF to dUMP to form

dTMP which is phosphorylated to thymidine diphosphate (dTDP) and thymidine pyrophosphate (dTTP) by dTMP kinase and nucleoside diphosphate kinase, respectively. TS activity depends on the availability of methylene-THF cofactors in order for the reaction to take place. The enzymes of the *de novo* dTMP pathway, serine hydroxymethyltransferase, TS and DHFR, translocate to the nucleus following sumoylation during S phase, and localize to the replication fork. [11].

5, 10 methylene-THF is also required to produce 5-methyl-THF, which can enter the methionine cycle. 5, 10 methylene-THF is irreversibly reduced to 5-methyl-THF by methylene-THF reductase (MTHFR). 5-methyl-THF is required for the methionine synthase (MS) catalyzed transmethylation of homocysteine to form methionine. In this reaction, 5-methyl-THF donates its methyl group to homocysteine producing THF and methionine [12] (Figure 1-2). Methionine can be adenosylated by methionine adenosyltransferase to S-adenosyl-methionine (AdoMet), which is the universal methyl donor for the many methylation reactions in the cell [9]. The transfer of the methyl group of AdoMet by cellular methyltransferases produces in S-adenosylhomocysteine (AdoHcy), which can be converted by S-adenosylhomocysteine hydrolase to homocysteine re-initiating the methionine cycle.

The conversion of homocysteine to methionine is vitamin B_{12} dependent since vitamin B_{12} is an enzyme cofactor for MS. Vitamin B_{12} deficiency results in low methionine synthase (MS) activity leading to a build-up of homocysteine and 5-methyl-THF. In the context of low MS activity, THF cannot be regenerated for use in the various metabolic endpoints of one-carbon metabolism. This is known as the methyl "trap" because one-carbons are "trapped" as 5-methyl-THF [12].

1.3 Folate and human health

Impairments in folate metabolism due to genetic polymorphisms or low folate intake have been linked to risk for gastrointestinal cancers, NTDs and cardiovascular disease, among other diseases and conditions [1]. The mechanisms that underlie these associations are not always clear. However, they presumably depend on the role of folate in nucleotide synthesis and/or maintenance of cellular methylation capacity.

1.3.1 Folate and megaloblastic anaemia

Folate deficiency is characterized by the development of megaloblastic anemia, a type of anemia defined by the development of large, abnormal erythroblasts. Folate is necessary in the production of three of the four nucleotides of DNA and therefore is required for proper DNA synthesis and cell proliferation. Red blood cell (RBC) production, without an adequate supply of folate, defective DNA synthesis leads to an arrest in the cell cycle at the growth phase. RNA and protein synthesis are relatively unaffected by the reduced folate, therefore the cells continue to grow but are unable to synthesize DNA and divide. The resulting erythroblasts have a nuclear/cytoplasmic asynchrony with a large cell volume and an immature nucleus with fine chromatin. These abnormal erythroblasts can be identified as megaloblasts [13,14]. Megaloblastic anemia caused by folate deficiency can be treated with physiological doses of FA [15].

1.3.2 Folic acid and neural tube defects

NTDs arise when the neural tube fails to close during embryogenesis early in the first trimester of pregnancy, between days 21-28 post conception [16]. The two most commonly observed NTDs in the human population are anencephaly and spina bifida. Anencephaly involves the cranial end of the neural tube and occurs when part of the brain

and skull fails to develop. Spina bifida is the most common NTD and involves the caudal end of the neural tube; it results in nerve and spinal cord damage in the lumbosacral region. Anencephaly results in death prior to or shortly after birth, whereas spina bifida is typically not lethal but can result in paraplegia and other co-morbidities [16].

In 1964, Bryan Hibbard was the first person to propose an association between FA and NTDs. The folate status of 1,484 pregnant women admitted to the Liverpool Maternity hospital was analyzed by the formiminoglutamic acid (FIGLU) excretion test and a bone marrow biopsy. Women with excessive FIGLU excretion in their urine, an indicator of folate deficiency, were twice as likely to have a baby with a congenital birth defect compared to those with a normal FIGLU excretion [17]. Hibbard's work led to a group of randomized controlled studies that further investigated the association between maternal folate status and NTDs in the offspring. The Medical Research Council (MRC) launched a randomized prevention trial to investigate the effects of periconceptional vitamin supplementation (either with 4 mg of FA or a mixture of seven other vitamins) in 1817 women at high risk of a pregnancy with a NTD due to a previous occurrence. In the cohort of 593 women who received FA supplementation there were 6 infants born with a NTD compared to 21 in the 692 women who did not receive FA supplementation, representing a 72 % protective effect of FA supplementation on the occurrence of NTDs [18]. Another randomised trial initiated by Szeizal and Dudas investigated the effects of vitamin supplementation in the prevention of primary occurrences of NTDs. Women were given a daily vitamin supplement from one month prior to conception until the third month of gestation containing 0.8 mg of FA along with multivitamins or a supplement of trace elements without FA. Among 2052 women who took a supplement without FA there were

6 NTD cases, compared to 0 NTD cases among the 2104 women who took a supplement containing FA [19].

Based on these findings, mandatory FA fortification of white flour and other enriched cereal products was implemented in 1998 in Canada and the United States. Following mandatory fortification, the prevalence of NTDs fell by 46% in Canada. The prevalence of folate deficiency in Canada has also been reduced to less than 1% of the population [20]. The current recommended intake is 400 µg/day of dietary folate equivalents (DFEs) (including fortified foods and natural sources of folate) for adult men and women. Women of child-bearing age are recommended to consume a prenatal vitamin with 400 µg FA/day in addition to the folate intake from natural sources and fortified foods (which is approximately 100 to 200 µg), to achieve an intake of 600 µg/day of DFEs [21,22]. Data from the Canadian Community Health Survey conducted in 2004, following mandatory FA fortification, indicate that only 17.7% of women of child-bearing age consumed ≥ 400 µg folic acid/d from supplements and fortified foods [23]. In addition, approximately 1.2-5 % of women and men had FA intakes above the UL of 1000 µg FA per day.

1.3.3 Folate and cancer Risk

The Canadian Cancer Society estimated for 2015 that there would be approximately 41,000 and 37,000 male and female cancer deaths, respectively, in Canada. Colorectal cancer (CRC) is the second and third most common cancers among men and women, respectively [24]. Cancer can be described as the survival and uncontrollable division of abnormal cells that manage to escape ordinary cell behavior and inherent cell proliferation restraints [7].

Cancer arises from the clonal expansion of a single cell. The proposed mechanism by which a normal cell transforms into a cancer cell is based on Darwinian evolution. Occasionally a spontaneous or induced genetic mutation in a normal cell, known as a driver mutation, can result in a cell with a selective advantage over adjacent cells allowing for preferential growth and/or survival of a clone. There remain some gaps in understanding the evolution of normal cells to cancerous cells however, for a normal cell to transform into a cancerous cell it must obtain multiple driver mutations by achieving hypermutability [7,25]. Although numbers vary depending on the cancer type, most cancers carry between 1000 to 20000 somatic point mutations and a few to hundreds of rearrangements, insertions and deletions with a small handful of these being driver mutations. Therefore genetic mutations have a causal role in the progression and development of cancer [7].

DNA damage left unrepaired or repaired incorrectly, and replication errors as a result of exogenous or endogenous factors lead to genetic mutations. Folate deficiency is associated with an increased risk for a number of cancers likely by predisposing normal tissues to neoplastic transformation [26]. Folate deficiency has the potential to induce genetic mutations through decreased nucleotide synthesis and decreased methylation capacity. The potential for folate to induce genetic mutations may increase the mutation rate of normal cells and increase the probability of a neoplastic transformation. It is also important to consider the role of folate in the progression of established cancer. Although folate adequacy could prevent the initiation of cancer, a supplemented FA intake could promote tumor progression by supporting the high rates of nucleotide synthesis required for the numerous cellular divisions and increased DNA synthesis of cancer cells [26,27].

Low folate intake and single nucleotide polymorphisms (SNPs) in folate related genes have been associated with the risk of gastrointestinal cancers with CRC being the most intensely studied [28]. In multiple published case-controlled studies, individuals with the highest dietary folate intake have a 35% reduction in the risk of CRC development compared to those with the lowest intake [29]. The most well studied association between folate and CRC risk is in individuals with two common SNPs in the MTHFR gene: C677T and A1298C. MTHFR is an enzyme responsible for mediating the flow of folate derived one-carbons between *de novo* nucleotide synthesis and methionine production [30]. The C677 TT and A1298 CC genotypes decrease enzyme activity and increase the availability of 5, 10 methylene-THF for *de novo* dTMP synthesis. Multiple meta-analyses have found a statistically significant inverse relationship between the two SNPs and CRC risk. The C677 TT genotype was associated with a 15-18% decrease in CRC risk [31,32].

1.3.4 Folate, the male germline and fertility

The use of assisted reproductive technologies (ART) has increased dramatically in recent years; it has approximately doubled from 2003 to 2012 [33,34,35,36]. Approximately 30% of male infertiliy issues are considered cases of unexplained sperm deficiencies or low sperm numbers [37]; this leaves the cause of about 70% of male infertility issues unexplained. Infertile men frequently have a high frequency of immature sperm in their ejaculate and sperm with DNA/chromatin damage [38,39,40,41]. In contrast to maternal folate status and effects on the offspring, the association between folate and male reproduction and fertility remains to be extensively studied. *De novo* dTMP and purine synthesis and methylation capacity are critical to any highly proliferative tissue. The process of spermatogenesis involves numerous cellular divisions and is highly dependent on DNA

synthesis and maintenance of methylation patterns. Because of its role in *de novo* nucleotide and methionine synthesis it is likely that folate can impact the male germline and fertility. In fact, low seminal plasma folate is associated with increased sperm DNA damage, decreased sperm counts and density and sperm aneuploidy in healthy men [42,43,44]. In contrast, FA supplementation can be beneficial when administered to males experiencing fertility issues. In a clinical trial, 65 males of couples experiencing infertility issues were treated with 15 mg/d of folinic acid (5-formyl-THF) for 3 months. Administration of 5-formyl-THF improved sperm motility and increased spermatozoa numbers [38]. Additionally, supplemental intake of FA and zinc increased sperm concentrations by 74% in subfertile men [45].

Folate deficiency has been associated with decreased cauda sperm counts and increased sperm DNA damage in mice *in vivo*[46]. Male mice fed a FA deficient diet (0 mg/kg) and kept on the diet for 15 weeks from weaning had significantly lower cauda sperm numbers, a 2-fold increase in expanded simple tandem repeat (ESTR) mutation frequency and an increased DNA fragmentation index in sperm compared to those weaned to a FA sufficient diet (2 mg/kg) [46]. In a separate study, mice fed a FA deficient diet (0.3 mg/kg) throughout life from weaning also showed a decrease in sperm numbers compared to those fed a control FA diet (2 mg/kg) [47].

The consequences of decreased sperm counts and lower sperm DNA quality can be further assessed by investigating male fertility and reproductive outcomes. An *in vivo* study investigated the effects of FA intake on male fertility and found that folate deficient males, fed a 0.3 mg/kg diet throughout life from conception to reproduction, had compromised fertility with a pregnancy rate of 52% compared to mice fed a folate sufficient diet (2

mg/kg) with a pregnancy rate of 85%. Resorption rates were also increased 2-fold in pregnancies sired by folate deficient males compared to pregnancies sired by folate sufficient males [47]. In contrast, pregnancy loss, measured by resorptions, was increased in males fed a supplemented FA diet (40 mg/kg) throughout life [48].

Detrimental effects of folate deficiency on sperm DNA damage, decreased sperm counts and fertility rates could be attributed to the potential of folate deficiency to induce genetic mutations. DNA mutations in the germline not only have direct effects on fertility but have an added risk associated because they can be passed down to offspring and can potentially affect future generations. Detrimental outcomes of inherited germline mutations may include transgenerational genetic instability, embryonic lethality and genetic disease [46]. Our lab observed that embryos from fathers fed a FA deficient diet (0 mg/kg) for 15 weeks from weaning were more likely to have developmental abnormalities such as delayed skeletal ossification and intestinal wall defects and were more likely to be small for gestational age [Moussa et al, unpublished data].

A reduced sperm number generally indicates impairments in spermatogenesis [46]. Spermatogenesis is the process by which mature haploid spermatozoa are produced from spermatids are produced from diploid spermatogonia which are further developed to form mature haploid spermatozoa [49]. Spermatogenesis in mice is initiated 3 to 7 days after birth and continues throughout life [50]. It can be divided into three main phases, a mitotic phase, a meiotic phase and a post-meiotic phase, lasting 10, 11 and 14 days, respectively [50]. During the mitotic phase, the stem cells undergo 6 divisions. The final division results in preleptotene spermatocytes, which proceed into the meiotic phase of spermatogenesis. During the meiotic phase, chromosomes condense and recombine followed by two

successive meiotic divisions that produce haploid spermatids. In the post-meiotic phase, also termed spermiogenesis, spermatids are transformed into mature spermatozoa, defined from spermatids by their flagella, acrosome and lack of the majority of the cytoplasm [50]. Another important modification within the process of spermiogenesis is the replacement of histones with protamines; this allows for tighter compaction of the DNA [50]. The many mitotic divisions throughout spermatogenesis provide windows of sensitivity to alterations in DNA synthesis and repair. Mutations accumulated through the process of spermatogenesis as a result of an inadequate supply of folate could be passed down to offspring and cause irreversible, permanent alterations in the genetic sequence.

It is important for these reasons to investigate possible factors that can induce DNA damage which could lead to genetic mutations and possibly the development of cancer, NTDs, megaloblastic anemia or male subfertility. It is also important to examine possible exogenous or endogenous factors that could prevent the induction of genetic mutations or decrease mutation rates.

1.4 Mutagenic potential of folate deficiency

One of the proposed mechanisms underlying the association of folate deficiency with cancer, NTDs and male subfertility is its potential to induce genetic mutations. Impaired nucleotide synthesis and dysregulated DNA methylation can result in DNA mutations [1]. Proposed pathways that might influence disease outcome include thymidylate (dTMP), methionine and purine biosynthesis, all of which are folate dependent reactions in folate-mediated one-carbon metabolism [1].

Folate is required for the *de novo* synthesis of dTMP catalyzed by TS. In conditions of low folate, TS activity is limited, which can lead to nucleotide imbalances such that

dUMP accumulates and dTMP is low [51]. dUMP can be converted to deoxyuridine diphosphate (dUDP) and then deoxyuridine triphosphate (dUTP) by sequential phosphorylation reactions. Deoxyuridine triphosphate nucleotidohydrolase (dUTPase) is an enzyme responsible for maintaining low cellular concentrations of dUTP by dUTP hydrolysis; however this homeostatic function can be overcome in the presence of high levels of dUTP [1]. The frequency of uracil misincorporation into DNA is largely dependent on the relative intracellular pool sizes of dUTP and dTTP. When concentrations of dUMP and dUDP are high, in cases of folate deficiency for example, dUTPase is unable to maintain a low dUTP: dTTP ratio [1,51]. Increased levels of dUTP lead to increased rates of uracil misincorporation into DNA. dUTP incorporation increases DNA mutations and the introduction of DNA double strand breaks (DSBs) as a result of improper repair, both leading to DNA instability [1]. Individuals with deficient serum folate levels (<4 ng/ml) have a significantly higher leukocyte DNA uracil content compared to those with normal serum folate levels (>6 ng/ml) [52]. A FA deficient diet can also lead to an increase in DNA uracil content in mice [53].

Uracil DNA glycosylase (UDG) is responsible for the repair of misincorporated uracil in DNA by initiating the base excision repair (BER) pathway. UDG cleaves the glycosidic bond and removes uracil forming an apyrimidinic (AP) site. To repair this AP site, the DNA sugar-phosphate backbone is broken creating a nick in the DNA followed by removal of the adjacent bases to the AP site. The number of bases removed depends on the type of BER that takes place, which is currently unknown in the case of uracil misincorporation repair. If short patch BER takes place, a single nucleotide base is removed; however if long patch BER takes place, 2 or more nucleotides are removed [54].

This is done by apurinic-apyrimidinic endodeoxyribonucleases. DNA polymerase then mends the DNA nick and replaces the AP site and adjacent removed bases with the appropriate nucleotides. An elevated uracil concentration increases the chance of uracil misincorporation at close proximity on opposing DNA strands. Simultaneous repair of nicks formed only 12 bp apart leads to DNA DSBs and gene deletions [51,55,56]. DNA DSBs can increase the chances of chromosomal rearrangements such as translocations, duplications and deletions and can ultimately lead to chromosomal breakage if left unrepaired [51]. DNA DSBs, if unrepaired, can lead to the formation of micronuclei. Micronuclei are extra-nuclear bodies that arise when chromosomal fragments or whole chromosomes do not navigate to polar ends of the cell during anaphase and do not get incorporated into the nucleus.

Folate is also responsible for the production of AdoMet, the major methyl donor of the cell [9]. DNA methylation occurs on approximately 1% of the cytosine residues of synthesized DNA. The dogma is that methylated genes are turned off or silenced, and unmethylated genes are turned on. DNA methylation can directly block transcription factors from accessing and binding the gene promoter, critical to gene expression [57]. DNA methylation can also regulate gene expression indirectly through DNA binding proteins that target methylated CpG sites. The DNA binding proteins can specifically bind to methylated cytosine residues and recruit transcriptional silencing complexes. DNA methylation is not only important in moderating gene expression but also in chromatin packaging, and rearranging. In cells with inhibited methyltranferase activity, chromosomal aberrations were observed including deletions, breaks, multibranched chromosomes, isochromosomes and translocations primarily in the pericentromeric region of chromosome

1 [58]. A reduced capacity to synthesize AdoMet as a result of folate deficiency results in a lower level of available methyl groups, which can potentially alter genomic methylation patterns in the cell thus altering gene expression and genomic instability. Many cancers in numerous tissues have been shown to have global DNA hypomethylation and site specific hypermethylation [59], indicating the importance of having an adequate supply of methyl groups for methylation reactions to take place.

Low folate status also has the potential to induce genetic mutations through its role in *de novo* purine synthesis. *De novo* purine synthesis is important in cell proliferation and maintaining genome stability. While altered *de novo* purine synthesis usually results in cell senescence or death, it can lead to nucleotide pool imbalances, and impaired DNA synthesis and repair [60,61]. In a Chinese hamster ovary (CHO) cell line auxotrophic for purines, purine starvation significantly decreased DNA replication, and UV-induced DNA damage repair was abnormal. UV damage induced lesions were left unrepaired and both mutagenicity and lethality in these cells were increased [60].

1.5 Supplemental folic acid intake

Folate deficiency has clear, detrimental effects on genome stability and human disease. However, the effects of long term FA supplementation have yet to be extensively studied, and the potential beneficial effects or risks associated with supplementation remain unclear. With the increased FA intakes in the Canadian population since mandatory fortification, it is important to investigate any potential benefits or risks associated with higher intakes of FA.

In terms of nucleotide synthesis, there is a possibility that an adequate level of FA may not be sufficient for maximum *de novo* purine and thymidylate synthesis especially in

tissues with high rates of cell proliferation. Thus, FA intake above adequate levels could ensure that purine and thymidylate synthesis is maintained and DNA synthesis is maximized, potentially resulting in fewer genetic mutations in highly proliferative cells. This may also pertain to other situations where the need for *de novo* synthesis is high; for example, mutagen induced damage requiring DNA repair may require higher capacity for DNA synthesis. *In vitro*, FA supplementation protects against oxidative stress and apoptosis induced by 7-ketocholesterol, shown to induce apoptosis in smooth muscle, endothelial and monocytic cells [62]. FA supplementation both in *in vitro* and *in vivo* also protects against benzo(a)pyrene (BaP)-induced mutations and chromosomal damage in human liver cells [63]. Similarly, FA supplementation reduced chromosomal aberrations in somatic and germ cells as well as reduced DNA fragmentation in mice treated with daunorubicin, an anticancer drug used in the treatment of multiple malignancies [64].

In contrast, concerns have been raised that FA supplementation might also have detrimental effects. A substantial proportion of the general population have a folate status indicative of FA intakes at or above the Upper Tolerable Intake Level (UL) [65]. It has been hypothesized that supraphysiological doses of FA could result in negative feedback on folate metabolism. High levels of FA could lead to a cellular accumulation of DHF. DHF has been shown to inhibit TS and MTHFR *in vitro*, thus its accumulation could inhibit the formation of dTMP and 5-methyl-THF and reduce DNA and methionine synthesis [66]. Concerns have also been raised regarding supplemental FA intake and the masking of vitamin B_{12} deficiency. MS is a vitamin B_{12} dependent enzyme responsible for transferring a methyl group from 5-methyl-THF to homocysteine to form methionine and regenerate THF. Without the regeneration of THF, nucleotide synthesis is inhibited which can lead to

megaloblastic anemia, an indicator of both folate and vitamin B_{12} deficiency. In the context of isolated vitamin B_{12} deficiency (without folate deficiency), increased intakes of FA can provide sufficient THF to bypass the metabolic block in DNA synthesis; therefore, FA treatment can mask vitamin B_{12} dependent anemia. Vitamin B_{12} deficiency also causes neurodegeneration through a pathway independent of folate. Therefore masking of B_{12} deficiency anemia could result in a delay in the diagnosis of vitamin B_{12} deficiency allowing for the progression of vitamin B_{12} deficiency associated neuropathies [57]. These potential benefits and concerns currently remain largely hypothetical and require further investigation.

1.6 Model for studying mutations *in vivo*

To measure the mutagenic potential of FA deficiency as well as a possible mitigation of induced mutations by FA supplementation, a mouse model specifically designed for studying mutation induction in a broad range of tissues *in vivo* is valuable. *In vivo* mutation analysis with the MutaMouse model is particularly useful because the mutagenic properties of folate deficiency have not been studied extensively *in vivo*. The MutaMouse has multiple copies of the lambda bacteriophage vector containing the *lacZ* gene (λgt10*lacZ*) in all of its cells, both germ and somatic. The reporter gene allows for detection of loss of function mutations that can be quantified and used to calculate a mutant frequency (MF). The *lacZ* assay is used to quantify mutations frequencies in the MutaMouse model and has been routinely used for genotoxicology studies over the past 20 years in order to analyze the risk of specific environmental exposures on MF in various tissues [67–70].

1.7 Hypothesis

Dietary FA intake will have an effect on germ and somatic mutations in the MutaMouse. Chronic FA deficiency will cause a higher mutation frequency in somatic and germ cells and exacerbate ENU-induced mutations. Supplemental levels of dietary FA intake will have a protective affect against mutations caused by ENU.

1.8 Objectives

1. I will examine the effect of male dietary FA intake on somatic DNA damage by measuring both mutation and micronucleus frequency in somatic DNA
2. I will examine the effect of male dietary FA intake on germline DNA damage by measuring mutant frequency in sperm DNA
3. I will determine the effect of a FA deficient or supplemented diet on the induction of DNA mutations by a known mutagen.

Chapter 2: General overview of the methods

Chapter 2 : General overview and rationale of methods

The following chapter outlines the animal study design, the background and rationale for the common methods and models used, and results from the study that are generalizable to both studies detailed in chapters 3 and 4.

2.1 Animal model: the MutaMouse

The MutaMouse transgenic strain was utilized for this study. The MutaMouse has multiple copies of the lambda bacteriophage vector containing the *lacZ* gene (lambdagt10*lacZ*) in all of its cells, both germ and somatic. These mice were created via pronuclear microinjection in which approximately 150 copies of the λgt10*lacZ* vector were injected in the male pronucleus of fertilized female BALB/c x DBA/2 CD2F1 eggs. The strain has been intercrossed for multiple generations. The MutaMouse strain has approximately 29.0 ± 4.0 copies of the vector in tandem repeats integrated in a head to tail manner at a single site on chromosome 3 [71]. Vector DNA extracted from any tissue of these transgenic mice can be packaged into phage and used to infect *E.coli*, leading to the formation of plaques following an overnight incubation. A selective media containing phenyl-β-D-galatopyranoside (P-gal) allows for positive selection and enumeration of phages harbouring a mutated copy of the *lacZ* gene. A non-selective media is used as a control for determining the number of background plaque forming units (PFUs) plated (Figure 2-1). Each plaque represents one genome isolated from one cell. Plaques on both media are counted and mutant frequencies can be calculated for a given tissue as follows: [72].

$$\text{PFUs/ µl} = \left(\frac{\#\ of\ titer\ plaques}{volume\ of\ cells\ plated\ (15\ \mu l)}\right)$$

Total PFUs = $(PFUs/\mu l \times (volume\ of\ cells + volume\ of\ packaged\ phage\ particles - volume\ of\ cells\ plated\ on\ titer\ plates))$

Mutant frequency = $\left(\frac{Total\ mutant\ plaques}{Total\ PFUs}\right)$

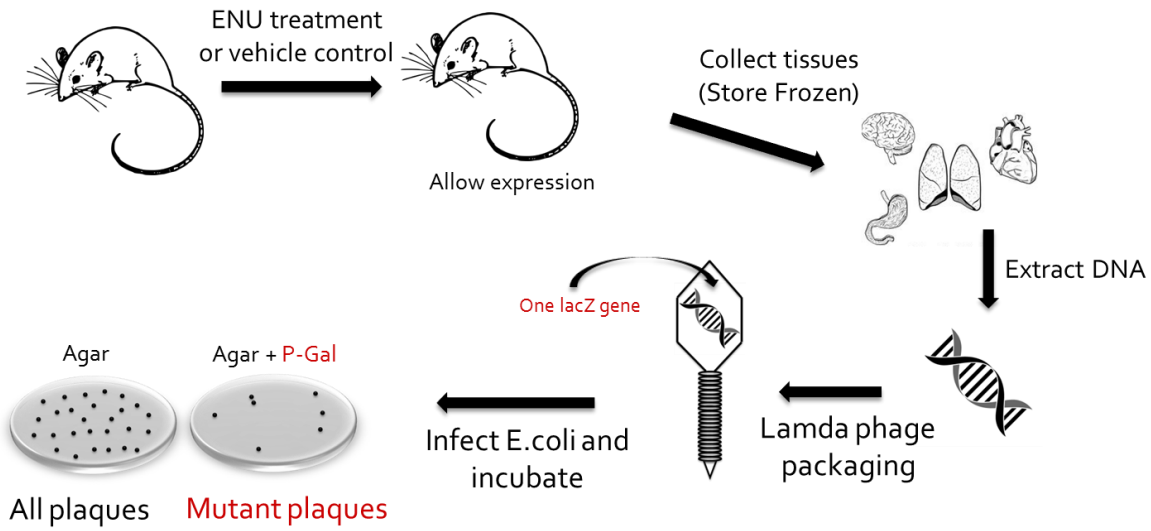

Figure 2-1 Schematic of the *lacZ* mutation assay

2.2 Animal study design

All mice were cared for in accordance with the Guidelines of the Canadian Council on Animal Care (CACC), described in the CACC Guide to the Care and Use of Experimental Animals [73]. The study was approved by the Health Canada Ottawa Animal Care Committee. Sixty weanling males derived from the Health Canada in-house MutaMouse colony were used and housed at standard humidity and temperature with a 12 hour light cycle. They had *ad libitum* access to food and water.

At 5 weeks of age, mice were fed one of three FA-defined diets based on the AIN-93G formula and kept on diet for 20 weeks *ad libitum* (Figure 2-2) [74]. The diets contained 0 mg/kg FA (deficient), 2 mg/kg FA (control) or 8 mg/kg FA (supplemented) (Dyets, Inc.; Bethlehem, PA). These diets have been routinely used in our lab and are physiologically relevant. The deficient diet (0 mg/kg) is used to represent an inadequate dietary FA intake, which in past studies has been shown to decrease both tissue and plasma folate but not render the rodents completely folate deplete, as folate can be synthesized by intestinal bacteria [75]. The control diet (2 mg/kg) corresponds with the Recommended Dietary Allowance for adult humans, 0.4 mg per day. It is representative of an adequate dietary FA intake for rodents as recommended by the American Institute of Nutrition [74]. The supplemented diet (8 mg/kg) is 4-fold the control diet and is used to represent a dietary FA intake of 1.6 mg per day in adult humans, representing a maximal folate intake, which can be achieved in extreme cases with the combined intake of natural folate and FA from natural dietary sources, FA fortified foods and FA containing supplements available over-the-counter in Canada. This would be approximately the maximal dose of FA intake.

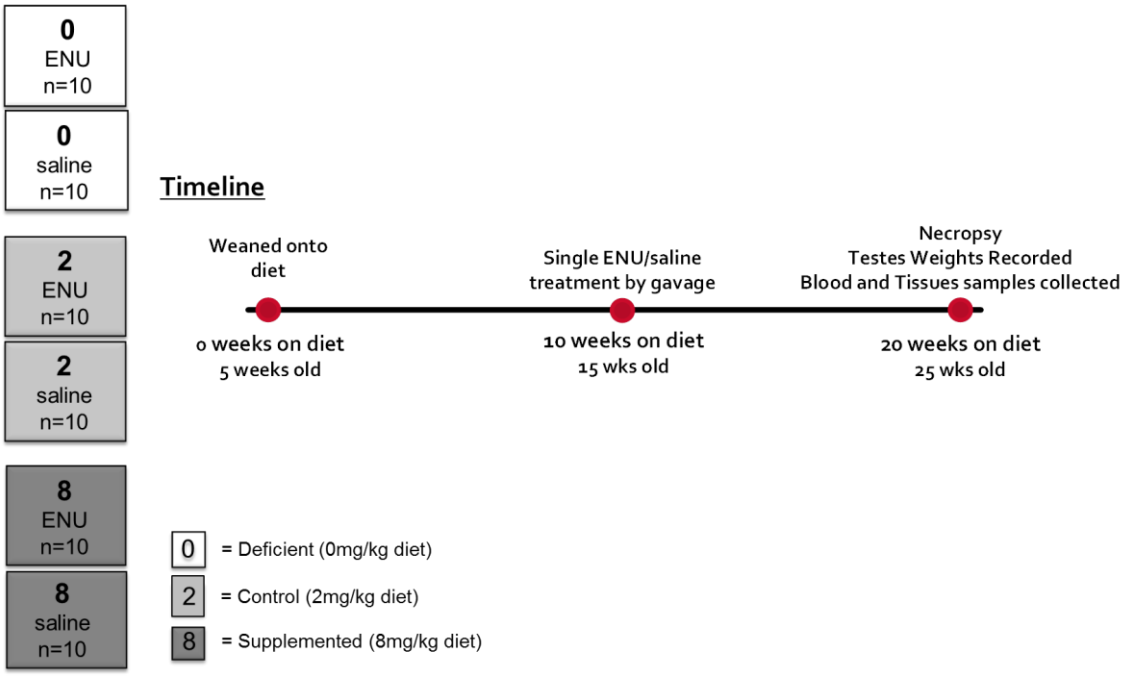

Figure 2-2 Animal study design. Male mice were fed a FA deficient, control or supplemented diet from weaning for 20 weeks ad libitum. Half the mice in each diet group were given a 5 µl/g dose of ENU at 50 mg/kg or saline by gavage.

Following 10 weeks on FA defined diets, half of the mice from each diet group were given a 50 mg/kg dose of ENU dissolved in phosphate buffer by gavage (n = 10) and the other half in each diet group (n = 10) were given saline by gavage. The mice were then kept on diet for another 10 weeks. The timing of ENU exposure was planned for two reasons. First, waiting 10 weeks ensured that tissue folate stores had reached homeostasis and were representative of the consumed FA diet at time of ENU exposure. In mice, highly proliferative tissues such as bone marrow and plasma can become folate deplete following in less than 5 weeks on a FA deficient diet. The liver and other tissues, however, can take up to between 5 and 10 weeks on a FA deficient diet to become folate deplete [76]. Second, the timeline ensured that the cauda sperm extracted from the mice were exposed to ENU as spermatogonial stem cells allowing for detection of induced mutations. The OECD guidelines recommends a sampling time (period of time without chemical exposure) of a minimum of 49 days to ensure mature cauda sperm collected were exposed to the chemical mutagen as spermatogonial stem cells [77].

2.3 Necropsy and tissue collection

Each mouse was weighed weekly from weaning until the week prior to necropsy for a total of 19 weeks. After 20 weeks on diet, the mice were killed under isoflurane anesthesia by cardiac puncture followed by cervical dislocation. Tissues collected that pertain to my studies include bone marrow, cauda and testes. These tissues are associated with folate-dependent pathologies including megaloblastic anemia and decreased cauda sperm counts. Bone marrow and sperm progenitor cells are highly proliferative and sensitive to FA deficiency owing to their high demand for *de novo* DNA synthesis.

The right and left cauda and right and left testes were dissected. The right and left testes were weighed separately. Bone marrow from the right and left femur was flushed out with phosphate buffered saline (PBS). All tissues were flash frozen in liquid nitrogen and stored at -80 °C until use. Blood was drawn via cardiac puncture into a heparin coated syringe (5:1 ratio of blood to heparin) and collected in K_2EDTA collection tubes and sent fresh overnight to Litron Laboratories. The remaining blood was separated into RBCs and plasma by centrifugation within an hour of collection and flash frozen in liquid nitrogen and stored at -80 °C until later use.

2.4 Folate extraction from red blood cells

Seventy-five microliters of RBC was placed into 200 µl of extraction buffer ((2% (w/v) sodium ascorbate (Sigma-Aldrich, St. Louis, MO), 0.2 M beta-ME, 0.05 M HEPES pH 7.85 (Sigma,), and 0.05 M CHES pH 7.85 (Sigma)). Samples were centrifuged at 1500 × *g* for 5 minutes at 4°C. Twenty-five microliters of the resulting supernatant was pipetted from each sample and stored at -80°C for use in protein content analysis described below. Samples were boiled at 100°C for 10 minutes then placed on ice for 10 minutes. Samples were then centrifuged at 21,000 × *g* for 5 minutes at 4°C and the exact volume of the resulting supernatant was recorded and collected into a 1.5ml tube. Rat serum conjugase (charcoal treated and dialyzed rat serum (Pel Freez)) was added at a volume of 0.25× of the second supernatant to each sample. Samples were placed in a 37°C waterbath (Fisher Scientific, Waltham, MA) for 3 hours, and then boiled for 5 minutes at 100°C. Samples were then centrifuged at 21,000 × *g* for 5 minutes at 4°C. The supernatant (folate extract) was collected into a 1.5 ml tube on ice and the exact volume was recorded. Extracts were stored at -80°C until use.

2.5 Plasma and red blood cell folate measurement

Folate extracted from RBCs as described above and plasma folate was measured for a subset of samples (n=5) from each of 6 experimental groups using the *Lactobacillus casei* (*L.casei*) microbiological assay as described [78]. In this assay, folate is extracted from the tissue of interest and plated with *L. casei*. *L.casei* are dependent on folate for growth, therefore their growth can be directly correlated with total folate content in a given sample. The bacteria are only able to metabolize monoglutamate forms of folate, for this reason sample folates were converted to the monoglutamate form by the addition of rat serum conjugase. *L. casei* has a higher affinity for folate monoglutamates in the form of 5-methyl-THF, the predominate form of folate in circulation [79]. Plasma folate is representative of recent folate intake and is the best indicator of recent folate status [80]. Tissue folate, such as that extracted from liver and RBCs, reflects long-term folate status in the body.

2.6 Red blood cell protein content

Folate in tissues is protein bound, therefore RBC folate concentrations must be normalised to total protein content. Proteins were quantified using the modified Lowry assay as previously described [81]. In this assay, two reactions take place that allow for protein content in a given sample to be determined. In the Biuret reaction, a purple complex is formed from the reaction between the peptide bonds of proteins with copper in an alkaline solution. The Cu^{+} complex reacts with Folin-Ciocalteau (FC) reagent in the FC reaction to produce an enhanced blue color which can be measured using a spectrophotometer [82].

2.7 Germline DNA damage analyses

In order to assess the effects of FA intake on the germline, testes weights were measured, manual sperm counts were performed and a *lacZ* mutation analysis was completed on DNA extracted from cauda epididymal sperm.

2.7.1 Testes weight and cauda sperm counts

Testes weight and cauda sperm counts are crude measures of male fertility. Smaller or larger testes are correlated with lower or higher sperm stores, therefore, testes weight can be an indicator of germ cell numbers [83,84]. Germline DNA mutations may have a negative effect on the process of spermatogenesis which could lead to decreased cauda sperm numbers and lower testes weights.

2.7.2 Cauda sperm *lacZ* mutant frequency analysis

The *lacZ* assay allows for the direct analysis of mutational events in sperm DNA as a result of a dietary intervention. The *lacZ* assay provides a means to measure germ cell MF and is sensitive enough to detect mutagenic effects in small sample sizes and low chemical doses. Mature sperm are stored in the cauda epydidimis prior to ejaculation. The process of spermatogenesis is approximately 49 days in mice and therefore a 70 day sampling time (time following exposure to a chemical mutagen or dietary intervention) is recomended prior to specimen collection [77]. A sufficient sampling time ensures that sperm collected from the cauda would have been exposed to the chemical mutagen (ENU) as spermatogonial stem cells allowing for the fixation of mutations in the germ line [77]. In the case of FA exposure, sperm collected from the cauda would have been exposed to FA diets for multiple rounds of divisions as primordial germ cells and throughout spermatogenesis.

Therefore, chronic FA exposure can be analyzed in the *lacZ* assay to allow for the fixation of mutations as well as the accumulation of mutations throughout spermatogenesis.

2.8 Somatic DNA damage

In order to assess the effects of FA on somatic DNA damage, *Pig-a* mutation frequency and micronucleus frequency in RBCs were measured as well as *lacZ* mutation analysis on DNA extracted from bone marrow. Bone marrow was used in the *lacZ* assay as it is a highly proliferative tissue with a high demand for folate, and because it is the source of RBCs, it is associated with megaloblastic anemia. It was assumed that this tissue would demonstrate folate-dependent DNA damage.

2.8.1 *Pig-a* mutant frequency analysis

Previously, our lab found that mice fed a FA deficient diet (0 mg/kg) for 18 weeks had a significantly higher *Pig-a* MF compared to mice fed a supplemented FA diet [85]. The *Pig-a* gene encodes an enzyme required for the production of the first intermediate in the biosynthetic pathway that produces the glycophosphatidylinositol (GPI) anchor. The *Pig-a* gene is located on the X chromosome and is the only X-linked gene of approximately 24 genes involved in GPI-anchor synthesis. A lack of GPI anchor synthesis is a result of either one mutation in the *Pig-a* gene or a mutation in both copies of one of the other autosomal genes. It is unlikely that a mutation would arise in both copies of an autosomal gene, therefore the *In Vivo* MutaFlow® assay assumes that a lack of GPI-anchor synthesis is a result of a mutation in the *Pig-a* gene [86,87]. In order to analyze the mutations in the *Pig-a* gene, an antibody against the GPI- anchored cluster of differentiation 24 (CD24) glycoprotein is used. Cells with a mutated copy of the *Pig-a* gene will not form GPI anchors and therefore will not express the CD24 glycoprotein (or other GPI-anchored proteins) on

the cell surface. A nucleic acid dye is used to allow differentiation between reticulocytes (RETs) and mature erythrocytes as reticulocytes contain RNA while mature erythrocytes do not. The frequency of RETs and erythrocytes lacking the CD24 surface marker (*Pig-a* mutants) can then be measured by flow cytometry in the *In Vivo* MutaFlow® assay [88]. The assay displayed a high sensitivity and specificity in the detection of five known mutagens and carcinogens including ENU. Background *Pig-a* mutant frequencies in RETs and RBCs of rodents are very low at approximately 5×10^{-6}, which may promote the sensitivity of the assay [89]. Further investigations are warranted to validate that GPI-marker-deficient cells harbor a mutation in the *Pig-a* gene, although a few studies have found that GPI-deficient cells consistently have a *Pig-a* gene mutation [90–92].

2.8.2 Micronucleus assay

The *In Vivo* MicroFlow® assay uses a staining technique to measure micronucleated cells by flow cytometry. Chromosomal breakage and damage can lead to fragmented DNA, which can form micronuclei. Micronuclei are small nuclei containing chromosomal fragments and/or whole chromosomes that had not moved to the polar ends of the cell during anaphase and are not incorporated into the nucleus. Micronuclei can form in a variety of cell types but are most easily observed in RBCs as they undergo the process of enucleation and can therefore be assessed by the presence (micronucleus) or absence (normal) of a nucleus. The frequencies of micronucleated erythrocytes (MN-RBCs) and micronucleated RETs (MN-RETs) are valid measures of chromosomal breakage in circulating cells. In splenectomised humans, who would have reduced capacity for clearance of MN-RBCs and MN-RETs from circulation, folate deficient individuals showed both higher uracil levels in DNA and a higher frequency of micronucleated cells

compared to folate adequate individuals. It was also found that FA supplementation of folate deficient-individuals lowered DNA uracil levels and MN-RETs [51]. Similarly, our lab recently found that male mice fed a FA deficient diet for 18 weeks showed a 1.8 fold and a 2-fold increase in MN-RETs, and a 1.5-fold and 1.6-fold increase in micronucleated MN-RBCs compared to male mice fed a control and supplemented diet, respectively [85].

The *In Vivo* MicroFlow® assay uses an antibody against the cluster of differentiation 71 (CD71) transferrin receptor to distinguish RETs (immature erythrocytes) from mature erythrocytes (RBCs). CD71 is present on RETs, but is lost following differentiation into mature erythrocytes. DNA is then stained allowing for the detection of micronuclei by flow cytometry by flow cytometry [93].

2.9 General results

2.9.1 Body weights

Body weights or weight gain did not differ significantly among the diet groups (Figure 2-3). Differences in means among diet groups were analyzed for significance by One-Way ANOVA. The data indicate that the mice consumed the given equally and any significant differences in other endpoints were likely not a result of differences in body weight but rather related to the difference in folate intake.

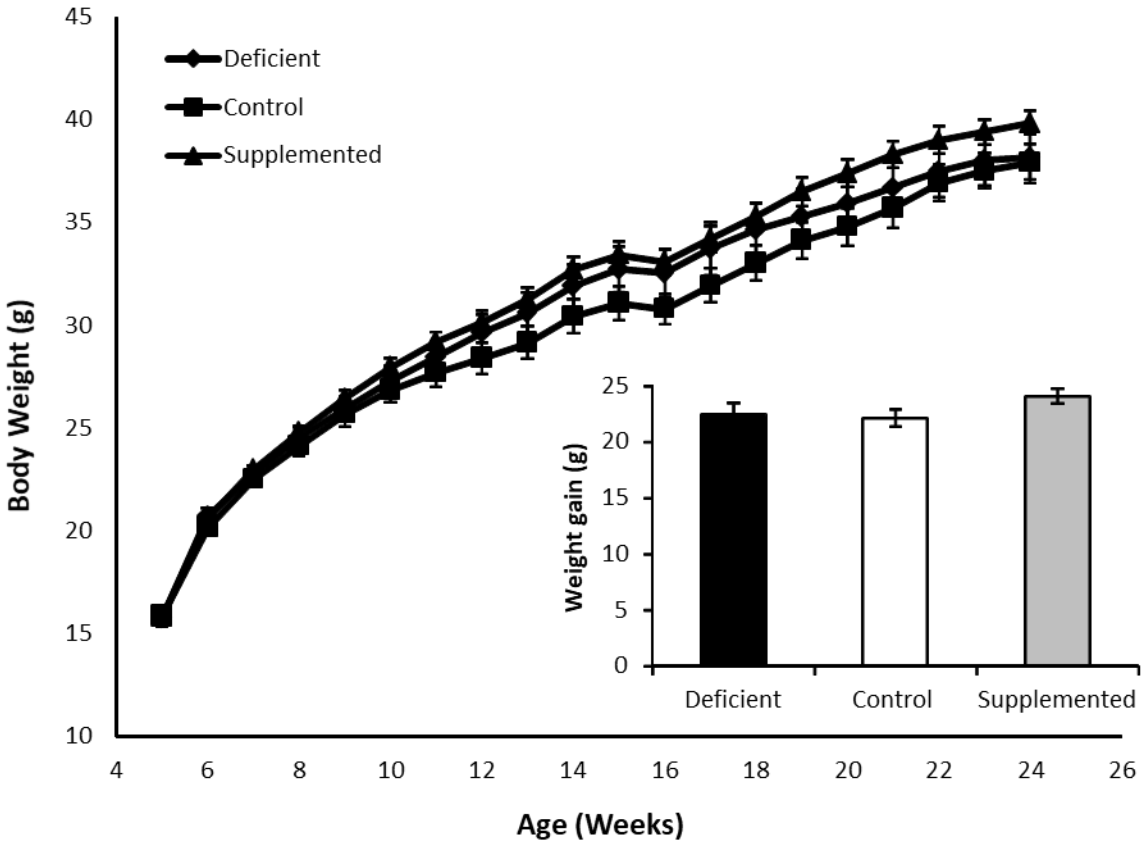

Figure 2-3 Body weights of male mice fed FA defined diets. Diamonds, deficient diet; squares, control diet; triangles, supplemented diet. *Inset*, total body weight gain from weaning. Black bar, deficient diet; white bar, control diet; grey bar, supplemented diet. Data are presented as mean ± SEM. Differences were assessed by One-Way ANOVA.

2.9.2 Plasma folate

Males fed a FA deficient and supplemented diet showed an 85% decrease and a 107% increase in plasma folate concentrations, respectively, compared to males fed the control diet ($p<0.05$) (Figure 2-4). Differences in means among diet groups were analyzed for significance by One-Way ANOVA and a Tukey's post-hoc analysis. These results indicate that the folate status of the male mice was reflective of their dietary intake.

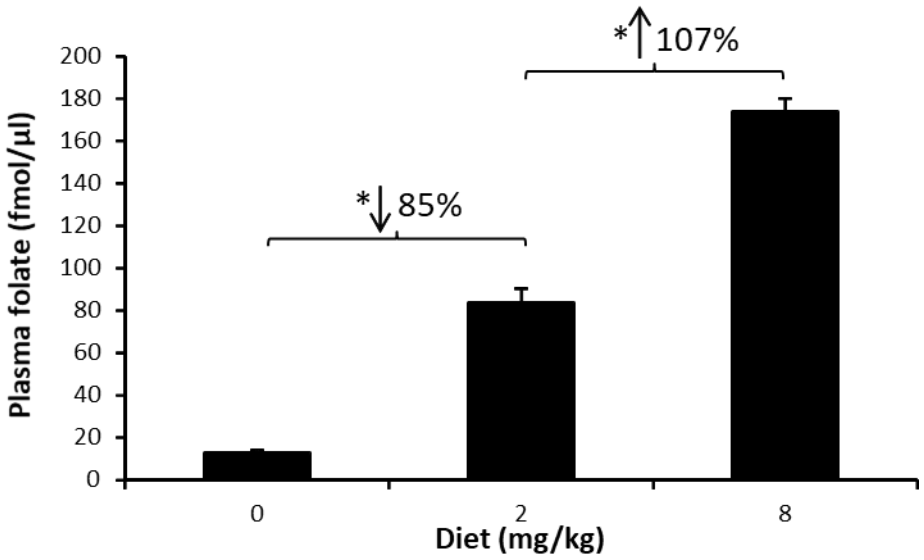

Figure 2-4 Plasma folate. *, significantly different than control, One-Way ANOVA, Tukeys post-hoc analysis ($p<0.05$). Data are presented as mean ± SEM.

2.9.3 RBC folate

Males fed a FA deficient and supplemented diet showed an 88% decrease and a 27% increase in RBC folate concentrations, respectively, compared to males fed the control diet ($p<0.05$) (Figure 2-5). Differences in means among diet groups were analyzed for significance by One-Way ANOVA and a Holm-Sidak post-hoc analysis. These results indicate that folate status in the tissues of the male mice was reflective of their dietary intake.

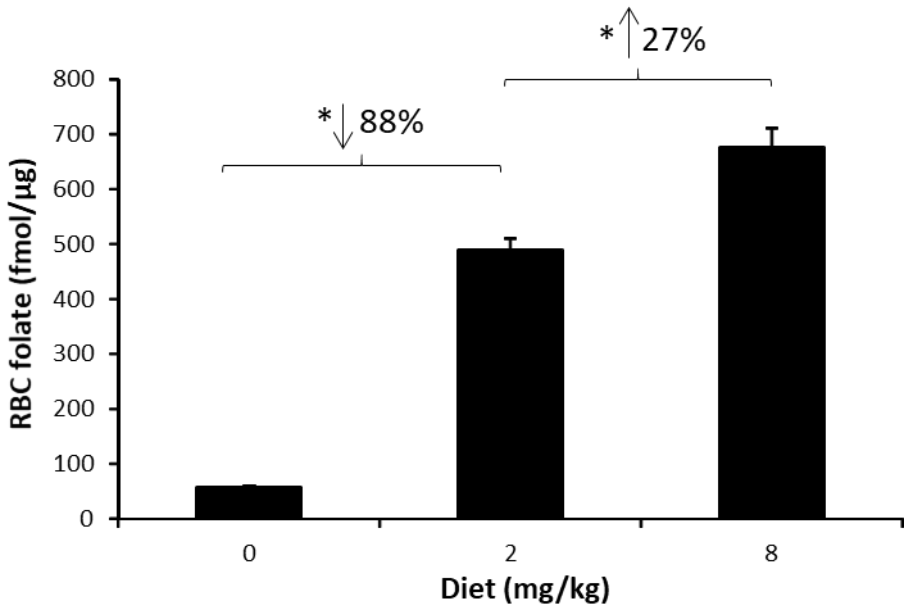

Figure 2-5 RBC folate. *, significantly different than control, One-Way ANOVA, Tukeys post-hoc analysis ($p<0.05$). Data are presented as mean ± SEM.

Chapter 3: Investigating the effects of dietary folic acid intake on parameters of male fertility and germline DNA damage

Chapter 3 : Investigating the effects of folic acid on parameters of male fertility and germline DNA damage

3.1 Abstract

Folate deficiency has been associated with decreased sperm counts and male subfertility. We propose that the detrimental effects of folate deficiency on parameters of male fertility are associated with the induction of genetic mutations in sperm. *In vitro*, folate deficiency has been shown to increase genome instability and DNA mutation frequency as well as exacerbate the mutagenic potential of known environmental mutagens. In addition, FA supplementation could protect against the effects of folate deficiency and perhaps protect against other environmental exposures that affect the germline. In this study, we employed the MutaMouse model to assess the effects of dietary FA intakes on parameters of male fertility and germline DNA point mutations. Male mice were fed one of three FA defined diets: deficient (0 mg/kg), control (2 mg/kg) and supplemented (8 mg/kg) from weaning for 20 weeks. To determine the effects of FA intake on the induction of germline mutations by a known mutagen, half of the mice from each diet group were gavaged with 50 mg/kg *N*-ethyl-*N*-nitrosourea (ENU) after 10 weeks on diet, followed by an additional 10 weeks on diet. In this mouse model, folate deficiency had no effect on sperm counts and testes weights, or germline DNA mutations. ENU exposure 10 weeks before necropsy did not affect sperm counts or testes weight or *lacZ* MF in sperm. FA supplementation did not modulate the effect of ENU-induced *lacZ* mutation frequency. The data suggests that FA intake has no effect on sperm or germline DNA point mutations in the male MutaMouse.

3.2 Introduction

Infertility issues are on the rise in Canada; in 2009-2010 the infertility rates had increased to 11.5 % -15.7 % from 8.5 % in 1992 [34]. The use of ART has also significantly increased; in fact, in 2003 10,656 ART cycles were reported to the Canadian ART Registry compared to 27,356 in 2012 [35,36]. Male infertility contributes to approximately 50% of couple infertilities worldwide [94]. Sperm parameters including: motility, concentration and morphology have been associated with male infertility as well as sperm DNA/chromatin integrity. In humans, sperm with DNA damage are observed more frequently in infertile than in fertile men, and can lead to negative reproductive outcomes. Genetic mutations and chromosomal alterations contribute to DNA damage. Germline DNA mutations can negatively affect reproductive success leading to subfertility or infertility and are of particular interest because of their potential to be passed down to the next generation. Transgenenerational mutations can lead to adverse health effects in the offpsring, including genetic disease and an increased predisposition to cancer [77]. In a human study, sperm with gamma radiation-induced DNA strand breaks were able to fertilize eggs but increased the likelihood of pregnancy loss and developmental delays in the embryo [95]. It is therefore important to investigate factors that may improve or protect against infertility as well as protect against the transmission of DNA damage to the offspring.

Folate is an essential water soluble B vitamin required for three main biosynthetic pathways: *de novo* dTMP, *de novo* purine, and methionine synthesis. *De novo* purine and dTMP synthesis are critical for DNA synthesis and consequently genome stability. Methionine can be converted to AdoMet, the universal methyl donor required for the many methylation reactions in the cell. Decreased thymidylate production leads to increased rates

of uracil incorporation into DNA, which can result in increased DNA mutation rates and DNA double strand breaks as a result of futile cycles of DNA repair [51]. Impairments in purine synthesis can result in altered DNA synthesis and genetic mutations. The lack of AdoMet reduces the availability of methyl groups that can alter cellular DNA methylation patterns thus potentially altering gene expression and causing genomic instability. Therefore decreased methylation capacity can affect genome stability by altering chromosomal rearrangement and potentially blocking access to gene promoters and/or indirectly promoting recruitment of transcriptional silencing complexes.

Low seminal plasma folate is associated with increased sperm DNA damage, decreased sperm counts and density and sperm aneuploidy in healthy men [42,43,44]. Recently it was found that male mice fed a FA deficient diet (0 mg/kg) for 15 weeks had decreased cauda sperm numbers, an increased ESTR mutation frequency and an increased DNA fragmentation index in sperm [46]. The many divisions throughout the process of sperm maturation (spermatogenesis) provide windows of sensitivity to alterations in DNA synthesis and repair, which could be influenced by folate intake. Mutations accumulated through the process of spermatogenesis as a result of an inadequate supply of folate could be passed down to offspring and cause irreversible, permanent alterations in the genetic sequence.

The MutaMouse transgenic rodent mutation (TGR) assay, also known as the *lacZ* assay allows for germline specific mutagen testing using a small sample size and low chemical/environmental doses. The MutaMouse has multiple copies of the lambda bacteriophage vector containing the *lacZ* gene (λgt10*lacZ*) incorporated into the genome of all of its cells, germ and somatic. Extracted DNA from any tissue, including sperm, of

these transgenic mice can be packaged into phage and used to infect *E.coli*, leading to the formation of plaques following an overnight incubation. Plaques on both a media selective for mutants and a non-selective media are counted and a MF can be calculated for a given tissue [72]. The MutaMouse model has been routinely used for genotoxicology studies over the past 20 years in order to analyze the risk of specific environmental exposures on MF in various tissues [67–70].

The growing issue of infertiliy is of concern as is the relationship between folate status and male infertility. Based on our previous findings demonstrating that folate deficiency induces ESTR mutation rates in cauda sperm in mice, and the opportunity to use a well established *in vivo* germline mutation assay with a dietary intervention, we proposed to evaluate the effect of dietary FA intake on germline DNA mutations in the transgenic MutaMouse model.

3.3 Methods

3.3.1 Testes weights

The left and right testis from each mouse were weighed separately on a Mettler Toledo analytical scale (Mettler Toledo, Columbus,OH) at time of tissue collection. Data are presented as the mean testis weight of both.

3.3.2 Cauda sperm counts

The left cauda from each mouse was used for sperm counts. Each cauda was minced with iris scissors in 1 ml of Triton X-100 solution (0.9% NaCl (Sigma) with 0.1% (v/v) Triton X-100 (Sigma-Aldrich)) until no intact piece of tissue was visible by eye. One hundred µl of each sample was diluted 1:8 with Triton X-100 solution (0.9% NaCl and 0.1% Triton-X-100) and sperm counted under the EVOS fl Fluorescence microscope

(AMG, Bothell, WA) at 40× using a haemocytometer. Each sample was loaded and counted twice and the mean was calculated.

3.3.3 Cauda sperm *lacZ* mutant frequency analysis

High molecular weight DNA was extracted from the right cauda of each mouse following the protocol described by O'Brien *et al*. [77]. In short, the cauda was finely minced in PBS and passed through a mesh filter. Cells were resuspended in 1 ml of 1× saline-sodium-citrate (SSC) and lysed with 0.15% sodium dodecyl sulfate. Sperm cells were resuspended in 940 µl of 0.2× SSC and digested overnight with 3.5×10^{-5} M Proteinase K. Following overnight incubation a standard phenol chloroform procedure followed by an ethanol precipitation was performed. DNA was spooled, rinsed in 70% ethanol and dissolved in TE-4 buffer (10mM Tris-HCL, pH 7.6, 10^{-4} M EDTA). DNA was stored at 4°C until use.

Extracted genomic DNA from cauda sperm was used in the *lacZ* assay as described by O'Brien *et al*. [77]. Briefly, the λgt10*lacZ* copies were rescued from 4 µl of extracted genomic DNA using the Transpack Packaging extract (Agilent Technologies, Santa Clara, CA). The host bacteria (galE$^-$ recA$^-$ *E.coli*) was infected with packaged phage particles and plated on minimal agar medium with and without the addition of 0.3% (w/v) P-gal. Plates without the addition of P-gal were used to estimate the total number of PFUs, while the plates with the added P-gal were used to estimate MF. Plates were incubated at 37°C overnight and plaques were counted the following morning. MF per mouse was calculated by dividing the number of mutants by the number of total PFUs. The mean MF was calculated per diet and treatment group. The results for each diet and treatment group were

summed together and the mean was calulcated. A minimum of 96,000 total PFUs were screened per animal.

3.3.4 Statistical analyses

Differences in means among diet and treatment groups for testis weight and cauda sperm counts were analyzed for significance by Two-Way ANOVA. Analyses were performed using SigmaPlot for Windows, version 13 (Systat Software, Inc.).

Cauda *lacZ* MF were fit to a generalized linear model with a binomial error distribution using the *glm* function. Overdispersion was permitted amongst biological replicates using the quasibinomial distribution for *glm*, as inter-individual variability is known to be high for the *lacZ* assay. Mice with less than 96, 000 total PFUs screened were removed from the analysis. Replicates that were outside of the expected binomial error distribution were considered outliers and also removed. Based on these criteria a total of 9 mice were removed out of the 41 mice considered, resulting in a final sample size of 32 mice with 4 or more mice in each of the 6 experimental groups. A 2-factor analysis, setting diet and ENU dose as factors, was performed to identify differences in group means. P-values were corrected for multiple-comparison using the Bonferroni-Holm method. Analyses were performed using R (version 3.2.0, www.r-project.org).

3.4 Results

3.4.1 Testis weights

There were no significant differences between left and right testes weights, therefore the mean weight of the left and right testes was used. A main effect of treatment was observed ($p=0.001$) in mean testis weight such that ENU treated male mice had significantly lower testis weights compared to saline treated males (Figure 3-1A). Among

the saline treated mice, mean testis weight was higher in males fed a FA supplemented diet compared to those fed a FA deficient diet ($p=0.043$) and a FA control diet ($p<0.001$). Testis weight was significantly lower in saline-treated male mice fed a FA control diet compared to those fed a FA deficient diet and exposed to saline, ($p=0.028$) (Figure 3-1A). Among the ENU treated mice, testis weight was significantly lower in males fed a FA control diet compared to those fed a FA supplemented diet (Figure 3-1A).

Given that testis size is proportional to body weight, we examined the effect of diet and ENU treatment on testis weight normalized to body weight; no significant differences were observed among diet groups however, ENU treated mice had lower testis weights compared to those exposed to saline ($p=0.044$) (Figure 3-1B).

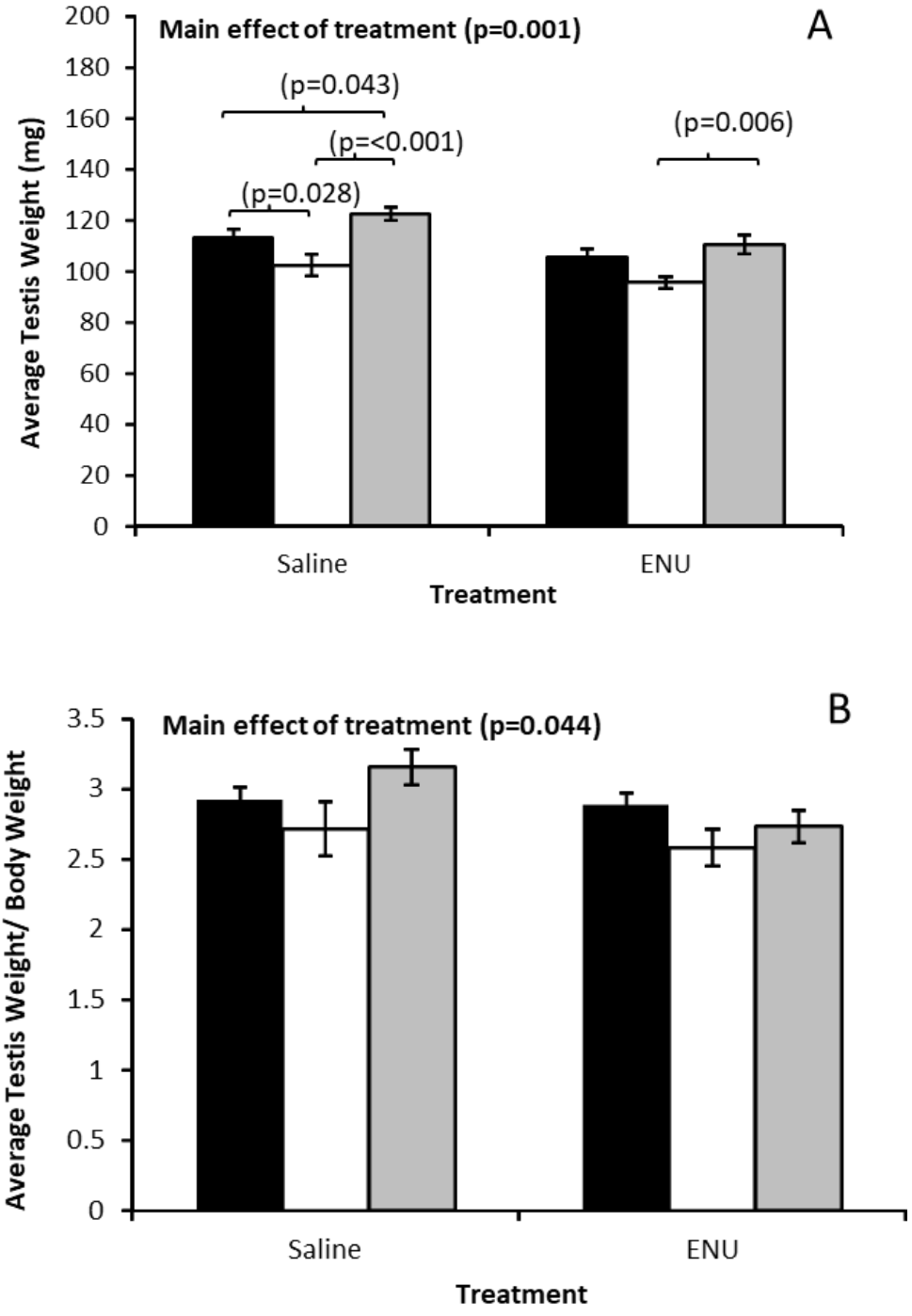

Figure 3-1 Mean testis weights of male mice fed FA defined diets and exposed to ENU or saline. (A) mean testis weights (B) mean testis weights over body weight. Black bar, deficient diet; white bar, control diet; grey bar, supplemented diet. Results analyzed using a Two-Way ANOVA, Holm-Sidak post-hoc analysis. Data presented as mean ± SEM.

3.4.2 Cauda sperm counts

Cauda sperm counts were measured for a subset of mice (n=4) from each experimental group. There were no significant effects of treatment (ENU vs. saline) or FA diet on cauda sperm counts (Figure 3-2).

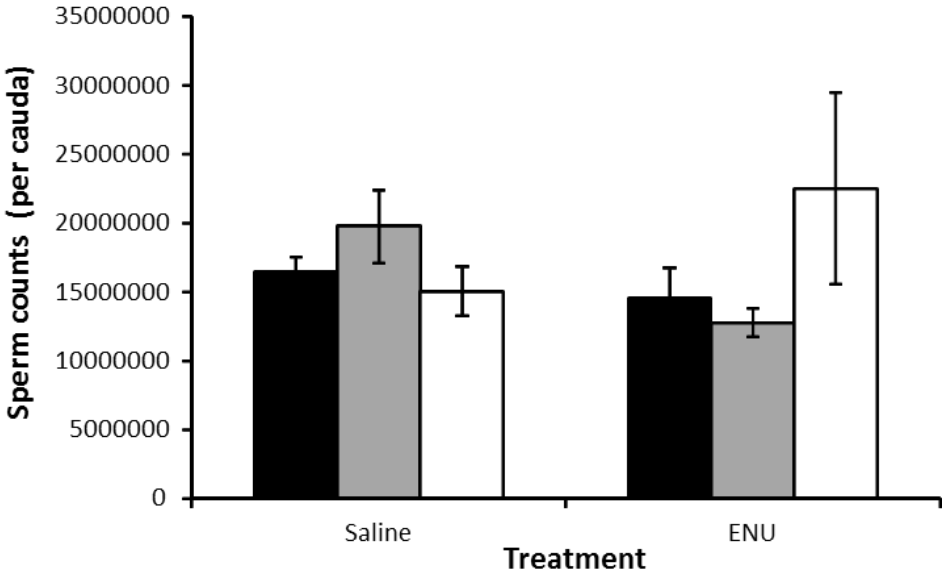

Figure 3-2 Cauda sperm counts of male mice fed FA defined diets and exposed to ENU or saline. Black bar, deficient diet; white bar, control diet; grey bar, supplemented diet. Results analyzed using a Two-Way ANOVA. Data presented as mean ± SEM.

3.4.3 Cauda sperm *lacZ* mutant frequency

lacZ mutant frequency was measured using DNA extracted from sperm. Mice treated with ENU showed significantly higher sperm *lacZ* mutant frequencies compared to those treated with saline ($p=0.006$). There were no significant differences based on diet (Figure 3-3A, B).

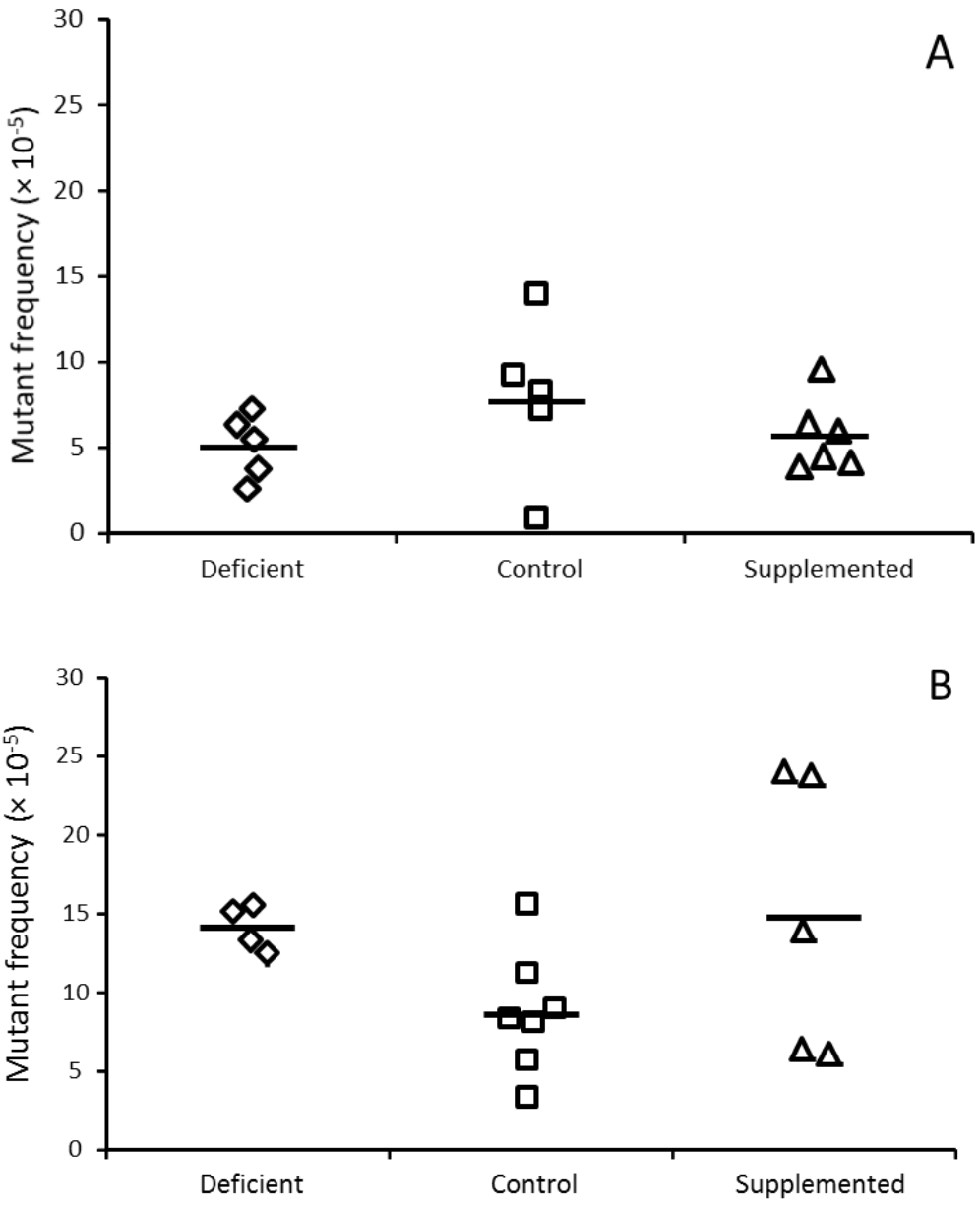

Figure 3-3 Cauda sperm *lacZ* mutant frequency of male mice fed FA defined diets and exposed to saline or ENU. (A) males exposed to saline (B) males exposed to ENU. Black line represents mean. Statistics based on 2-factor binomial regression with a Bonferroni-Holm post-hoc analysis. Main effect of ENU ($p = 0.006$), no other significant differences.

3.5 Discussion

Folate deficiency has been associated with decreased sperm counts and male subfertility [46]. Infertility is a growing concern in Canada and other countries. Male infertilities can be a result of low sperm numbers and poor sperm quality although most infertility cases are idiopathic (no known cause), as assessed by current methods [37]. Current methods for assessing male fertility factors are restricted to sperm number, motility and, in some cases, DNA fragmentation; however, many other characteristics of sperm, such as DNA damage including mutations, likely contribute to male infertility. The germline is not only important to study in the context of fertility but also because of the potential for germline mutations to be passed down to future generations and induce detrimental effects in the offspring. In this study we aimed to investigate the effects of a low, adequate and high dietary FA intake on germline DNA damage, by measuring sperm counts, testis weight and sperm *lacZ* mutant frequency.

Sperm counts and testes weights are crude proxy measures of male fertility and can indicate the efficiency of spermatogenesis. Impairments in spermatogenesis can lead to decreased sperm quality as well as decreased sperm counts. Low seminal plasma folate is associated with increased sperm DNA damage, decreased sperm counts and density and sperm aneuploidy in healthy men [42,43,44]. In mice, folate deficiency results in decreased cauda sperm counts and an increased sperm DNA fragmentation index, an indicator of DNA strand breaks [46]. In this study, we observed no effect of dietary FA intake on testis weight and, contrary to previous findings we found that folate deficiency had no effect on cauda sperm counts. Differences in our findings regarding sperm counts could be due to differences in study design or mouse strain. In the previous study, the BALB/c mouse strain

was used whereas in the current study the MutaMouse transgenic strain was used, a DBA and BALB/c cross. The BALB/c strain has reduced expression of DNA protein kinase (DNA PKs), a protein critical for repairing DNA DSBs. As such the BALB/c strain has inefficient repair of ɣ- ray-induced DSBs, due to reduced DNA DSB end joining [96].

Based on our previous results showing decreased cauda sperm numbers accompanied by an increase in DNA strand breaks in BALB/c mice fed a FA deficient diet, we propose that the mode of action of folate deficiency in sperm is through DNA DSBs. Given that we observed what appears to be a strain effect, our data suggest that the phenotype is dependent on the presence of a susceptible genetic background. Folate deficiency has the potential to induce DNA DSBs through two routes: decreased *de novo* dTMP synthesis and decreased methionine synthesis. A decrease in dTMP synthesis can lead to increased frequency of uracil misincorporation into DNA. The initiation of base excision repair (BER) creates an abasic site, or nick in the DNA. If two nicks are formed on opposing sides of the DNA less than 12 bp apart, DNA DSBs can be generated [1]. A decrease in methionine synthesis consequently leads to a decrease in AdoMet production and thus a decrease in available methyl donors for the many methylation reactions in the cell. Inhibited methyltransferase activity has been shown to lead to chromosomal aberrations such as breaks, deletions, duplications and translocations [58]. It is possible that the repair deficiency in BALB/c mice permits the accumulation of unrepaired DSBs. The presence of DSBs could disrupt the process of spermatogenesis and reduce cauda sperm numbers making the BALB/c strain particularly susceptible to FA deficiency. In contrast, the MutaMouse strain does not have a DSB repair deficiency and therefore could be relatively more efficient at repairing DNA DSBs than BALB/c mice. This strain difference

could explain the differential effects of folate deficiency on spermatogenesis and sperm numbers.

Previously, we showed that mice fed a FA deficient diet for 15 weeks had a significantly higher sperm ESTR mutation frequency [46]. ESTRs are non-coding, 4-10 bp repeat units of DNA that are unstable in the germline [97]. ESTR mutations, which are characterised as the gain or loss of repeat units, are proposed to occur by an indirect mechanism involving secondary structure formation during DNA repair and replication in pre-meiotic stages of spermatogenesis [98]. ESTR mutations may therefore be affected by changes in chromatin conformation as a result of changes in methylation that can prevent access to and repair of secondary structures formed across ESTR loci [98]. The loss of repeat units may also be a result of the formation of DNA DSBs. This mechanism is consistent with the proposed mechanism for the mode of action of folate deficiency on sperm. A low folate intake decreases AdoMet production which could alter the methylation patterns and chromosomal stability. Indeed, changes in genomic methylation patterns have been shown to lead to chromosomal aberrations including breaks and deletions [58]. A low folate intake also decreases dTMP synthesis, which leads to uracil misincorporation into DNA and may lead to DNA DSBs [51]. DNA DSBs are one of the most detrimental forms of DNA damage and are repaired by one of two pathways; homologous recombination (HR) or non-homologous end-joining (NHEJ). HR involves the use of an undamaged sister chromatid or a homologous chromosome as a template for repair. In contrast, NHEJ involves the direct ligation of broken ends without the need for a homologous template [99,100].

In contrast to the ESTR analysis, the *lacZ* mutation assay detects DNA point mutations as well as small insertions and deletions up to 3 bp long [72]. Point mutations are

repaired by excision repair, which involves removing damaged or mispaired single base(s) or nucleotide pair(s) followed by replacement of the correct bases by DNA polymerase [101]. Types of excision repair include: BER, nucleotide excision repair (NER) and mismatch repair (MMR) [101]. The likely mechanism underlying the induction of point mutations is a decrease in *de novo* purine synthesis. DNA DSBs would likely not be detected by the *lacZ* assay and therefore would not contribute to a higher MF in folate deficient mice, explaining the lack of significant effect of folate deficiency on cauda sperm *lacZ* MF. Based on previous findings in BALB/c mice and the findings in the current study, we propose that folate deficiency induces DNA DSBs but it has little or no effect on point mutations in the germline.

Of note, the spontaneous MF of 7.9×10^{-5} observed in the saline treated, control fed mice was over double the expected MF of 3×10^{-5} in MutaMouse sperm [77]. A higher baseline mutation frequency could have masked any effect of folate. It is possible that increasing the number of animals screened and correcting for clonal expansion (daughter cells arising from a single mutated cell) could improve the statistical reliability of this assay and improve the results.

ENU induced a significantly higher sperm *lacZ* MF, as expected, at 12.6×10^{-5} compared to saline at 6.2×10^{-5}. ENU works as an alkylating agent and is able to induce DNA mutations at a frequency of $1.5\text{- } 6 \times 10^{-5}$ in pre-meiotic spermatogonial stem cells [102]. ENU primarily induces A to T and T to A transversion mutations. Other studies have suggested that FA supplementation can mitigate the effects of chemical toxins and mutagens [43,62,63]. Theoretically with an increased amount of FA, both *de novo* dTMP and purine synthesis could be maximized and decrease errors in DNA synthesis and increase

efficient repair of DNA damage. However, FA supplementation did not affect ENU-induced mutations in sperm in this study. The dosage of 50 mg/kg ENU was possibly too high to observe a protective effect of FA supplementation; perhaps a lower dose of ENU would allow for an effect of FA intake to be observed.

Despite hypothetical concerns raised about FA supplementation, we did not see any negative effects of a supplemented diet on sperm counts, testes weights or cauda sperm *lacZ* MF. Supraphysiological doses of FA could lead to an intracellular build-up of DHF. DHF has been shown, *in vivo,* to inhibit TS and MTHFR which could negatively impact folate metabolism by inhibiting *de novo* nucleotide and methionine synthesis and potentially induce genetic mutations. In this study, FA supplementation did not have an effect on mutations in sperm either with or without the induction of mutations by ENU. FA supplementation also appeared to have no protective effects in the male germline compared to the folate adequacy.

3.6 Conclusion

In this study, we determined that folate deficiency had no effect on parameters of male fertility as measured by cauda sperm counts and testis weights in the MutaMouse model. Neither FA deficiency nor FA supplementation had an effect on mutations in the *lacZ* transgene in cauda sperm. Contrary to hypothetical concerns, FA supplementation did not have a negative effect on these proxy measures of male fertility or MF in cauda sperm, but also did not mitigate the effects of ENU-induced germline mutations at a 50 mg/kg dose. In the context of this and other studies, we suggest that folate deficiency may cause DSB but not DNA point mutations in the germline.

Chapter 4: Folate deficiency increases chromosomal damage and DNA mutations in bone marrow

Chapter 4 : Folate deficiency increases chromosomal damage and DNA mutations in bone marrow

4.1 Abstract

Folate deficiency causes megaloblastic anemia and neural tube defects, and is also associated with some cancers. While the specific mechanisms are unknown, the association of folate with these diseases likely reflects the impact of altered folate metabolism on nucleotide synthesis and/or cellular methylation capacity in rapidly dividing tissues. *In vitro*, folate deficiency increases DNA mutation frequency and genome instability, as well as exacerbates the mutagenic potential of known environmental mutagens. Conversely, it remains unclear whether or not elevated FA intakes are beneficial or detrimental to the induction of DNA mutations and by proxy human health. We used the MutaMouse, a transgenic mouse model routinely used for genotoxicology studies, to examine the *in vivo* effects of FA deficient, control and supplemented diets on somatic DNA MF and genome instability in hematopoietic cells. We also examined the interaction between FA intake and exposure to the known mutagen ENU on DNA mutation frequency and genome instability. Male mice were fed the experimental diets for 20 weeks from weaning. Half of the mice from each diet group were gavaged with 50 mg/kg ENU after 10 weeks on diet and remained on diet for an additional 10 weeks. Mice fed a FA-deficient diet had a 130% increase in RBC micronucleus frequency, a measure of chromosomal damage ($p=0.034$), and a doubling of bone marrow *lacZ* MF ($p=0.035$), a measure of DNA mutagenesis, compared to mice fed the control FA diet. However, *Pig-a* MF did not differ among the diet groups. Neither micronucleus nor *lacZ* or *Pig-a* MF differed between the mice fed the FA control and supplemented diets. Mice exposed to ENU showed significantly higher

bone marrow *lacZ* and *Pig-a* MF, but there was no effect of FA intake on ENU-induced MF. The data indicates that FA deficiency increases DNA mutations and chromosomal damage in highly proliferative somatic cells, but that FA intake does not mediate the effect of a strong mutagen.

4.2 Introduction

Folate is an essential water soluble B-vitamin required for *de novo* dTMP, purine and methionine synthesis. Its role in *de novo* dTMP and *de novo* purine synthesis make it important for DNA synthesis and consequently cell proliferation and genome stability (Figure 1). Folate is also required for the production of AdoMet, the major cellular methyl donor required for DNA and histone methylation and therefore important for gene expression and genome stability. Folate deficiency has been associated with disease in somatic tissues including non-alcoholic fatty liver disease, megaloblastic anemia and colon cancer [6]. Induced genetic mutations and genomic instability are proposed mechanisms by which folate deficiency is associated with folate-related diseases.

Folate deficiency resulting from either a low folate intake or impairments in folate metabolism, decreases *de novo* nucleotide and AdoMet production, which can lead to genome instability. A decreased availability of purine nucleotides leads to nucleotide pool imbalances, increased mutation rates, decreased cell growth rates, alterations in DNA synthesis and improper DNA repair of UV-induced lesions [60,61]. TS transfers a methylene group to dUMP from dTMP. Folate deficiency inhibits the activity of TS which leads to a build-up of dUMP, the precursor for uracil. An increase in the intra-cellular pools of dUMP can lead to increased uracil misincorporation into DNA, the repair of which requires the formation of an abasic site in the DNA, also called a nick. If two DNA nicks are formed in

close proximity it can lead to DNA DSBs [1,52]. Decreased AdoMet production resulting from folate deficiency reduces available methyl groups ; lower cellular methylation capacity *in vivo* can alter DNA methylation patterns which can lead to chromosomal aberrations including breaks, deletions, translocations and duplications [58]. Thus folate deficiency has the potential to be mutagenic as a result of decreased purine synthesis and clastogenic as a result of decreased dTMP and AdoMet synthesis. For these reasons it is important to examine the effect of FA intake on DNA mutations and chromosomal damage and instability.

A substantial proportion of the Canadian population has folate status indicative of FA intake potentially at or above the UL (1 mg FA/day) [65]; this higher intake of FA may have unknown benefits or detriments. *In vitro*, FA supplementation protects against oxidative stress and apoptosis induced by 7-ketocholesterol, as shown in smooth muscle, endothelial and monocytic cells [62]. FA supplementation both in *in vitro* and *in vivo* also protects against induced mutations and chromosomal damage in human liver cells caused by BaP, a strong mutagen and carcinogen[63]. Similarly, FA supplementation reduced chromosomal aberrations in somatic and germ cells, as well as reduced DNA fragmentation in mouse spleen cells treated with daunorubicin, an anticancer drug used in the treatment of multiple malignancies [64]. Therefore, it is possible that FA intake above adequate levels could maximize DNA synthesis and repair in conditions of exposure to environmental toxicants, potentially resulting in fewer genetic mutations and clastogenic effects.

Conversely, hypothetical concerns have been raised about higher than required intakes of FA. High levels of intracellular FA have been proposed to lead to an accumulation of DHF, the product of the first step in FA bioactivation. Accumulation of

DHF has been shown to inhibit TS and MTHFR *in vitro*, thus inhibiting the formation of dTMP and 5-methyl-THF, respectively, and therefore inhibiting normal DNA and methionine synthesis [66]. Therefore FA supplementation could potentially mirror the clastogenic effects of folate deficiency by inducing DNA DSBs and chromosomal instability. These concerns remain hypothetical and require further investigation.

Recently our lab demonstrated that male BALB/c mice fed a FA deficient diet had a significantly higher *Pig-a* mutant frequency and micronucleus frequency in RETs and RBCs compared with mice exposed to a control diet [85]. For the current study, we used the MutaMouse model to measure mutations in a non-expressed gene target, the *lacZ* transgene, in addition to the *Pig-a* gene, as well as to measure micronucleus frequency in RETs and RBCs.

4.3 Methods

4.3.1 Micronucleus frequency

Blood was drawn via cardiac puncture into a heparin coated syringe (5:1 ratio of blood to heparin) and collected in K_2EDTA collection tubes following the instructions for the *In Vivo* MicroFlow® kit (Litron Laboratories, Rochester, NY). Samples were sent fresh on the day of necropsy to Litron Laboratories for analysis. Micronucleus frequency was measured using a propidium iodide DNA stain, to identify the presence of micronuclei. An antibody against the CD71 RET surface expressed protein was used to differentiate RETs and RBCs. Cells were sorted according to RETs, RBCs, MN-RETs and MN-RBCs by flow cytometry, as described [93]. The mean number of cells analyzed for %RETs was 1.05×10^6 cells/sample with a minimum of 6.6×10^5 cells/sample. For MN-RBCs, the mean number of cells analyzed was 1.03×10^6 cells/sample with a minimum of $6.6 \times$

10^6 cells/sample. For MN-RETs, the mean number of cells analyzed was 1.99×10^4 cells/sample with a minimum of 1.98×10^4 cells/sample.

4.3.2 *Pig-a* mutant frequency

Blood samples were drawn as above following instructions for the *In Vivo* MutaFlow® kit and sent fresh to Litron Laboratories for analysis. *Pig-a* mutation frequency was measured using an antibody against the CD24 GPI-anchored protein which can be detected and used to sort *Pig-a* mutants by flow cytometry, as described [88]. The mean number of cells analyzed for %RETs was 1.58×10^5 cells/sample with a minimum of 1.45×10^5 cells/sample. For mutant RBCs, the mean number of cells analyzed was 1.64×10^8 cells/sample with a minimum of 1.31×10^8 cells/sample and for mutant RETs the mean number of cells analyzed was 5.72×10^6 cells/sample with a minimum of 3.72×10^6 cells/sample.

4.3.3 Bone marrow *lacZ* mutant frequency

Bone marrow from the right and left femur was flushed out with phosphate PBS, frozen in liquid nitrogen and stored at -80 ºC until use. DNA was extracted from bone marrow following the protocol described by Gingerich *et al* [103]. Briefly, bone marrow tissue (from one femur) was incubated overnight in lysis buffer containing 3.5×10^{-5} M proteinase K. Following overnight incubation, a standard phenol chloroform procedure followed by an ethanol precipitation was performed. DNA was spooled, rinsed in 70% ethanol and dissolved in TE-4 buffer (10mM Tris-HCL, pH 7.6, 10^{-4} M EDTA). The extracts were stored at 4°C until use.

Extracted genomic DNA was used in the *lacZ* assay as described by O'Brien *et al* [77]. Briefly, the λgt10*lacZ* copies were rescued from 4 µl of extracted genomic DNA using

the Transpack Packaging extract (Agilent Technologies, Santa Clara, CA). The host bacteria (galE$^-$ recA$^-$ *E.coli*) was infected with packaged phage particles and plated on minimal agar medium with and without the addition of 0.3% (w/v) P-gal. Plates without the addition of P-gal were used to estimate the total number of plaque forming units PFU, while the plates with the added P-gal were used to estimate MF. Plates were incubated at 37°C overnight and plaques were counted the following morning. MF per mouse was calculated by dividing the number of mutants by the number of total PFUs. The mean MF was calculated per diet and treatment group. The results for each diet and treatment group were summed together and the mean was calculated. A minimum of 125,000 total PFUs were screened per animal, following the OECD test guidelines recommendation [104].

4.3.4 Statistical analyses

Differences among diet groups for body weight, and plasma and RBC folate concentration were analyzed for significance by Two-Way ANOVA to determine a diet by treatment effect. If no effects of ENU were observed a One-Way ANOVA was performed. In cases where the data failed equal variance or normality, data were log transformed for analysis. Differences in *Pig-α* MF and micronucleus frequency among diet and ENU treated groups were analyzed for significance by Two-Way ANOVA. Correlation analyses were determined using the Pearson Product Moment correlation test. Analyses were performed using SigmaPlot for Windows, version 13 (Systat Software, Inc.).

Analysis of bone marrow *lacZ* MF was performed using R (version 3.2.0, www.r-project.org). Bone marrow *lacZ* MF data were fit to a generalized linear model with a binomial error distribution using the *glm* function. Overdispersion was permitted amongst biological replicates using the quasibinomial distribution for *glm*, as inter-individual

variability is known to be high for the *lacZ* assay. Mice with less than 125, 000 total PFUs screened were removed from the analysis. Replicates that were outside of the expected binomial error distribution were considered outliers and also removed. Based on these criteria a total of three mice were removed out of the 60 mice considered, resulting in a final sample size of 57 mice with 8 or more mice in each of the 6 experimental groups. A 2-factor analysis, setting diet and ENU dose as factors, was performed to identify differences in group means. P-values were corrected for multiple-comparison using the Bonferroni-Holm method.

4.4 Results

4.4.1 Micronucleus frequency

ENU treatment had no effect on micronucleus frequency due to timing of the ENU treatment which allowed for a wash out period. As such, data from both saline and ENU treated groups were combined for analysis. The %RETs did not differ among the diet groups (Figure 4-1A). The frequency of MN-RETs was significantly higher in male mice fed a FA deficient diet compared to those fed a FA control ($p=0.04$) and supplemented diet ($p=0.034$) (Figure 4-1B). The frequency of MN-RBCs was significantly higher in male mice fed a FA deficient diet compared to those fed a FA control diet ($p=0.034$), and tended to be higher compared to those fed a FA supplemented diet ($p=0.071$) (Figure 4-1C). The %RETs, MN-RET and MN-RBC frequencies did not differ between mice fed the control and supplemented diets.

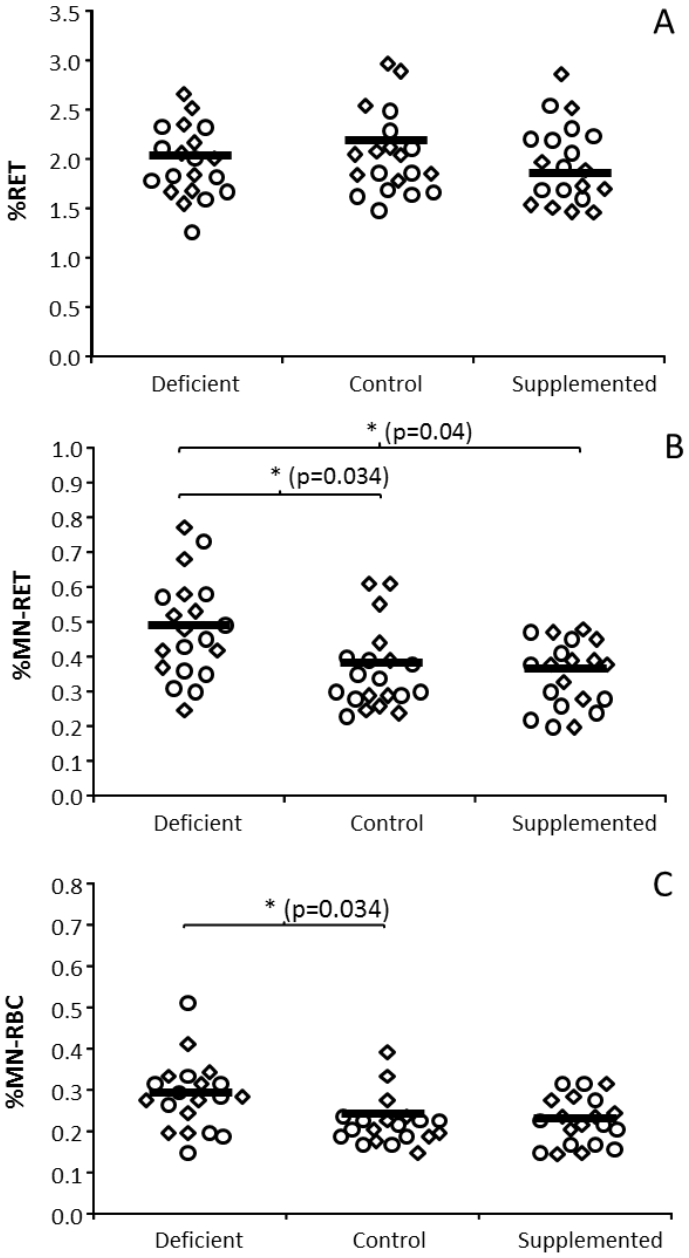

Figure 4-1 Percent reticulocytes and micronucleus frequency in male mice fed folic acid defined diets. No treatment effect was seen; therefore ENU and saline data were combined for analysis. A main diet effect on % MN-reticulocytes and %MN-RBCs was observed ($p<0.05$), using a Two-Way ANOVA, Holm-Sidak post-hoc analysis. (A) % RETs (B) MN-RET frequency (C) MN-RBC frequency. The black line represents the mean. Diamonds, ENU exposure; circles, saline exposure. *, significantly different based on a Two-Way ANOVA, Holm-Sidak post-hoc analysis. A minimum of 6.6×10^5, 1.98×10^4 and 6.6×10^6 cells/sample was measured for %RETs, MN-RETs and MN-RBCs, respectively.

4.4.2 Micronucleus frequency and red blood cell folate correlation

MN-RETs (Correlation coefficient, $r = -0.492$, $p = 0.006$) and MN-RBCs (Correlation coefficient, $r = -0.405$, $p = 0.026$) were negatively correlated with RBC folate concentration (Figure 4-2).

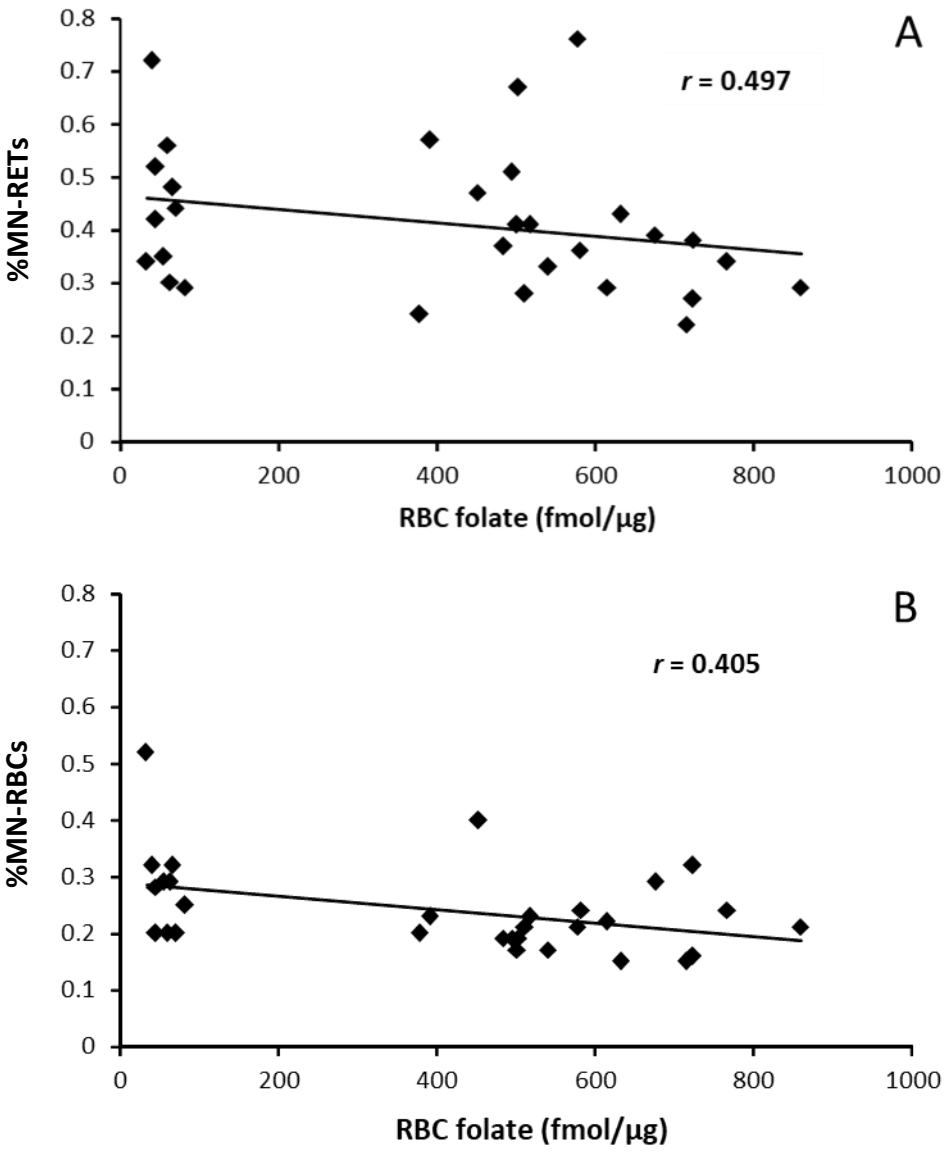

Figure 4-2 Correlation between red blood cell folate and micronucleus frequency. (A) MN-RETs (B) MN-RBCs. Correlations were determined using the Pearson Product Moment correlation test, (p=0.006) and (p=0.026) respectively.

4.4.3 *Pig-a* mutant frequency

A main effect of treatment (ENU vs. saline) was seen ($p<0.001$) for *Pig-a* RET MF and *Pig-a* RBC MF, such that ENU treated mice had higher MF than saline treated mice. Diet had no effect on %RETs, RET MF and RBC MF, and did not interact with ENU treatment (Figure 4-3).

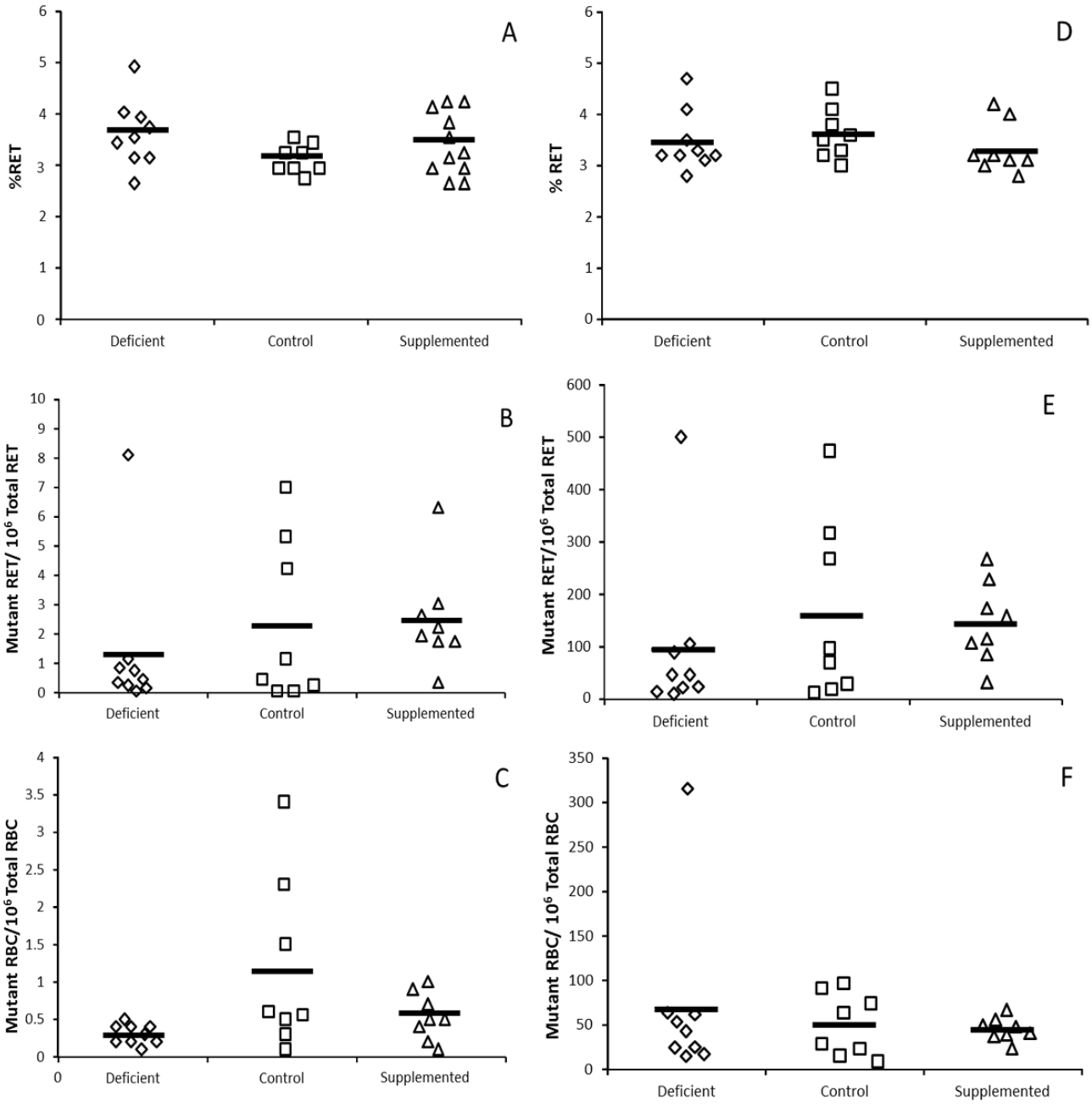

Figure 4-3 Percent reticulocytes and *Pig-a* mutant frequency in male mice fed folic acid defined diets. A main effect of ENU treatment was observed ($p<0.001$) using a Two-Way ANOVA, Holm-Sidak post-hoc analysis such that ENU induced higher mutation frequency compared to saline treatment. Therefore, graphs were separated by treatment: saline (A, B, C) and ENU (D, E, F). (A) and (D) %RETs; (B) and (E), *Pig-a* RET MF; (C) and (F), *Pig-a* RBC MF. The black line represents the mean. Diamonds, deficient diet; squares, control diet; triangles, supplemented diet. Note the difference in y-axis in (E) and (F) compared to (B) and (C). A minimum of 1.45×10^5, 3.72×10^6 and 1.31×10^8 cells/sample was measured for %RETs, mutant RETs and mutant RBCs, respectively.

4.4.4 Bone Marrow *lacZ* mutant frequency

Mice treated with ENU showed significantly higher bone marrow *lacZ* MF compared to mice treated with saline. In ENU treated mice, there were no significant differences based on diet. In saline treated mice, those fed the FA deficient diet had a significantly higher bone marrow MF compared to those fed the control FA diet ($p= 0.035$) (Figure 4-4).

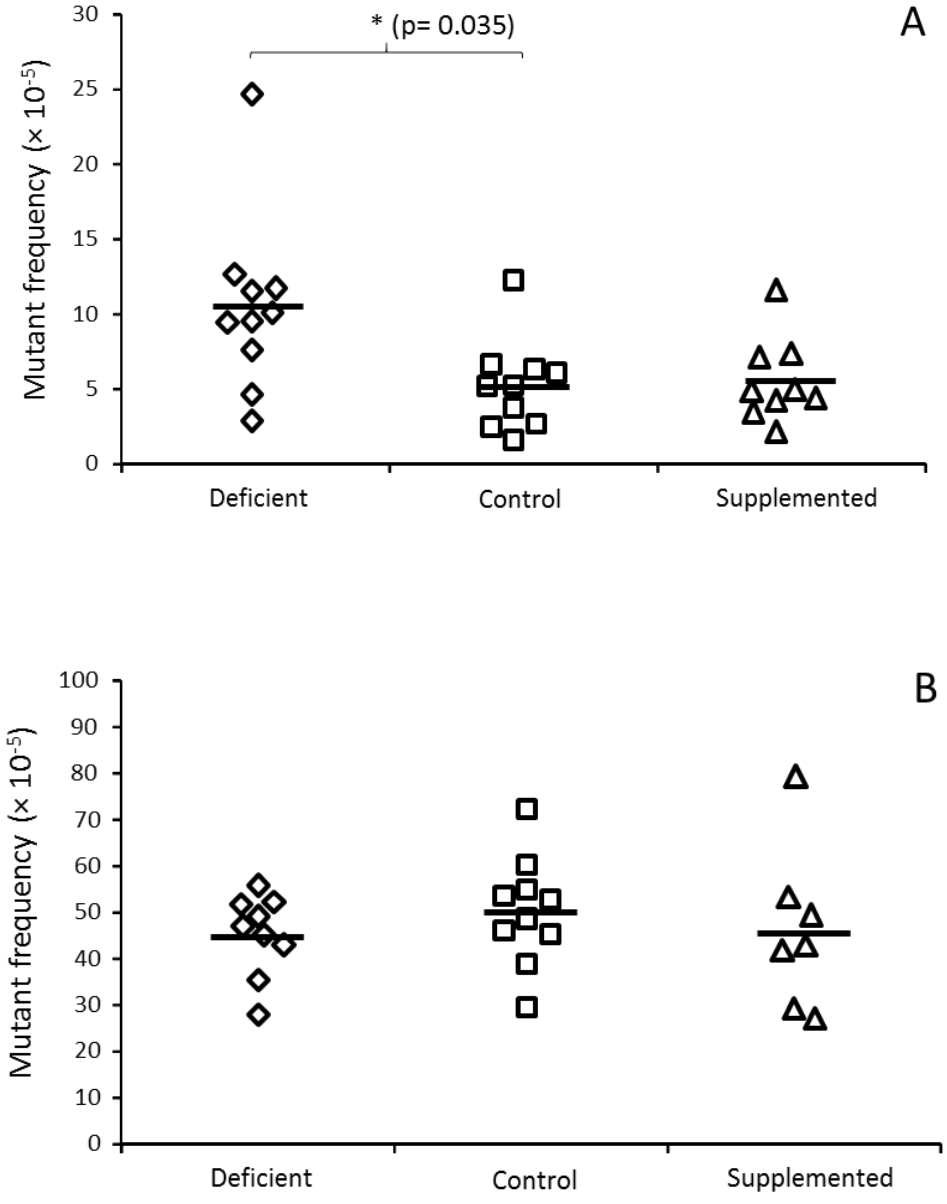

Figure 4-4 Bone marrow *lacZ* mutant frequency of male mice fed folic acid defined diets and exposed to saline or ENU. (A) Males exposed to saline (B) males exposed to ENU. *, statistically different based on 2-factor binomial regression with a Bonferroni-holm post-hoc analysis. Note the difference in y-axis between (A) and (B).

4.4.5 Bone marrow *lacZ* and *Pig-a* mutant frequency correlation

Pig-a mutant RETs (Correlation coefficient: 0.621, p = 0.002) and *Pig-a* mutant RBCs (Correlation coefficient: 0.811, p = 0.000003) were positively correlated with bone marrow *lacZ* MF (Figure 4-5).

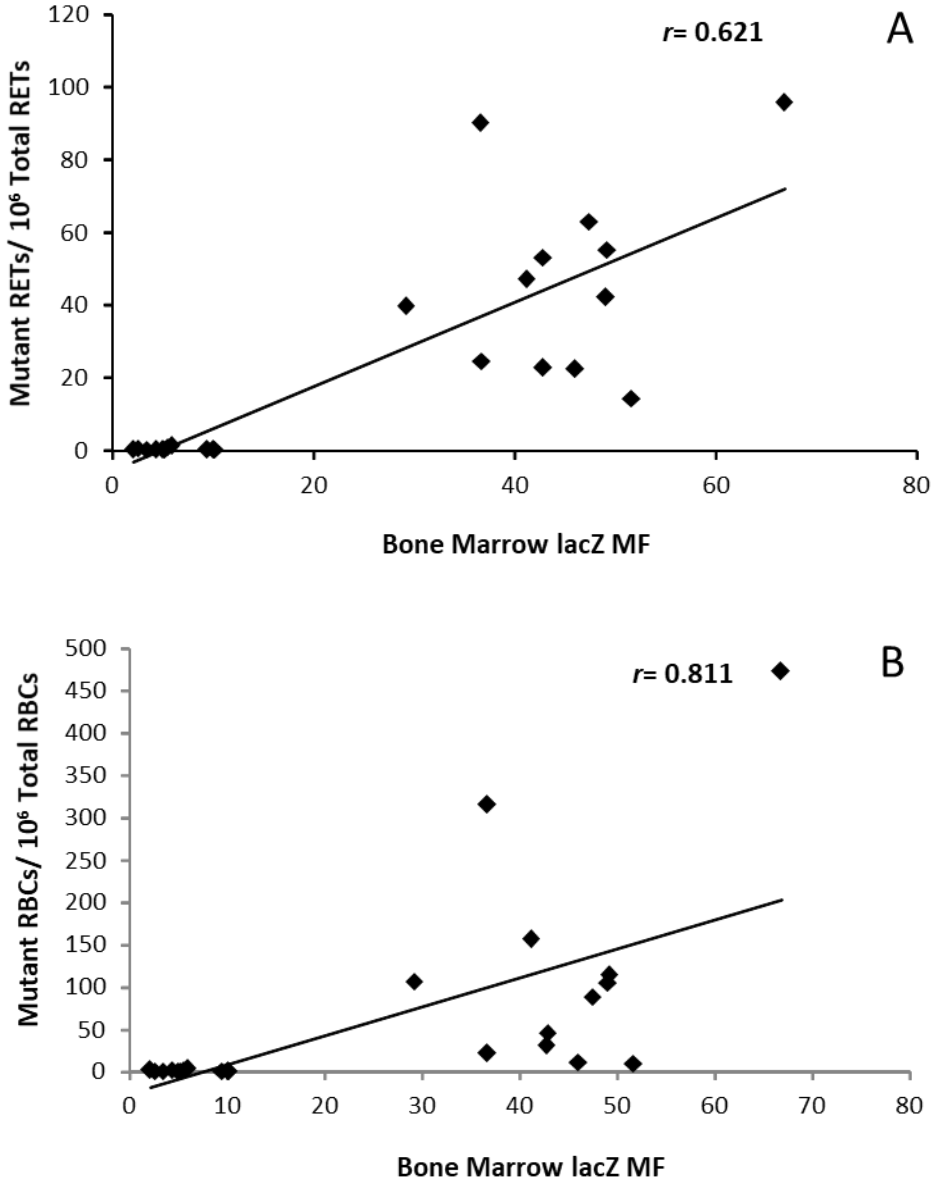

Figure 4-5 Correlation between bone marrow *lacZ* mutant frequency and *Pig-a* mutant frequency. (A) Mutant RETs (B) Mutant RBCs. Correlations were determined using the Pearson Product Moment correlation test, (p=0.002) and (p=0.000003) respectively.

4.5 Discussion

While folate deficiency has been associated with cardiovascular disease, gastrointestinal cancers, and is known to cause NTDs, the specific underlying mechanisms remain unclear. In this study, we demonstrate that highly proliferative somatic cells such as bone marrow are susceptible to the induction of DNA mutations and chromosomal instability in conditions of folate deficiency. However, our data also indicate that at doses used in this study, ENU induced DNA mutations are not mitigated by FA supplementation. Overall, FA supplementation provided no additional protection from DNA mutations and chromosomal instability when compared to adequate FA intake.

DNA DSBs can lead to chromosomal loss or amplification and in some cases translocations, which can promote tumorigenesis [105]. In a large international study, individuals with an increased MN frequency in peripheral blood lymphocytes had an increased incidence of cancer, most commonly urogenital and gastro-intestinal cancers [106]. Folate deficiency can induce DNA DSBs through two pathways: *de novo* dTMP synthesis and methionine synthesis. Decreased *de novo* dTMP can lead to uracil misincorporation into DNA, the repair of which can result in DNA DSBs [1]. A decrease in methionine synthesis consequently leads to a decrease in AdoMet production. A decreased availability of methyl donors and subsequent modification of DNA methylation patterns can lead to chromosomal aberrations such as breaks, duplications, deletions and translocations. Based on the potential of folate deficiency to induce DNA DSBs, we wanted to investigate the effects of dietary FA intake on chromosomal damage (clastogenic) or chromosomal loss (aneugenic) in RBCs.

Micronuclei are an indicator of DNA DSBs and form when either whole chromosomes or chromosomal fragments do not navigate to polar ends of the cell during anaphase. Although micronuclei can indicate aneugenic activity, we assume that folate-mediated micronuclei measured in this study are due to DNA DSBs and not whole chromosome loss. As RBCs undergo the process of enucleation, MN-RBCs and MN-RETs can be scored by flow cytometry using a DNA stain as marker for the presence of micronuclei. We found that FA deficiency induced a 1.3 and 1.4-fold increase in MN-RETs compared to a FA control and supplemented diet, respectively. Similarly, FA deficiency induced a 1.3-fold increase in MN-RBCs compared to the FA control diet. These results are comparable to our previous findings where FA deficiency induced a 1.8–2.5-fold and 1.5–1.6-fold higher MN-RETs and MN-RBCs frequencies, respectively, compared to mice fed FA control or supplemented diets [85]. These results indicate that folate deficiency consistently induces chromosomal damage, likely DNA DSBs, in precursors of RETs and RBCs. We propose that folate deficiency induced DNA DSBs arise through either decreased dTMP or methionine synthesis. FA supplementation had no effect on micronucleus formation in RETs or RBCs compared to adequacy; however, micronucleus frequency did correlate with RBC folate concentration, such that as RBC folate concentration increased micronucleus frequency decreased.

We noted a slightly lower fold increase in MN-RET and MN-RBCs in the current study in comparison with our previous study. We suggest that the variation can be explained by the different mouse strains used. In the previous study, the BALB/c inbred strain was used whereas here we used the MutaMouse, a DBA and BALB/c cross, transgenic strain. The BALB/c strain has reduced expression of DNA protein kinases (PKs)

and inefficient repair of ɣ- ray-induced DSBs, specifically in the end joining process [96]. It is possible that this repair deficiency makes the BALB/c mouse strain more susceptible to DNA damage due to a deficiency in DSB repair. The differences in strain susceptibility can be compared to the genetic diversity within the human population. Individuals within the human population, possibly due in part to polymorphisms in folate related genes, may be more or less susceptible to folate deficiency and /or folate deficiency induced DNA damage.

In addition to DSBs and chromosomal instability, we propose that another mechanism that contributes to the association between folate deficiency and cancer and other diseases is its ability to induce genetic mutations. Folate deficiency can induce DNA point mutations by reducing *de novo* purine synthesis. We recently showed that male BALB/c mice fed a FA deficient diet (0 mg/kg) for 18 weeks from weaning had a 2.2 fold higher *Pig-α* MF in RBCs compared to those fed a FA supplemented diet (6 mg/kg). The mutagenic effect of FA deficiency was modest in comparison to that of ENU, a potent chemical mutagen, with MFs of 1.6×10^{-6} and 46×10^{-6}, respectively. The *Pig-α* gene is one of 24 involved in GPI-anchor synthesis; GPI-anchors are important for anchoring proteins to the cell surface. The *Pig-a* assay is based on the lack of GPI-anchor synthesis, which may occur due as a result of a loss of function mutation or a deletion in the *Pig-a* gene on the X chromosome.

Here we investigated the effect of dietary FA intake on the induction of genetic mutations using the *lacZ* assay in addition to the *Pig-a* assay. Bone marrow tissue was selected because folate deficiency causes megaloblastic anemia as well as it is a highly proliferative tissue with a high demand for folate. The *lacZ* mutation assay, typically used

for genotoxicology studies, is able to detect point mutations and small insertions and deletions up to 3 bp long [72]. In saline exposed mice, it was found that folate deficiency doubled the bone marrow *lacZ* MF compared to folate adequacy. Mice fed the deficient diet had a mean MF of 10.5×10^{-5} compared to a MF of 5.2×10^{-5} in mice fed the control diet. The spontaneous MF for bone marrow observed in mice fed the control diet was comparable to the historical control spontaneous MF of $5.3 \pm 1.7 \times 10^{-5}$ in the Health Canada MutaMouse colony (Marchetti, personal communication). Mice fed the FA supplemented diet did not have a different MF than the control fed mice. Based on the types of mutations the *lacZ* assay is able to detect, we propose that the likely mechanism contributing to the increased MF in mice exposed to a FA deficient diet is *de novo* purine synthesis. Indeed, the most common mutations in control *lacZ* mice involve guanine [107]. Mutation induction in the *lacZ* transgene is comparable to that in endogenous loci and thus mutations in the *lacZ* transgene can serve as a proxy for mutation induction across the genome [108,109]. In addition to the *lacZ* gene point mutations may be induced in other regions of the genome and may contribute to folate-related cancers. In CRC, for instance, point mutations in the p53 tumor suppressor gene occur in approximately 50% of cases [110–112]. It is possible that FA deficiency could contribute to the induction of point mutations in the p53 tumor suppressor gene or other cancer related genes.

In a previous study, the BigBlue transgenic mouse was used to assess the effects of FA intake on DNA mutations in the colon. The BigBlue transgenic strain contains the *lacI* reporter gene in the lambda phage construct, as opposed to the *lacZ* reporter gene in the MutaMouse. Both transgenic strains also can be used to screen for mutations in the *cII* lambda phage gene in the *cII* positive selection assay. A FA deficient diet with less than

0.05 ppm FA administered for 32 weeks to BigBlue mice did not have an effect on c*II* mutant frequency compared to those on a folate adequate diet at 4 ppm. The colon is a highly proliferative tissue much like bone marrow and has been shown to become folate depleted quickly compared to other tissues such as liver [76]. However studies have shown that different tissues, even if highly proliferative, have variable responses to chemical treatment in the MutaMouse and Big Blue assay [113–116]. The size of the *cII* gene is ~ 300 bp long compared to the 3000 bp of the *lacZ* gene; its size makes it a less sensitive target to mutation induction [72]. The mouse strain could also have an influence on the differing results since the BigBlue mouse is on a C57BL/6J background and the MutaMouse is on a DBA × BALB/c background.

In contrast to our previous study, we did not observe an increase in *Pig-a* MF in mice fed a FA deficient diet. In human studies, mutations in the *Pig-a* gene are typically a result of small base-pair substitutions or frameshift mutations, and rarely a result of large deletions [117]. Therefore it is unlikely that the *Pig-a* assay will detect clastogenic or aneugenic DNA damage. This could explain why folate deficiency induced a higher MN frequency in RBC and RETs, a result of DNA DSBs, but did not induce an effect in the *Pig-a* gene. While we previously saw a statistically significant increase in *Pig-a* MF in folate deficient mice, the magnitude of the effect was small indicating that other factors such as genetic background could mitigate its effects.

We expected that a dietary FA intervention would induce comparable results in the *Pig-a* and bone marrow *lacZ* assay, as mutations in the bone marrow, which contain RBC precursors as well as RET and RBCs, would likely appear in RET or RBCs. *Pig-a* mutant RETs (Correlation coefficient: 0.621, $p = 0.002$) and *Pig-a* mutant RBCs (Correlation

coefficient: 0.811, p = 0.000003) were positively correlated with bone marrow *lacZ* MF (Figure 4-5); supporting that the mode of action of folate deficiency is likely the same in both gene targets. Therefore, the difference in results comparing the *Pig-a* MF and bone marrow *lacZ* MF, can be attributed to the differences in the assays themselves, the respective mutational targets of the assays and the tissues in which the mutational events are being measured. In a previous study, the sensitivity of the *lacZ* and *Pig-a* assays in detecting BaP-induced mutations in bone marrow were compared. The *lacZ* assay had a higher mutagenic efficiency with the magnitude of the *lacZ* response in bone marrow 25-fold higher than that of the *Pig-a* assay [118]. One of the proposed explanations for the difference was the type of cells used. The *Pig-a* assay analyzes mutations in the *Pig-a* gene in RETs or RBCs, while the *lacZ* in bone marrow measures mutations in the *lacZ* gene in RETs and RBCs present in the bone marrow as well as various stem cells types not all of which are destined to become RETs an RBCs. Other things to consider when comparing the two assays are the differences in genomic location of the two target genes, the sequence context, the gene size and the fact that the *lacZ* transgene is not transcribed in the mouse while the *Pig-a* gene undergoes transcription and is therefore subject to transcription-coupled repair [118], all of which may contribute to an amplified response in the *lacZ* gene in somatic tissues.

Supplemental FA intake was investigated in this study to address concerns related to the potentially high intake of FA in the Canadian population. However, FA supplementation had no effect, positive or negative, on chromosomal damage, bone marrow *lacZ* MF, or *Pig-a* MF. Previously, in a similar study, our lab observed that FA supplementation did not have an effect of *Pig-a* or micronucleus frequency [85]. Our data

indicate that FA supplementation provides no additional benefit compared to adequacy, but also has no negative impact on DNA mutagenesis or chromosomal instability.

We were also interested in whether FA intake interacted with an environmental mutagen on mutation induction. ENU is an alkylating agent, from which an ethyl group can be transferred to nitrogen or oxygen radicals of adenine, guanine, cytosine and thymidine. This transfer can result in base mispairing, which can lead to a single base pair substitution following two rounds of replication [102]. For example, the transfer of an ethyl group to oxygen in position 4 of thymidine forms ethylthymidine which DNA polymerase can mistake for cytosine and result in a GT base pair as oppose to an AT base pair. As such, ENU predominantly induces point mutations, primarily AT to GC transitions, but has also been shown to induce small deletions [102]. Male mice exposed to ENU had significantly higher *Pig-a* mutant frequencies in both RETs and RBCs compared to those exposed to saline, as would be for a known potent chemical mutagen. A higher MF in ENU exposed mice is expected given that it is a known potent chemical mutagen that at a dose of 250 mg/kg can induce point mutations at a magnitude of ~150×10^{-5} [119]. The ENU-induced *lacZ* MF is comparable to the ENU- induced *lacZ* MF found in a previous study in bone marrow at 20.1×10^{-5} (2.2-fold increase over baseline) where mice were treated with an acute ENU treatment of 15.5 mg/kg [120]. We saw no effect of FA intake on ENU-induced MF using either assay. It is possible that a dose of 50 mg/kg ENU induces mutations at too high a frequency for FA intake to exacerbate or mitigate its mutagenic potential. Perhaps a lower dose of ENU could allow for any potential protective effect of FA supplementation or negative effect of FA deficiency to be observed.

Although ENU can induce micronuclei formation [121], we did not see any significant effects of ENU treatment on micronucleus frequency or %RETs. This can be explained by the 10 week time period between ENU exposure and collection of blood. The life span of a RET is ~15h, prior to transformation into NCEs which have a lifespan of ~30-40 days [122,123]. The 70 day (10 wk) time period between ENU exposure and blood collection allowed for any MN-RET and MN-RBCs to be washed out and replenished by RETs and RBCs that were unexposed to ENU as bone marrow precursors. In a previous study, a 50 mg/kg ENU exposure via oral gavage in MutaMouse males 2 days prior to blood collection induced a 5.2 and 1.2 fold increase in MN-RET and MN-RBCs respectively, compared to controls. A lower %RETs was found in mice exposed to ENU at 0.5% compared to controls at 1.6% [124]. In the current study, as ENU has already been shown to induce MN, we were primarily interested in the effects of chronic FA diet on MN formation, therefore blood was taken after 20 weeks on diet, (10 weeks after ENU exposure).

%RET is an indicator of bone marrow toxicity. In contrast to our previous study, we did not see any differences among the diet groups in %RET; %RET for all diet groups was in the expected range between 1 and 6% in adult mice [125]. Previously folate deficiency was associated with a lower % RET in comparison to folate adequacy [85]. This difference may also be explained by the repair deficiency in the BALB/c strain not found in the MutaMouse strain. Percent RETs was slightly lower at 1.97 % in the *In Vivo* MicroFlow® assay compared to 3.47 % in the *In Vivo* MutaFlow® assay. The difference can be explained by the difference in RET staining methods. In the *In Vivo* MutaFlow® assay, RETs are

stained for RNA content, whereas in the *In Vivo* MicroFlow® assay the RETs are stained for the CD71 surface expressed protein.

In order to ensure that any significant differences among diet groups were a result of folate intake plasma and RBC folate status were measured. Plasma folate is the optimal indicator of current circulating folate status [126]. Mice fed a deficient and supplemented diet had an 85% decrease and a 107% increase in plasma folate concentrations respectively, compared to those fed a control FA diet. In addition, we looked at folate status in RBCs to obtain a tissue folate status reflective of long term folate intake. We observed that mice fed a deficient and supplemented diet had an 88% decrease and a 27% increase in RBC folate concentrations, respectively. Therefore plasma and RBC folate concentrations were reflective of FA intake.

4.6 Conclusions

In this study, we determined that folate deficiency increases chromosomal damage in RBCs as well as increases DNA mutations in non-transcribed regions of the bone marrow in *lacZ* mice. Neither folate deficiency nor FA supplementation had an effect on mutations in the *Pig-a* gene of RBCs. Contrary to hypothetical concerns, FA supplementation did not have a negative effect on micronuclei formation or somatic DNA mutations. Neither did FA supplementation protect or exacerbate the induction of somatic DNA mutations by ENU. These studies emphasize the importance of maintaining a folate adequate diet to minimize genomic instability in somatic tissues.

Chapter 5: Discussion

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5.1 Summary of results and implications for human health

Folate deficiency has been associated with male subfertility, increased risk for gastro-intestinal cancers and has a causal role in the development of NTDs and megaloblastic anemia. We propose that the ability of folate deficiency to induce genetic mutations and chromosomal instability underlies, at least in part, its association with these conditions. Therefore the purpose of this study was to investigate the effects of low and high dietary FA intake on somatic and germline DNA mutations and chromosomal aberrations *in vivo*.

5.1.1 Cancer

Mutations, chromosomal aberrations and genomic instability all play a role in the development and progression of cancer. Cancer can be described as the survival and uncontrollable division of abnormal cells that manage to escape the restraints of ordinary cell behavior and inherent cell proliferation [7]. It is proposed that the early stages of carcinogenesis involve the induction of genomic instability, allowing for transformation of a normal cell into a cancerous cell [127]. Low folate intake and SNPs in folate related genes have been associated with the risk of gastrointestinal cancers such as CRC [28]. It is likely that folate deficiency predisposes normal tissues to neoplastic transformation [26]; for instance, FA deficiency, in this study, induced micronuclei formation and DNA mutations in somatic cells.

We found that inadequate FA intake increased chromosomal damage in RETs and RBCs by inducing a significantly higher micronucleus frequency compared to an adequate FA intake. Similarly in a previous study, folate deficiency induced a higher MN-RET and

MN-RBC frequency compared to folate adequacy [85]. Chromosomal breakage and damage can lead to fragmented DNA, which can form micronuclei. Micronuclei are small nuclei containing chromosomal fragments and/or whole chromosomes that had not moved to the polar ends of the cell during anaphase and are not incorporated into the nucleus. We present evidence that folate deficiency is capable of inducing clastogenic or aneugenic DNA damage in RBCs, suggesting that this could also occur in other highly proliferative tissues. Folate deficiency has the potential to induce DNA and chromosomal breaks through two pathways involved in one-carbon metabolism: *de novo* dTMP and methionine synthesis.

In a large international study, individuals with an increased micronucleus frequency in peripheral blood lymphocytes had an increased incidence of cancer, most commonly urogenital and gastro-intestinal cancers [106]. This human study supports an association between micronucleus frequency and cancer development. The results from the current study therefore support an association between folate deficiency and risk of cancer development.

In addition to clastogenic or aneugenic damage, measured by micronucleus formation, an indicator of DNA DSBs, FA deficiency also induced DNA mutations in bone marrow cells. Mice fed a FA deficient diet had a significantly higher bone marrow *lacZ* MF compared to those fed a FA adequate diet. The *lacZ* mutation assay detects point mutations and small insertions and deletions up to 3 bp long [72], therefore it is likely that folate deficiency induces DNA mutations through the *de novo* purine synthesis pathway. *De novo* purine synthesis is decreased in cases of low folate intake, which can lead to nucleotide pool imbalances and DNA point mutations [60]. It is assumed that mutations in the *lacZ* reporter gene are a proxy for mutagenesis across the genome; therefore point

mutations may be induced by folate deficiency in other regions of the genome. Mutations in the p53 tumor suppressor gene are found in many tumors and occur in approximately 50% of CRCs [110–112]. FA deficiency could contribute to cell transformation by inducing point mutations in the p53 tumor suppressor gene or other cancer related genes.

In addition to CRC, folate deficiency has been associated with leukemia, a group of cancers of blood forming cells including bone marrow. Polymorphisms in folate related genes have been associated with an increased susceptibility to acute lymphoblastic leukemia (ALL), potentially due to a reduced cellular folate uptake or metabolism [128]. ALL is a cancer initiated in white blood cell precursors (lymphocytes) in the bone marrow. Therefore our findings in bone marrow and RBCs may also be relevant in the context of leukemia and specifically ALL and inform a potential mechanism for the induction of mutations by folate deficiency.

FA intake did not have an effect on *Pig-a* MF in RBCs in the MutaMouse model. This was unexpected given that in a previous study, mice fed a FA deficient diet for 18 weeks had a significantly higher *Pig-a* MF compared to those fed a FA supplemented diet. *Pig-a* mutants are scored based on a lack of GPI-anchor synthesis and consequently a lack of cell surface expressed proteins. In humans, a lack of surface expressed proteins are typically a result of small mutations, such as point mutations, and rarely large deletions, in the *Pig-a* gene [87]. Therefore it is likely that DNA DSBs are not detected in the *Pig-a* assay, which could explain why DNA damage was observed in the MN assay and not in the *Pig-a* assay. Differences in the *Pig-a* assay results between the current and previous study may be attributed to the difference in mouse strain used. While we previously saw a statistically significant increase in *Pig-a* MF in folate deficient mice, the magnitude of the effect was

small indicating that other factors such as genetic background could mitigate its effects. In the previous study, the BALB/c inbred strain was used whereas the MutaMouse, a transgenic DBA and BALB/c cross strain was used in the current study. The BALB/c strain has reduced expression of DNA PKs and inefficient repair of ɣ- ray-induced DSBs, specifically in the end joining process [96]. The repair deficiency in the BALB/c strain, however, does not address differences in point mutation susceptibility or repair between the two strains as DNA DSBs are repaired by a different mechanism than DNA point mutations. It is possible, however, that the BALB/c strain may also be more susceptible to DNA point mutations by an alternate mechanism.

We expected that a dietary FA intervention would induce comparable results in the *Pig-a* and bone marrow *lacZ* assay, as mutations in the bone marrow, which contain RBC precursors, would likely appear in RET or RBCs. However, folate deficiency induced a higher *lacZ* MF in bone marrow and did not induce an effect on *Pig-a* MF. A positive correlation between *Pig-a* mutant RETs (Correlation coefficient: 0.621, $p = 0.002$) and RBCs (Correlation coefficient: 0.811, $p = 0.000003$) and bone marrow *lacZ* MF (Figure 4-5); supports that the mode of action of folate deficiency is likely the same in both gene targets but induces an amplified response in bone marrow *lacZ* MF. In a previous study, the sensitivity of the *lacZ* and *Pig-a* assays in detecting BaP-induced mutations in bone marrow were compared. The *lacZ* assay had a higher mutagenic efficiency with the magnitude of the *lacZ* response in bone marrow 25-fold higher than that of the *Pig-a* assay [118]. One of the proposed explanations for the difference was the type of cells used. The *Pig-a* assay analyzes mutations in the *Pig-a* gene in RETs or RBCs, while the *lacZ* in bone marrow measures mutations in the *lacZ* gene in various stem cells types not all of which

are destined to become RETs and RBCs. Other possible explanations are the differences in genomic location of the two target genes, the sequence context, and the fact that the *lacZ* transgene is not transcribed in the mouse while the *Pig-a* gene undergoes transcription and is therefore subject to transcription-coupled repair [118]; these may contribute to an amplified response in the *lacZ* gene in somatic tissues.

A substantial proportion of the general population have a folate status indicative of FA intake at or above the UL (1 mg FA/d) [65]. Based on the current folate intakes of the general population, FA supplementation was an important consideration in this study. Concerns have been raised regarding the effects of increased FA intakes on folate metabolism, the promotion of cancer and the masking of vitamin B_{12} deficiency, allowing the progression of vitamin B_{12} associated neuropathies. FA is metabolized by the enzyme DHFR to form DHF which is in turn metabolized to the bioactive form of folate THF. High levels of FA could theoretically lead to a cellular accumulation of DHF, an inhibitor of TS and MTHFR *in vitro*. Thus the accumulation of DHF could inhibit the formation of dTMP and 5-methyl-THF, and consequently reduce normal DNA and methionine synthesis [66].

MS is a vitamin B_{12} dependent enzyme responsible for transferring a methyl group from 5-methylTHF to homocysteine to form methionine and regenerate THF. Without the regeneration of THF, nucleotide synthesis is inhibited which can lead to megaloblastic anemia, an indicator of both folate and vitamin B_{12} deficiency. Increased intakes of FA can provide sufficient THF to bypass the metabolic block in DNA synthesis, in effect treating vitamin B_{12} dependent anemia without treating the vitamin B_{12} deficiency. Another effect of chronic vitamin B_{12} deficiency is neurodegeneration. Therefore a delay in the diagnosis of vitamin B_{12} deficiency due to the absence of anemia could allow the progression of

vitamin B_{12} deficiency associated neuropathies [57]. In addition, high FA intake could promote the growth of established neoplasms by providing sufficient nucleotides for the increased DNA synthesis demand characteristic of highly proliferative cancer cells. Similar to our previous findings, a supplemented FA intake did not have any adverse effects on chromosomal damage, mutations in the *lacZ* or *Pig-a* gene [85]. Although the topic of FA supplementation warrants further investigation, our preliminary data help alleviate the hypothetical concerns of increased FA intakes and disease progression and/or negative feedback on folate metabolism as they relate to the induction of DNA mutations or chromosomal instability.

5.1.2 Male fertility

Infertility issues have increased in Canada; in 2009-2010, rates had increased to 11.5 % -15.7 % from 8.5 % in 1992. In parallel, the use of ART has dramatically increased [34,35,36]. Folate deficiency is associated with male subfertility, and in previous studies has led to an increased sperm DNA fragmentation index, increased ESTR mutations in sperm and decreased cauda sperm counts in mice [46]. Low seminal plasma folate is associated with increased sperm DNA damage, decreased sperm counts and density and sperm aneuploidy in healthy men [42,43,44]. In chapter 3 we examined the effects of dietary FA intake on germline DNA damage by measuring cauda sperm counts, testis weights and *lacZ* MF in sperm. Unlike our previous study, folate deficiency had no effect on testis weight or cauda sperm counts. In addition, a FA deficient diet had no effect on sperm *lacZ* MF.

Sperm DNA fragmentations index, as measured in the previous study, is an indicator of DNA strand breaks which can be induced if one of two pathways in folate one-carbon metabolism are altered: *de novo* dTMP and methionine synthesis. In conditions of

low folate, TS activity is reduced leading to a build-up of intracellular levels of dUMP, which can be converted to dUTP by sequential phosphorylation reactions. An increase in dUTP levels increases uracil misincorporation into DNA, the repair of which can lead to DNA DSBs and genomic instability. Similarly in cases of low folate, methionine synthesis, and consequently AdoMet production is decreased leading to a decrease in available methyl donors for cellular methylation reactions. A decreased methylation capacity and altered methylation patterns in the genome can lead to chromosomal aberrations including breaks, deletions, duplications and translocations [1,58].

The *lacZ* mutation assay, however, is only able to detect point mutations and small insertions and deletions up to 3 bp long, thus a large deletion that would result from DNA DSBs would likely go undetected [72]. Therefore we propose that FA deficiency induces DNA DSBs in sperm that are undetectable by the *lacZ* assay but captured in in the previous study as a higher DNA fragmentation index. Differences in results from the ESTR mutation analysis and the *lacZ* assay may also be attributed to differences in what is being measured. ESTRs are non-coding, unstable, 4-10 bp repeat units in the genome. Mutations are thought to arise at ESTR loci through an indirect mechanism involving secondary structure formation resulting in the gain or loss of repeat units, which may result from DNA DSBs. The *lacZ* assay, in contrast, measures mutations in a coding region and is able to detect point mutations and small insertions and deletions [72]. Overall, in this study, FA deficiency does not appear to affect parameters of male fertility or sperm DNA damage. It is important to note that these mice were not mated to directly assess male fertility and reproductive outcomes. Given the impact of folate on other metabolic endpoints, including cellular methylation reactions, we cannot say whether these male mice were fertile.

Where FA deficiency may have negative impacts on male fertility, FA supplementation may help prevent or treat sub-fertile or infertile men. In a clinical trial, 65 men from couples experiencing infertility were treated with 15 mg/d of folinic acid (5-formyl-THF) for 3 months. Administration of 5-formyl-THF improved sperm motility and increased spermatozoa numbers [38]. Additionally, supplemental intake of FA and zinc increased sperm concentrations by 74% in subfertile men [45]. We did not observe an effect of FA supplementation on sperm *lacZ* MF, cauda sperm numbers or ENU- induced mutations in sperm. Supplemental FA intake also did not have any adverse effects on the male germline, similar to a previous study in which supplemental FA intake had no effect on cauda sperm counts, sperm DNA fragmentation index, or sperm ESTR mutations [46]. FA supplementation in the BALB/c and MutaMouse strain does not appear to have any effect on mutations in the germline or measures of male fertility.

The results from the current and previous studies inform a potential mechanism for folate deficiency in sperm, namely that sperm may be susceptible to folate-mediated DSBs. Our results also suggest that genetic background likely interacts with diet making some individuals more or less susceptible to diet induced effects. Understanding the mechanisms underlying the development of male infertility can help in the treatment and prevention of the condition.

5.1.3 Mitigation of genomic instability induced by environmental exposures

In vitro, FA supplementation protects against oxidative stress and apoptosis induced by 7-ketocholesterol, a chemical that induces apoptosis in smooth muscle, endothelial and monocytic cells [62]. FA supplementation both in *in vitro* and *in vivo* also protects against the induction of mutations and chromosomal damage in human liver cells

by benzo(a)pyrene (BaP), a strong mutagen and carcinogen [63]. Similarly, FA supplementation reduces chromosomal aberrations in somatic and germ cells as well as reduced DNA fragmentation in mice treated with Daunorubicin (DNR), an anticancer drug used in the treatment of multiple malignancies [64]. These data suggest that increased FA intake could maximize DNA synthesis and methylation potential by upregulating *de novo* nucleotide and methionine synthesis, which could decrease susceptibility to DNA mutations and altered methylation patterns. Therefore we hypothesized that FA supplementation would mitigate the effects of ENU-induced mutations in somatic cells and the germline. However, as shown in in chapter 3 and 4 we observed no effect of supplemented FA intake on the induction of mutations by ENU. However, it is possible that a 50 mg/kg dose of ENU was high enough to counteract any benefit of FA intake on the induction of mutations. These results indicate that an increased FA intake may not provide any additional benefit in the prevention and/ or treatment of somatic mutations induced by environmental toxicants.

5.1.4 Comparison of folate-mediated effects on genomic stability in somatic and germline cells

We observed that inadequate FA intake led to an increased bone marrow *lacZ* MF, likely due to decreased *de novo* purine synthesis and consequently the induction of DNA point mutations. In contrast, we did not see an effect of FA intake on sperm *lacZ* MF. It is possible that there was no effect of folate deficiency on *lacZ* mutations in cauda sperm. However, bone marrow *lacZ* MF was positively correlated with sperm *lacZ* MF (Correlation coefficient, $r = 0.478$, $p = 0.002$, $y = 0.1173x + 5.6122$; Figure 5-1). Given the slope, sperm appears to be less susceptible to the induction of point mutations in the *lacZ*

gene compared to bone marrow by a factor of 2 to 4. It is possible that in addition to DNA DSBs in sperm, proposed based on results from previous studies, FA deficiency may have also induced DNA point mutations but at a level that was not detectable by the *lacZ* assay. A larger sample size may be needed to detect what might be small changes in MF in sperm. Differences between our observations in bone marrow and sperm *lacZ* MF may therefore be explained either by a difference in mode of action of FA deficiency on sperm and bone marrow, a difference in susceptibility of the two tissues to mutations in the *lacZ* gene or simply a lack of mutation induction in sperm induced by folate deficiency.

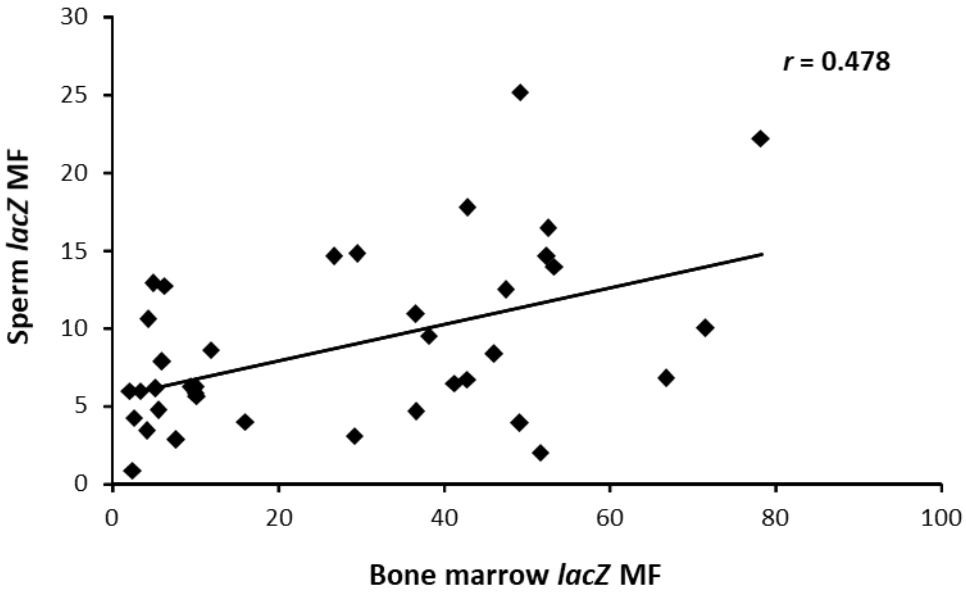

Figure 5-1 Correlation between sperm *lacZ* mutant frequency and bone marrow *lacZ* mutant frequency. Correlation was determined using the Pearson Product Moment correlation test, ($p = 0.002$, $y = 0.1173x + 5.6122$).

5.1.5 Mouse strain differences

The results presented in chapter 3 and 4 based on studies of the MutaMouse strain partly contradict our results observed in the BALB/c inbred mouse strain. A FA deficient diet induced detrimental effects on the male germline and an increase in *Pig-a* MF in BALB/c mice, results that were not repeated in the current study. The difference in susceptibility of the two strains to the effects of FA intake provides a means to place our findings in the context of the human population. In contrast to inbred mouse strains, genetic diversity is high within the human population; thus individuals within the population have a varied susceptibility to environmental/ dietary influences based on their genetic makeup. In the case of nutrition, there is often an interaction between genetic susceptibility and nutrient intake. For example, individuals that are homozygous for a common SNP in the *MTHFR* gene (677 C>T) are less likely to have CRC than individuals that are heterozygous (CT) or homozygous (CC) for the common allele [28]. However, in conditions of folate deficiency, they are all equally likely to have colorectal cancer. Similar observations have been made in animal studies. For example, *Apc* $^{min/+}$ *Shmt1*$^{-/+}$ mice did not develop more intestinal tumors than *Apc* $^{min/+}$ *Shmt1*$^{+/+}$ (*Shmt1* wildtype) mice when both were fed a control diet. It was only after *Apc* $^{min/+}$ *Shmt1*$^{-/+}$ mice were fed a choline and FA deficient diet that they developed 50% more intestinal tumors than Shmt1 wildtype mice. The FA deficient diet alone had no effect on mice without the *Apc* $^{min/+}$ *Shmt1*$^{-/+}$ genotype indicating that FA deficiency was not enough to induce the development of tumors. SHMT1 is an enzyme that regulates the partitioning of one carbon units between the dTMP and methionine biosynthetic pathways; *Apc* is a tumor suppressor gene altered expression of which and has been associated with CRC [76]. These data suggest that the association of

folate deficiency with disease endpoints including cancer and male subfertility, is likely not a one-factor association but instead a gene by diet interaction; a genetic background with increased susceptibility to folate deficiency or genetic mutations may be required for FA intake to elicit an effect. This is an important consideration when investigating the effects of FA intake on somatic and germline mutations and the implications for human health as scientific results pertaining to the effects of FA intake cannot be applied to the entire population.

Overall the detrimental effects of folate deficiency on chromosomal damage and DNA mutations found in this study and others highlight the importance and additional benefits of bringing the Canadian population to folate adequacy. These findings also illustrate the importance of folate adequacy for the sub-populations within the 1 % of the Canadian population that remain folate deficient. A lack of significant effects of FA supplementation at a physiologically relevant dose, equivalent to 1600 µg/day, on somatic and germline DNA damage alleviate concerns associated with high intakes of FA in the population.

5.2 Study strengths and limitations

A strength of this study was our ability to measure MN frequency, *Pig-a* MF, sperm counts, testes weight and MF in bone marrow and sperm simultaneously. The use of the MutaMouse strain and the *lacZ* assay allowed us to simultaneously measure MF across both somatic and germ cells. Although the use of the MutaMouse strain was a strength in this study, its decreased susceptibility to DNA damage in the form of DNA DSBs, compared to that of the BALB/c inbred strain, made it more challenging to detect effects of FA on mutations.

In our study design, ENU was administered 10 weeks prior to blood and tissue collection. The 10 week wash out period prevented the observation of changes in MN frequency between saline exposed and ENU exposed mice. The timing also prevented the opportunity to analyze a protective effect of FA supplementation on MN induced by ENU exposure. In the future a separate blood sample should be taken 1 week after ENU exposure for the MN endpoint, as well as 10 weeks following ENU exposure for the *Pig-a* and *lacZ* endpoints.

Another important measure that could have been investigated in this study is DNA uracil content. We proposed that DNA DSBs were the mechanism underlying the effect of folate deficiency on the male germline. Folate deficiency can induce DNA DSBs through two pathways: *de novo* dTMP and methionine synthesis. Decreased *de novo* dTMP synthesis leads to uracil misincorporation into the DNA. Measuring the uracil content in DNA could therefore help elucidate the pathway in folate metabolism responsible for DNA DSBs in sperm and confirm the hypothesis.

5.3 Future directions

The results of this study highlight the effects of FA intake on somatic mutations in bone marrow and germline mutations in cauda sperm *in vivo*. Future investigations are warranted to increase the statistical power as well as determine the mechanisms underlying the significant effects of FA intake on mutations. In future studies we would like to investigate the contrasting results found between bone marrow and sperm *lacZ* mutation induction and perhaps identify the mechanism underlying the differences.

The mutational spectra can be used to determine the mode of action of folate deficiency on mutations in bone marrow. In Chapter 4 a significantly higher bone marrow

lacZ MF was found for mice fed a FA deficient diet compared to control. It was proposed that the increased MF was a result of DNA point mutations accumulated due to a lack of *de novo* purine synthesis. In order to ensure the statistical significance of this result and possibly bring forward other significant results the mutant plaques should be sequenced in order to correct for clonal expansion. Clonal expansion can result in what are known as "jackpot" mutations which are mutations that arose in an early precursor cell prior to division which results in many daughter cells carrying an identical mutation. The *lacZ* assay provides the opportunity to sequence mutant plaques (used to quantify *lacZ* MF) to correct for clonal expansion and identify the mutational spectra. Once sequenced, any identical mutations that arose from clonal expansion can be corrected for. The mutational spectra can also be used also help determine the mode of action of folate deficiency on mutations in bone marrow.

In addition to the work presented in Chapter 4, the *lacZ* assay could be performed on other somatic tissues including the colon epithelium, a tissue collected at the time of necropsy, to investigate the association between folate deficiency and colorectal cancer. This analysis could help inform the mechanisms underlying the role of folate in the development of gastrointestinal cancers.

5.4 Conclusions

In conclusion, this thesis demonstrates that FA deficiency is detrimental to somatic DNA integrity, and could potentially lead to adverse health effects including cancer and other diseases. This thesis also identifies possible mechanisms underlying the association between folate deficiency and disease risk and development. In combination with data from previous studies, the current study indicates that DNA DSBs and point mutations arise in

somatic and germ cells and a gene by diet interaction likely underlies the association of folate deficiency with the development of disease. Supplemental FA intake did not have any beneficial or detrimental effects on somatic or germ cell mutations, suggesting that FA intakes at the current levels in the population are not detrimental to DNA integrity.

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