**Investigating the effects of folic acid deficiency and supplementation on somatic and
germline mutation frequencies in male mice**

by

Breanne Swayne

A thesis submitted to the Faculty of Graduate and Postdoctoral Affairs

In partial fulfillment of the requirements for the degree of

Master of Science

in

Biology

Carleton University

Ottawa, Ontario

© 2012

Breanne Swayne

Library and Archives
Canada

Published Heritage
Branch

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque et
Archives Canada

Direction du
Patrimoine de l'édition

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence

ISBN: 978-0-494-93537-8

Our file Notre référence

ISBN: 978-0-494-93537-8

NOTICE:

The author has granted a non-exclusive license allowing Library and Archives Canada to reproduce, publish, archive, preserve, conserve, communicate to the public by telecommunication or on the Internet, loan, distribute and sell theses worldwide, for commercial or non-commercial purposes, in microform, paper, electronic and/or any other formats.

The author retains copyright ownership and moral rights in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

AVIS:

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque et Archives Canada de reproduire, publier, archiver, sauvegarder, conserver, transmettre au public par télécommunication ou par l'Internet, prêter, distribuer et vendre des thèses partout dans le monde, à des fins commerciales ou autres, sur support microforme, papier, électronique et/ou autres formats.

L'auteur conserve la propriété du droit d'auteur et des droits moraux qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

In compliance with the Canadian Privacy Act some supporting forms may have been removed from this thesis.

While these forms may be included in the document page count, their removal does not represent any loss of content from the thesis.

Conformément à la loi canadienne sur la protection de la vie privée, quelques formulaires secondaires ont été enlevés de cette thèse.

Bien que ces formulaires aient inclus dans la pagination, il n'y aura aucun contenu manquant.

Canada

Abstract

Folate is required for both DNA synthesis and methylation. Folic acid deficiency results in increased DNA damage and can initiate cancer. However, most Canadians have high blood folate concentrations and little is known about the potential effects of diets high in folate. The effects of folic acid deficiency and supplementation on somatic mutation and germ cell integrity were examined in this project. Folic acid deficiency caused an increase in somatic chromosome damage, decreased sperm counts, increased sperm chromatin damage and increased tandem repeat mutation. This highlights the importance for men to ensure their diets have sufficient folic acid prior to conceiving children. Supplementation did not cause an increase in somatic or germ cell mutation frequencies. Interestingly, folic acid supplementation during early development prevented ethylnitrosourea-induced mutagenesis in the sperm of adult mice, suggesting that supplementation during development may protect the germline of male mice from mutagenic exposures later in life.

Acknowledgements

I would like to thank Dr. Carole Yauk and Dr. Amanda MacFarlane for developing this project and for guidance and support throughout my time as a graduate student. Thank you to my committee members; Dr. Iain Lambert and Dr. Bruce McKay for helpful suggestions and input. I would also like to thank Nathalie Behan, Thomas Shipley, Caitlin Ritz, Andrea Rowan-Carroll, Cheryll Perry and Julie Todd for technical assistance. A BIG thanks to Andrew Williams for conducting all of the statistical analyses. Thanks to the Canadian Institute of Health Research training program in Reproduction, Early Development and the Impact on Health for supporting me financially, and for allowing me to partake in informative training sessions. Finally, a huge thank you goes to my family, friends and loved ones for supporting me and motivating me throughout the last two years, I couldn't have done it without you!

Table of Contents	
Abstract	ii
Acknowledgements	iii
Table of Contents	iv
List of Tables	vi
List of Figures	vii
List of Abbreviations	viii
Statement of contribution	ix
Chapter 1: Introduction	1
1.1 Folate/Folic acid	2
1.2 The effects of dietary folic acid on somatic DNA damage and mutation	8
1.3 The effects of dietary folic acid on germline mutation	9
1.4 Hypothesis and Objectives	12
1.4.1 Hypotheses	12
1.4.2 Objectives	12
1.5 Endpoints	13
1.5.1 Somatic DNA damage/mutation	13
1.5.2 Germline DNA damage/mutation	14
Chapter 2: Supplemental dietary folic acid has no effect on chromosome damage in erythrocyte progenitor cells of mice	16
2.1 Abstract	17
2.2 Introduction	18
2.3 Methods	20
2.3.1 Animal studies	20
2.3.2 Sample collection	25
2.3.3 Red blood cell folate	25
2.3.4 Micronucleus assay	25
2.3.5 <i>Pig-a</i> assay	25
2.3.6 Uracil content in DNA	26
2.3.7 Global DNA methylation	26
2.3.8 Statistical analysis	26
2.4 Results	27
2.4.1 Weaning diet study	27
2.4.2 Multi-generational study	28
2.5 Discussion	32
2.6 Conclusions	37
Chapter 3: Investigating the effects of dietary folic acid on sperm count, DNA damage and mutation in Balb/c mice	38
3.1 Abstract	39
3.2 Introduction	40
3.3 Methods	43

3.3.1 Mice	43
3.3.2 Red blood cell folate concentrations	46
3.3.3 Cauda epididymal sperm counts	46
3.3.4 Sperm chromatin structure assay	46
3.3.5 Isolation of DNA for mutation analysis	47
3.3.6 Single-molecule PCR for detection of mutants	48
3.3.7 Statistical analysis	49
3.4 Results	49
3.4.1 Red blood cell folate concentrations	49
3.4.2 Cauda epididymal sperm counts	52
3.4.3 Sperm chromatin structure assay	52
3.4.4 Tandem repeat mutations	54
3.5 Discussion	56
3.6 Conclusions	61
 Chapter 4: <i>In utero</i> supplementation with folic acid may protect the germline of male offspring from ENU-induced damage	62
4.1 Abstract	63
4.2 Introduction	64
4.3 Methods	66
4.3.1 Mice	66
4.3.2 Red blood cell folate	70
4.3.3 Cauda epididymal sperm counts	70
4.3.4 Sperm chromatin structure assay	70
4.3.5 Single-molecule PCR for detection of mutants	70
4.3.6 Statistical analysis	70
4.4 Results	71
4.4.1 Red blood cell folate	71
4.4.2 Sperm counts	73
4.4.3 Sperm chromatin structure assay	75
4.4.4 Tandem repeat mutations	77
4.5 Discussion	79
 Chapter 5: Discussion	84
5.1 Summary of results	85
5.2 Relevance of findings to human health	86
5.3 Limitations/Improvements that could be made in future studies	87
5.4 Future directions	90
5.5 Conclusions	92
 References	94

List of Tables

Chapter 2:

Table 2-1: RBC folate, micronucleus frequencies, uracil content in nuclear DNA and global DNA methylation.	31
---	-----------

Chapter 3:

Table 3-1: Effects of post-weaning and early developmental dietary folic acid deficiency and supplementation on sperm count and sperm chromatin damage in male mice.	53
---	-----------

List of Figures

Chapter 1:	
Figure 1-1: Folate-mediated one-carbon metabolism.	4
Chapter 2:	
Figure 2-1: Pedigree of the weaning and multi-generational diet studies.	23
Chapter 3:	
Figure 3-1: Folate-mediated one-carbon metabolism.	41
Figure 3-2: Pedigree depicting the dietary groups analyzed in the present study.	45
Figure 3-3: Red blood cell folate in male offspring.	51
Figure 3-4: Effect of folate status on tandem repeat mutation frequencies in the sperm of male offspring.	55
Chapter 4:	
Figure 4-1: Pedigree depicting exposures and diets for analysis of ESTR mutation frequencies in mouse sperm.	69
Figure 4-2: Red blood cell folate in male offspring from female mice fed diets containing 0 mg/kg folic acid (deficient), 2 mg/kg folic acid (control) or 6 mg/kg folic acid (supplemented) during pregnancy/postpartum and lactation (early developmental), or from weaning to maturity (post weaning).	72
Figure 4-3: Effects of ENU treatment on cauda sperm counts from male offspring from the post-weaning and early developmental folic acid diets.	74
Figure 4-4: Effects of ENU treatment in males from the five dietary groups on (A) % sperm with abnormal DNA fragmentation index, and (B) standard deviation of DFI in sperm.	76
Figure 4-5: Effects of ENU treatment on tandem repeat mutation frequencies in the sperm of male offspring with different post-weaning and early developmental dietary folic acid intakes.	78

List of Abbreviations

DFI: DNA fragmentation index

DHF: dihydrofolate

DHFR: dihydrofolate reductase

dUMP: deoxyuridine monophosphate

dTMP: deoxythymidine monophosphate

ENU: ethylnitrosourea

ESTR: expanded simple tandem repeat

GPI: glycoposphatidylinositol

MN-NCE: micronucleated normochromatic erythrocyte

MN-RET: micronucleated reticulocyte

NCE: normochromatic erythrocyte

NTD: neural tube defect

OECD: The Organisation for Economic Co-operation and Development

RBC: red blood cell

RET: reticulocyte

SAM: s-adenosylmethionine

SCSA: sperm chromatic structure assay

SM-PCR: single molecule PCR

THF: tetrahydrofolate

TS: thymidylate synthase

Statement of Contribution

Chapter 1: Introduction

- **Writing of Introduction:** Breanne Swayne

Chapter 2: Supplemental dietary folic acid has no effect on chromosome damage in erythrocyte progenitor cells of mice

- **Study design:** Dr. Amanda MacFarlane and Dr. Carole Yauk
- **Sample collection:** Breanne Swayne
- **DNA isolation:** Breanne Swayne
- **Micronucleus and *Pig-a* assays:** Blood samples were shipped by Breanne to Litron Laboratories for analysis. Breanne received the raw data and analyzed them.
- **Uracil incorporation:** Breanne shipped bone marrow DNA samples to Dr. Patrick Stover's laboratory, where uracil incorporation was measured. Raw data were sent to Breanne for analysis.
- **Global DNA methylation:** Breanne Swayne
- **Writing of Manuscript:** Breanne Swayne

Chapter 3: Investigating the effects of dietary folic acid on sperm count, DNA damage and mutation in Balb/c mice

- **Study design:** Dr. Amanda MacFarlane and Dr. Carole Yauk
- **Sample collection:** Breanne Swayne
- **DNA isolation:** Breanne Swayne
- **Sperm counts:** Breanne Swayne, Alice Kawata, Dr. Mike Wade
- **SCSA:** Was measured in Dr. Mike Wade's lab by Alice Kawata. Breanne received the raw data for analysis.
- **ESTR mutation analysis:** Breanne Swayne
- **Writing of Manuscript:** Breanne Swayne

Chapter 4: *In utero* supplementation with folic acid may protect the germline of male offspring from ENU-induced damage

- **Study design:** Dr. Amanda MacFarlane and Dr. Carole Yauk
- **Sample collection:** Breanne Swayne
- **DNA isolation:** Breanne Swayne
- **Sperm counts:** Breanne Swayne, Alice Kawata, Dr. Mike Wade
- **SCSA:** Was measured in Dr. Mike Wade's lab by Alice Kawata. Breanne received the raw data for analysis.
- **ESTR mutation analysis:** Breanne Swayne
- **Writing of Chapter:** Breanne Swayne

Chapter 5: Discussion

- **Writing of Discussion:** Breanne Swayne

Chapter 1:

Introduction

1.0 Chapter 1: Introduction

1.1 Folate/Folic Acid

Folate is a B vitamin (B9) that mammals must obtain from their diet and is naturally found in fruits, nuts and leafy green vegetables [1]. Folic acid is the more stable synthetic form of folate that is used in the fortification of foods; natural folate loses its activity in days to weeks, whereas folic acid remains stable in food for months to years [2]. Research has shown that a diet deficient in folic acid during pregnancy can lead to neural tube defects (NTD), specifically during the first trimester of pregnancy [3]. NTD arise during embryogenesis when the neural tube fails to close [3].

The link between folic acid and neural tube defects was first described in 1964, and since then extensive research has demonstrated that maternal supplementation with folic acid reduces the prevalence of NTD [3]. For example, 4 mg of folic acid per day administered in a randomized, double blind prevention trial to women at high risk of having a NTD affected birth reduced the prevalence of NTD by 72% relative to a non-supplemented cohort [4]. In order to reduce the incidence of NTD, fortification of cereal products with folic acid was mandated in Canada and the United States in 1998 [5, 6]. De Wals *et al.* (2007) subsequently examined the effects of fortification on the prevalence of NTD [5]. They reported a 46% decrease in the prevalence of NTD post-fortification [5]. However, since the introduction of fortification, a substantial portion of the population has red blood cell folate concentrations that indicate consumption of high levels of folic acid, and the health effects of these elevated levels are not understood.

Folate acts as a methyl donor in both DNA synthesis and DNA methylation (Figure 1-1) [7]. In the DNA synthesis pathway, the production of both purines and

thymidylate rely on products of one-carbon metabolism [7]. 10-formylTHF is required for the *de novo* synthesis of purines. In this reaction, carbon 2 and 8 of the purine ring are obtained from 10-FormylTHF by phosphoribosylaminoimidazole carboxamide formyltransferase and glycinamide ribonucleotide formyltransferase [8]. For thymidylate synthesis, deoxyuridine monophosphate (dUMP) is methylated by thymidylate synthase to become deoxythymidine monophosphate (dTMP) [7]. The methyl group for this conversion comes from 5,10-methylenetetrahydrofolate, an intermediate of one-carbon metabolism [7]. Folate is also necessary for the appropriate methylation of DNA. In this process, 5-methyltetrahydrofolate (a product of one-carbon metabolism) donates a methyl group to homocysteine, converting it into methionine, which is consequently metabolised into s-adenosylmethionine (SAM) [7]. SAM is the methyl donor for cellular methylation reactions.

Figure 1: Folate-mediated one-carbon metabolism. Folate is required for the synthesis of purines, thymidylate and for the methylation of methionine. AdoMet is a methyl donor used for cellular methylation reactions. AdoHcy, S-adenosylhomocysteine; AdoMet, S-adenosylmethionine; DHF, dihydrofolate; DHFR, dihydrofolate reductase; FTHFS, formyltetrahydrofolate synthetase; MTHFC, methenyltetrahydrofolate cyclohydrolase; MTHFD, methylenetetrahydrofolate dehydrogenase; MTHFR, methylenetetrahydrofolate reductase; SHMT1, cytoplasmic serine hydroxymethyltransferase; THF, tetrahydrofolate, TS, thymidylate synthase.

Folate deficiency affects the synthesis of purines, thymidylate, and methionine, and can lead to DNA strand breaks, instability and hypomethylation [1, 9-12]. The synthesis of purines is inhibited by folate deficiency, causing nucleotide pool imbalance and the misinsertion of DNA bases [13]. Folate deficiency also prevents the conversion of dUMP to dTMP causing uracil misincorporation [7]. Uracil that is incorporated into DNA instead of thymidine is quickly removed by uracil glycosylase, producing a single strand break [7]. If folate deficiency persists during DNA repair of the single strand break, the gap may be filled by another uracil and the DNA repair cycle repeated [7]. Ultimately, this process results in the accumulation of single strand breaks in the DNA, which can also lead to double strand breaks [7]. Folate deficiency can also prevent the methylation of homocysteine to form methionine, result in low levels of SAM and prevent proper DNA methylation, and ultimately cause global hypomethylation of DNA [7]. DNA methylation regulates chromatin structure, gene expression, and DNA stability [14]; thus, it is of critical importance that DNA is properly methylated for normal cellular functioning. Hypermethylation of DNA can lead to heterochromatin formation and the inappropriate silencing of genes [15]. For example, tumour suppressor genes are hypermethylated in various cancers [15]. DNA hypomethylation has been linked to the inappropriate activation of genes, including the activation of transposable elements and proto-oncogenes [7].

Although various adverse effects of a diet deficient in folate have been demonstrated, the effects of a diet supplemented with high levels of folic acid are unclear. The folate status of the Canadian population was determined in the Canadian health measures survey for the 2007-2009 time period and was published by Colapinto *et al.*

(2011) [16]. The survey found that 40% of the Canadian population had red blood cell folate concentrations above 1360 nmol/L, which is considered high folate status [16].

Clinical trials have been performed to determine whether folic acid supplementation can prevent the onset of cardiovascular disease and cancer in high risk individuals [2]. Of the many studies that have been performed, only one has shown that supplementation might be beneficial [17]. Wang *et al.* (2007) conducted a meta-analysis of eight randomized trials and found that folic acid supplementation may reduce the risk of stroke in individuals with no history of stroke [17]. Instead of discovering that folic acid supplementation can be preventative for cancer and cardiovascular disease, these studies found that folic acid supplementation can lead to adverse effects in certain individuals. For example, high levels of folic acid supplementation promote the progression of cancer when precancerous lesions are present in the colon [2].

It has also been proposed that high intake of folic acid could saturate one-carbon metabolism resulting in the accumulation of dihydrofolate, which inhibits the enzymes methylenetetrahydrofolate reductase (MTHFR) and thymidylate synthase (TS) [2]. Inhibition of MTHFR would prevent the formation of 5-methyltetrahydrofolate, the methyl donor involved in the formation of SAM [2]. TS is the enzyme that converts dUMP to dTMP; inhibition of this enzyme would prevent thymidylate synthesis causing higher levels of uracil incorporation [2]. Pickell *et al.* (2011) showed that folic acid supplementation may delay embryonic development in mice [18]. They fed pregnant wild-type (MTHFR +/+) and MTHFR +/- mice a 40 mg/kg folic acid diet (20 times the control diet) and examined embryonic development [18]. They found that folic acid supplementation during pregnancy resulted in delayed embryonic development in mice

fed the supplemented diet, and that this effect was more severe in the wild-type mice than the MTHFR +/- mice [19]. The authors proposed that higher folate levels are transferred to wild type embryos, causing inhibition of enzymes in the folate pathway and leading to delayed embryonic development [19]. In contrast, MTHFR +/- mice have reduced production of 5-methylTHF and therefore transfer less folate to the embryo, preventing the negative effects [19].

In contrast to the potentially adverse effects of folic acid supplementation on DNA damage and mutations, it is also possible that supplementation provides some protection to the genome by mitigating the adverse effects of specific toxicants. For example, exposure to various environmental toxicants leads to the production of reactive oxygen species (ROS), which is associated with carcinogenesis [20]. ROS can damage DNA, RNA, proteins and lipids, cause chromosomal instability and mutation, and alter gene expression [20]. Folic acid is oxidized by ROS and in this manner acts as a free-radical scavenger, thereby providing some protection to the genome from ROS [21]. Thus, folic acid may mitigate the genotoxic effects of external agents that operate via ROS production.

The studies described above demonstrate that folic acid deficiency causes DNA damage and can affect DNA methylation. However, relatively few people in developed countries consume diets deficient in folic acid. In contrast, a substantial portion of the population may be experiencing high folic acid intakes. The adverse or beneficial effects of high folate levels on DNA damage and mutation are unclear, and are the focus of this thesis.

1.2 The effects of dietary folic acid on somatic DNA damage and mutation

Cancer development and progression involves the accumulation of gene mutations, epigenetic changes and pathway alterations. Every day we are exposed to millions of agents that can damage our DNA and induce somatic mutations. The accumulation of somatic mutations in our genome is one mechanism that initiates cancer development [22]. The study of dietary and environmental factors that can cause somatic mutation and that may promote the development of cancer is thus important. Conversely, the identification of dietary variables that could possibly decrease mutation rates, or mitigate induced mutation, would also be of great benefit to public health.

Folate deficiency has been found to induce DNA strand breaks leading to DNA instability and micronuclei formation in somatic cells both *in vitro* and *in vivo* [7]. For example, Blount *et al.* (1997) found increased levels of uracil in DNA and increased chromosome damage (micronuclei) in humans with deficient levels of folic acid; supplementation with folic acid reversed these effects [9]. This has also been demonstrated *in vitro*. Duthie *et al.* (1998) exposed human lymphocytes to folic acid deficient medium for 10 days and then examined DNA strand breaks using the comet assay [10]. An increase in DNA strand breaks was observed in cells grown in media lacking folic acid [10]. Similar effects have also been observed in mouse studies. MacGregor *et al.* (1990) and McDorman *et al.* (2002) fed mice a folic acid deficient diet for 7 weeks and observed a doubling in red blood cell micronucleus frequency [23, 24]. Knock *et al.* (2006) examined the frequency of intestinal tumours in mice fed folic acid deficient diets for one year [25]. One quarter of the mice fed the folic acid deficient diet developed an adenoma or adenocarcinoma in the duodenum and none of the mice fed the

control diet developed tumors [25]. Linhart *et al.* (2009) showed that folic acid deficiency does not lead to DNA sequence mutations, but does cause uracil incorporation, indicating that increased DNA strand breaks is likely the mechanism behind tumour development [12]. These studies show that folic acid deficiency induces DNA damage and may play a role in cancer development.

It is well established that folic acid deficiency causes somatic DNA damage and may be carcinogenic. However, the potentially mutagenic effects of supplementation, or the mitigating effects of supplementation on chemically induced genotoxicity, are unknown. Therefore, the effects of diets deficient or supplemented with folic acid on background and induced somatic DNA damage and mutation warrant investigation.

1.3 The effects of dietary folic acid on germline mutation

Germline mutations are important as they can be passed on to offspring. Inherited mutations may lead to various detrimental outcomes including embryonic lethality, genetic disease, or transgenerational genetic instability, thereby influencing the health of future generations. Infertility rates in Canada have increased from 8.5% in 1992 to between 11.5-15.7% in 2009-2010, and the number of babies born with a birth defect is between 2-3% [26, 27]. An association between infertility and birth defects has been shown for increased maternal age, obesity, smoking and alcohol use [26, 27]. However, other variables underlying the increasing rates of infertility and birth defects effects have been difficult to identify. We are exposed daily to complex mixtures of environmental chemicals, some of which have been shown to affect germ cell quality [28]. It is possible that exposure to toxic environmental chemicals that cause DNA damage or mutation in

germ cells plays a role in the observed declines in fertility and healthy births. However, although animal research strongly supports the existence of germ cell mutagens, no agent has ever been declared to be a human germ cell mutagen [28].

The study of heritable mutation is difficult because of the extremely low rate of spontaneous germline mutation [29]. As such, large numbers of animals and high chemical doses are required to assess the mutagenicity of a toxicant on the germline [29]. A handful of studies have explored the role of folate in germ cell DNA damage, mutation or genetic stability. Low folate concentration in seminal plasma is associated with low sperm count and increased sperm DNA damage in humans [30, 31]. Decreased daily folate intake is also associated with aneuploid sperm in humans [32]. Padmanabhan *et al.* (2008) treated male mice with methotrexate to evaluate germ cell toxicity [33]. Methotrexate is a folate antagonist that inhibits the enzyme dihydrofolate reductase and as such mimics folic acid deficiency [33]. They found that methotrexate treated mice had decreased sperm counts and increased sperm DNA damage [33]. These studies suggest that folic acid deficiency is mutagenic to germ cell DNA, though no study has quantified DNA sequence mutation rates in the germ cells or offspring of folic acid deficient animals. Nothing is known about the effects of diets rich in folic acid on the germline.

Currently, the most efficient means to study germline DNA sequence mutations in mice is through the analysis of non-coding expanded simple tandem repeats (ESTRs). ESTRs are made up of short 4-10 bp repeat units that are tandemly repeated to form long homogeneous arrays that can be up to 16 kb in length [34, 35]. They have high rates of both spontaneous and induced mutations in somatic and germ cells, allowing for the determination of mutation frequencies at environmentally relevant doses using small

sample sizes [36]. ESTRs are thought to mutate through an indirect mechanism involving the formation of secondary structures that result in the gain or loss of repeat units during DNA repair or replication [34, 35]. Various agents have been demonstrated to cause ESTR mutations in sperm including radiation, well-established chemical mutagens, anti-cancer drugs, tobacco smoke and air pollution [36-41].

The high rates of spontaneous mutation at ESTRs makes them useful for the study of induced mutation at environmentally relevant doses [36]. For example, Yauk *et al.* (2008) measured DNA damage, global DNA methylation and germline mutation rates in mice exposed to ambient air near two steel mills and a major highway [39]. They found a 1.6 fold increase in ESTR mutation frequency in the sperm of mice exposed to ambient air compared to mice breathing HEPA filtered air. In addition, they found that sperm DNA in the mice breathing ambient air was hypermethylated. The results suggest that exposure to chemicals or particles in the air results in DNA methylation changes that contribute to ESTR destabilization [39]. The findings support a role for perturbations in DNA methylation in ESTR mutation induction [39].

Yauk *et al.* (2008) conducted experiments to specifically examine the relationship between DNA methylation and ESTR instability. They exposed mouse embryonic fibroblast cells to 6 chemicals with different modes of genotoxic action. Global DNA methylation and DNA methyltransferase expression were measured in these cells to examine whether changes in methylation correlate with ESTR destabilization [35]. The chemical 5-azacytidine was used as it is known to cause DNA hypomethylation and is generally not mutagenic. Cells treated with this chemical showed an increase in ESTR mutation frequency demonstrating that altered methylation may affect ESTR instability

[35]. No relationship was found between global DNA methylation, DNA methyltransferase expression and ESTR mutation frequency; however, altered methylation was detected in cells treated with 4 of the chemicals [35]. Interestingly, the only chemical that did not cause changes in global DNA methylation was cisplatin, which also did not affect ESTR mutation frequency [35]. It was proposed that chemical exposures lead to changes in DNA methylation and subsequent modifications in chromatin conformation that affect the ability of secondary structures to be repaired at ESTR loci [35]. Since folic acid is known to cause global DNA hypomethylation and DNA instability, ESTR mutation is an interesting and relevant endpoint for the study of folate and mutation.

1.4 Hypotheses/Objectives

1.4.1 Hypotheses

1. If male mice are fed maternal and/or weaning diets deficient in folate then it may cause somatic and germline DNA damage and mutation
2. Supplemented folic acid diets during early development or post-weaning may lead to increased chromosome damage and germline mutation
3. Increased maternal and/or weaning folic acid intake may protect offspring from ethylnitrosourea (ENU)-induced mutagenesis

1.4.2 Objectives

1. Examine the effect of dietary folic acid intake from weaning to maturity on DNA mutation frequencies and DNA damage in somatic DNA.

2. Examine the effect of maternal and weaning dietary folic acid intake on tandem repeat mutation frequencies in the germline
3. Determine whether increased folic acid intake protects offspring from ENU-induced mutagenesis in germ cells

1.5 Endpoints

1.5.1 Somatic DNA damage/mutation

In this thesis, the micronucleus assay was used to quantify chromosome damage [42]. Micronuclei are pieces of damaged chromosomes or whole chromosomes that lag behind during anaphase and are not included in the nuclei when the nuclear envelope reforms [9, 43]. In this study, micronuclei were measured in both reticulocytes and erythrocytes using flow cytometry [42]. During erythropoiesis, the nucleus is expelled from the blood cell, leaving behind micronuclei that can be stained using a nucleic acid dye and measured [42]. Micronuclei were measured in both reticulocytes (immature blood cells) and erythrocytes (mature blood cells), differentiated from one another using an antibody against the transferrin receptor, that is only expressed in reticulocytes. Propidium iodide was used to stain DNA and the sample was injected into a flow cytometer to measure the number of cells containing micronuclei. This is a widely accepted assay that has The Organisation for Economic Co-operation and Development (OECD) guidelines and is considered a standard *in vivo* genotoxicity assay, which is why it was used in this project.

The *Pig-a* assay is a new *in vivo* technique for measuring DNA sequence mutation in reticulocytes and erythrocytes based on glycosylphosphatidylinositol (GPI) anchor

deficiency [44]. GPI anchor biosynthesis involves 12 genes, 11 of which are autosomal and one (*Pig-a*) that is X-linked [44]. Therefore GPI deficiency would be caused by either a mutation in the single copy of *Pig-a*, or would result from mutations in both copies of one of the autosomal genes [44]. Since mutations in both copies of a gene is extremely rare, GPI deficiency is thus assumed to be overwhelmingly associated with *Pig-a* mutation [44]. A method for measuring the number of GPI deficient cells using fluorescent antibodies and flow cytometry has been developed, and was used here to test the effects of folic acid deficiency on *Pig-a* mutation induction. The *Pig-a* assay used an antibody against the GPI anchor (CD59) to sort cells containing a *Pig-a* mutation using a flow cytometer. Mutation frequency for the *Pig-a* assay was expressed as the number of GPI deficient cells per one million cells. Although the assay is relatively new, there are already cross-laboratory validation exercises going on to develop OECD guidelines for the global acceptance of this assay [45].

1.5.2 Germline DNA damage/mutation

The Sperm chromatin structure assay (SCSA) uses acid-induced denaturation to determine the DNA fragmentation index (DFI) of sperm DNA [46]. A low pH solution is added to the sperm that denatures DNA that is damaged (sperm that contains many strand breaks or sperm with incomplete replacement of histones with protamines) [46]. Acridine orange is then added, which stains double stranded DNA green and denatured single stranded DNA red [46]. The sample is analyzed by flow cytometry and DFI is calculated by comparing red fluorescence to the total fluorescence for each sperm [46]. % DFI can then be calculated and represents the % of all cells that have an abnormal DFI. This assay

is emerging as a valuable tool for measuring chromatin damage and DNA strand breaks in sperm for the diagnosis and treatment of infertility [47]. 60-75% of male factor infertility is unexplained, but 40% of unexplained infertility cases have high DFI values [47]. SCSA can be used in combination with other traditional sperm assessments to predict fertility and determine treatment options [47].

Mutations at ESTR alleles arise by gains or losses of repeat units, which can be measured using a pedigree approach (studying parental band length relative to offspring band length) or using single molecule PCR (SM-PCR) [34]. SM-PCR permits an analysis of mutations in both sperm and somatic cells [48]. For germ cell analysis, PCR is conducted on dilute samples of DNA containing, on average, a single genome (i.e., approximately 3 pg of DNA) [48]. Comparing the length of ESTR alleles in hundreds of sperm cells allows for the detection of germline mutations (size shifts relative to the most common band) in a given animal [48]. SM-PCR was used in this project to determine ESTR mutation frequencies resulting from different maternal or weaning folic acid diets. This assay is only used by a handful of laboratories, and it does not have an OECD guideline. However, PCR amplification of these GC-rich repeats, especially at the single molecule level, is technically challenging and thus few laboratories are set-up to run this assay.

Chapter 2:

**Supplemental dietary folic acid has no effect on
chromosome damage in erythrocyte progenitor
cells of mice**

This data has been published in Swayne *et al.* (2012): Swayne, B.G., Behan, N.A., Williams, A., Stover, P.J., Yauk, C.L., MacFarlane, A.J. (2012) Supplemental Dietary Folic Acid Has No Effect on Chromosome Damage in Erythrocyte Progenitor Cells of Mice. *The Journal of Nutrition*, 142(5):813-7.

2.0 Chapter 2: Supplemental dietary folic acid has no effect on chromosome damage in erythrocyte progenitor cells of mice

2.1 Abstract

Folate deficiency can cause chromosome damage, which could result from reduced *de novo* thymidylate synthesis or DNA hypomethylation. High folic acid intake has been hypothesized to inhibit folate-dependent one carbon metabolism, which could also lead to DNA damage. A significant proportion of the general population may have high folic acid intakes. In this study, two experiments were conducted to examine the effects of folate on chromosome damage. First, male mice were fed folic acid deficient (0 mg folic acid/kg diet), control (2 mg/kg) or supplemented (6 mg folic acid/kg diet) diets from weaning to maturity. Second, female mice were fed the deficient, control or supplemented diet throughout pregnancy, lactation and breeding for three generations; male mice from the F3 generation were fed the same diet as their mothers at weaning, producing 3 groups of F3 male mice. Red blood cell (RBC) micronucleus frequencies, a measure of chromosome damage or aneuploidy, were determined for both experimental groups. In mice fed diets from weaning to maturity, erythrocyte micronucleus frequency increased by 24% in deficient compared to control fed mice. F3 mice fed diet deficient showed a 260% and 174% increase in reticulocyte and erythrocyte micronucleus frequency, respectively, compared to F3 control mice. The supplemented diets did not affect micronucleus frequency, suggesting that excess folic acid at this level does not promote or protect against chromosome damage. The results suggest that chronic exposure to folic acid at the levels similar to those achieved through fortification is unlikely to be clastogenic or aneugenic.

2.2 Introduction

Folate is an essential B vitamin required for *de novo* purine, thymidylate and methionine biosynthesis [49]. Folate deficiency therefore decreases *de novo* nucleotide synthesis and cellular methylation potential, which can cause chromosome damage, altered chromatin structure and ultimately genome instability and aberrant gene expression [49]. For example, folate deficiency in humans leads to increased micronucleus frequency (a measure of chromosome damage) in RBCs, an effect that is reversible with folic acid supplementation [9, 50]. In addition, mice fed a folic acid deficient diet for 7 weeks exhibit increased micronuclei (4). Folate deficiency may cause micronucleus formation as a result of impaired *de novo* thymidylate synthesis [9]. A methyl group from 5,10-methylenetetrahydrofolate (methyleneTHF) is transferred to dUMP by thymidylate synthase (TS) to form thymidylate. When *de novo* thymidylate synthesis is limited, cellular levels of dUTP increase resulting in increased incorporation of uracil into DNA [10, 51, 52]. DNA repair removes the uracil, but in the absence of improved folate status, a cycle of increased uracil incorporation and repair can cause DNA double strand breaks [51, 53]. Alternatively, methyleneTHF can be reduced by the enzyme methylenetetrahydrofolate reductase (MTHFR) to form 5-methylTHF, which is used to remethylate homocysteine to produce methionine. Methionine is converted to S-adenosylmethionine (SAM), the major cellular methyl donor [49]. Folate deficiency can result in reduced cellular methylation potential and DNA hypomethylation. Hypomethylation of pericentromeric DNA can cause centromere dysfunction, and thus produce micronuclei due to chromosome loss [54].

Perhaps counter-intuitively, folic acid supplementation has also been hypothesized to inhibit folate-dependent one carbon metabolism [2]. Folic acid is the synthetic form of folate used in vitamin supplements and for food fortification. Normally, folic acid is taken up by intestinal enterocytes and sequentially reduced to dihydrofolate (DHF) and THF by dihydrofolate reductase (DHFR). THF can be metabolized to 5-methylTHF, the major form of folate in circulation. However, high folic acid intake can overwhelm enterocyte metabolism and result in unmetabolized folic acid in circulation [55, 56]. Folic acid must then be metabolized by the tissues, which could result in the accumulation of cellular DHF. DHF is an inhibitor of MTHFR [57] and may therefore reduce the synthesis of 5-methylTHF required for homocysteine remethylation. Thus, DHF accumulation could cause decreased methionine synthesis and consequently decreased availability of methyl groups for cellular methylation. DHF is also an inhibitor of TS [58] and its accumulation could also theoretically decrease the capacity for *de novo* thymidylate synthesis and lead to chromosome breaks. As such, high folic acid intake, might also lead to genome instability and micronucleus formation.

Canada and the US mandated fortification of white flour with folic acid in 1998 to reduce the prevalence of neural tube defects [6]. As a result, folate deficiency is virtually non-existent in the Canadian population [16]; however, the folate status of a significant proportion of the general population is indicative of high folic acid intakes at or above the tolerable upper intake level [59]. Thus, it is important to evaluate the effects of folic acid supplementation on DNA damage.

Canadians can be divided into two groups: those born before or after mandatory fortification. Future generations will also be exposed to dietary folic acid from

conception; therefore it is necessary to determine the impact of high dietary folic acid intake on health within and across multiple generations. In the present study, I investigate the effect of folic acid deficiency and supplementation from weaning to maturity on RBC micronucleus frequency in adult male mice. In addition, I examine RBC micronucleus frequency in male mice fed folic acid deficient or supplemented diets for multiple generations. The folic acid deficient diet was used as a positive control for clastogenic/aneugenic events. The control diet approximates the recommended dietary allowance of folic acid for adults, which is 0.4 mg per day. The supplemented diet is an environmentally-relevant 3x increase above the recommended daily allowance. Daily vitamin supplements containing up to 1.0 mg folic acid are available without prescription in Canada, therefore, adults consuming supplements in addition to fortified foods can, and do, achieve this level of folic acid consumption [60, 61].

2.3 Methods

2.3.1 Animal Studies

All mice were cared for in accordance with the Guidelines of the Canadian Council on Animal Care, described in the CACC Guide to the Care and Use of Experimental Animals [62] and were approved by the Health Canada Animal Care Committee prior to the initiation of the study. Mice were pair-housed in plastic, HEPA-filtered cages at $22 \pm 2^{\circ}\text{C}$ and room humidity of a minimum of 40% and a maximum of 60%, with a 12-h-light/-dark cycle.

Colony founders: 52 female and 26 male Balb/c mice (7 week old) were purchased from Jackson Laboratories (Bar Harbor, ME) to establish a breeding colony and to produce

mice for the F0 generation. At 8 weeks of age, breeding trios were set-up with two female mice and one male mouse. Breeding colony mice were fed a fixed-formula, non-purified diet (Teklad Global 14% Protein Rodent Maintenance Diet; protein, 14%; energy, 2.9 kcal/g; fat, 4%; and fiber, 18%; Teklad Diets, Madison, WI).

Diets: The control diet was AIN-93G [63], which contains 2 mg folic acid/kg diet (Dyets, Inc, Bethlehem, PA). The folic acid deficient diet was modified AIN-93G containing 0 mg folic acid/kg diet (Dyets, Inc.) and the folic acid supplemented diet was modified AIN-93G containing 6 mg folic acid/kg diet (Dyets, Inc.).

Weaning diet study (Figure 2-1A): F0 female Balb/c mice were fed the control diet at weaning and throughout breeding and lactation. F0 females were bred at 7 weeks of age with age-matched, control diet-fed male Balb/c mice to produce the F1 generation. At day 21, F1 male mice were weaned to either the deficient, control or supplemented diet (n=10 for each diet) and fed the diet for 12 weeks. For 3 weeks prior to euthanasia, the F1 male mice were fed the control diet.

Multi-generational study (Figure 2-1B): Female Balb/c mice (F0) were weaned at day 21 to either the deficient, control or supplemented diet (n=32/diet) and fed the diet throughout breeding and lactation. F0 females were bred at 7 weeks of age with age-matched, control diet-fed male Balb/c mice to produce the F1 generation. F1 and F2 female mice were weaned to the same diet as their mother and fed the diet throughout breeding and lactation. F1 and F2 females were bred as described above. F3 male offspring were weaned to the same diet as their mothers (n=3, 6 and 6 for deficient, control and supplemented, respectively) and fed the diet for 32 weeks.

A single 24 week old female mouse that had been fed the control diet was intraperitoneally injected with 40 mg/kg of ethylnitrosourea (ENU) (Sigma-Aldrich, Oakville, Ontario) 48 hours prior to euthanasia to act as a positive control for the micronucleus analysis.

lactation and breeding for three generations; male mice were weaned onto the same diet as their mother (n=3, 6 and 6 for the deficient, control and supplemented diets, respectively).

2.3.2 Sample collection

Mice were weighed 4 and 18 days prior to sample collection for the weaning diet study and multi-generation study, respectively. Mice were euthanized by live decapitation, and 120 μ l of blood was collected into a tube containing 350 μ l of anticoagulant (provided by the manufacturer, Litron Laboratories, Rochester, NY) for the micronucleus analysis. The remaining blood was collected into a heparin-coated tube for RBC folate determination. Livers were dissected and weighed.

2.3.3 Red blood cell folate

RBC folate was measured using the *Lactobacillus casei* microbiological assay [64]. Folate content was normalized to total protein, which was determined using the Lowry assay [64].

2.3.4 Micronucleus Assay

Blood samples were fixed in methanol on the day of sample collection in accordance with the MicroFlow® kit (Litron Laboratories, Rochester, NY) instructions. Fixed blood samples were then shipped to Litron Laboratories for micronucleus analysis. A three-color labelling method was used to stain the blood samples as described by Dertinger *et al.* [65].

2.3.5 Pig-a Assay

Blood samples were prepared as previously described [66], and shipped to Litron Laboratories for *Pig-a* mutation analysis. An antibody against the GPI anchor (CD59) is

used to sort cells with a *Pig-a* mutation using a flow cytometer, as described by Phonethepswath *et al.* [66].

2.3.6 Uracil Content in DNA

DNA was extracted from bone marrow cells using a standard phenol:chloroform technique. A mass spectrometry based method was used to analyze uracil content in nuclear DNA, as previously described [67].

2.3.7 Global DNA methylation

Global DNA methylation in bone marrow cells was analyzed using two different methods. The cytosine extension assay uses a methyl-sensitive restriction enzyme *HpaII* (New England Bio Labs, Ipswich, MA) (5'-CCGG-3') to measure changes in global DNA methylation at cytosine residues, and was performed as previously described [68]. DNA treated with the CpG methyltransferase (*M.SssI*) (New England Bio Labs, Ipswich, MA) was used as a positive control for the cytosine extension assay. The Methylamp Global DNA Methylation Quantification kit (Epigentek, Farmingdale, NY) was also used to quantify global DNA methylation, according to the manufacturer's protocol. Positive control DNA and a negative control were included with the Methylamp kit.

2.3.8 Statistical Analysis

Differences among the diet groups for body weight, liver weight, liver weight as % body weight, RBC folate, DNA uracil content and global methylation were identified using a two-sample bootstrap test (t-Pivot method) assuming unequal variances [69, 70]

in R [71]. This test is distribution free; the null distribution of the test is simulated by sampling the residuals with replacement and re-calculating the test statistic. The Bonferroni-Holm method was applied to the p-values to control for the familywise error rate. Standard errors for the mean difference for each comparison were estimated using the bootstrap method [70]. Generalized estimating equations [72, 73], assuming a Poisson distribution for the error, were used to model the counts of the %RET, %MN-RET and %MN-NCE. Generalized estimating equations are semi-nonparametric, and provide an alternative to generalized linear models. Generalized estimating equations only require specification of the first two moments, the mean and the variance. In this analysis a log link function was used and the results were back transformed to the original scale. The delta method was used to estimate the back transformed standard errors for the relative difference. Correlations were determined using the Pearson Product Moment correlation test. All analyses, except correlations, were conducted in R using the `geeglm()` function in the `geepack` library [74, 75]. Correlations were determined in SigmaPlot for Windows, version 11.0 (Systat Software, Inc.). Data are presented as mean $\pm$ SEM.

2.4 Results

2.4.1 Weaning diet study

RBC folate was decreased 24% in mice fed the deficient diet compared to mice fed the control diet ($P=0.003$; Table 2-1), even after being fed the control diet for three weeks prior to killing. In contrast, mice fed the supplemented diet containing 3x more folic acid did not have increased RBC folate in comparison with mice fed the control diet (Table 2-1).

Liver weights did not differ between the deficient, control and supplemented diet groups (1.49 ± 0.03 g). Body weights for mice weaned to the deficient, control and supplemented diets did not differ (34.0 ± 0.7 g). There were no significant differences for liver weight expressed as a percentage of body weight among the deficient, control and supplemented diet groups ($4.42 \pm 0.07\%$).

In the weaning diet study a total of 20 million normochromatic erythrocytes (NCE) and 577,383 reticulocytes (RET) were analyzed for all mice, respectively. There were no significant differences in the frequency of micronucleated reticulocytes (MN-RET) among mice fed the deficient, control or supplemented folic acid diets (Table 2-1). However, there was a significant 24% increase in micronucleated normochromatic erythrocytes (MN-NCE) for mice fed the deficient diet compared to mice fed the control diet ($P < 0.0001$) or the supplemented diet ($P < 0.0001$; Table 2-1). Micronucleus frequencies for control and supplemented groups did not differ. The ENU-treated positive control mouse exhibited an 11-fold increase in micronucleus frequency compared to the control group.

Pig-a mutation frequency did not differ between any of the diet groups (Table 2-1). There were no significant diet-dependent changes in bone marrow uracil content (Table 2-1). Similarly, global DNA methylation did not differ between the diets (Table 2-1). These metrics tended to have large amounts of biological variability and there were no apparent trends in the data.

2.4.2 Multi-generational diet study

Three groups of F3 male mice were generated. These mice were the descendants of mothers fed deficient, control or supplemented diets for three consecutive generations and were weaned onto their respective maternal diets and fed the diets for 32 weeks prior to sample collection.

F3 mice from the deficient group had RBC folate concentrations that were decreased by 90% relative to mice from the control group ($P=0.004$; Table 2-1). In addition, RBC folate was increased by 33% in F3 mice from the folic acid supplemented group compared to mice from the control group ($P=0.03$; Table 2-1).

Liver weights between the F3 deficient, control and supplemented mice did not differ (1.65 ± 0.08 g). There was no change in body weights between the deficient, control and supplemented mice (37.9 ± 0.9 g). The deficient mice had a significant increase in liver weight as a percentage of body weight (5.21 ± 0.08 %) compared to the control (4.19 ± 0.14 %) ($P=0.03$) mice and supplemented mice (4.07 ± 0.28 %) ($P=0.02$).

Folic acid deficient F3 mice demonstrated a significant 260% and 174% increase in the frequency of MN-RET and MN-NCE, respectively, in comparison with the control and supplemented groups ($P<0.0001$; Table 2-1). A total of 13.5 million NCE and 300,000 RET were analyzed across all mice, respectively. The MN-RET and MN-NCE frequencies did not differ between the control or supplemented mice.

Uracil content in nuclear DNA declined with increasing dietary folic acid, but there were no statistically significant differences among the dietary groups (Table 2-1). The uracil content in nuclear DNA for the supplemented group tended to be 27% lower than the control group ($P=0.11$) and 40% lower than the deficient group ($P=0.01$; Table 2-1). A significant correlation was observed between bone marrow DNA uracil content

and MN-RET frequency ($r = 0.57$, $P=0.03$), and uracil and MN-NCE frequency ($r = 0.57$, $P=0.03$) with all diet groups included. Absolute DNA uracil content in mice from the weaning study was 4-fold higher than in mice from the multi-generation study. There were no significant changes in bone marrow global DNA methylation among the groups (Table 2-1).

Table 2-1: RBC folate, micronucleus frequencies, uracil content in nuclear DNA and global DNA methylation. Samples from the weaning diet study (male mice fed the deficient, control or supplemented diet for 12 weeks from weaning, all mice were fed the control diet for 3 weeks prior to sacrifice) and the multi-generational study (dams fed the deficient, control or supplemented diet throughout pregnancy and lactation for 3 generations; male mice from the third generation were weaned to the same diet as their mother and fed the diet for 32 weeks).

	Weaning Diet Study			Multi-generational Study		
	D	C	S	D	C	S
RBC folate, <i>fmol/mg protein</i>	724±31 *	905±21	907±20	90.8±8.6 *	793±19	1059±30 *
RET Micronucleus Frequency, %	0.42±0.02	0.47±0.02	0.47±0.02	1.38±0.29 *	0.38±0.02	0.40±0.03
NCE Micronucleus Frequency, %	0.31±0.01 *	0.25±0.01	0.25±0.01	0.73±0.06 *	0.27±0.01	0.28±0.01
<i>Pig-a</i> No. Mutant RET, <i>per 10⁶</i>	1.2±0.6	1.7±0.8	1.1±0.6	N/A	N/A	N/A
<i>Pig-a</i> No. Mutant RBC, <i>per 4x10⁶</i>	6.8±2.0	6.4±2.5	5.1±1.3	N/A	N/A	N/A
Uracil content, <i>pg</i> <i>uracil/ug DNA</i>	0.48±0.03	0.38±0.05	0.48±0.07	0.14±0.02	0.12±0.01	0.09±0.01
Incorporation of [H ³]dCTP in DNA, <i>fold C</i>	1.12±0.84	1.00±0.68	0.80±0.93	0.96±1.03	1.00±0.56	0.64±0.35
Global DNA methylation, % of <i>positive control</i>	3.31±2.61	3.70±1.87	5.55±3.24	8.87±1.87	7.13±5.47	5.18±2.57

¹ Values are mean ± SEM, *Significantly different from control, *P* < 0.05. C, control; D, deficient; NCE, normochromatic erythrocyte; RET, reticulocyte; S, supplemented.

2.5 Discussion

Micronuclei are pieces of damaged chromosomes or whole chromosomes that lag behind during anaphase and are not included in the nuclei when the nuclear envelope reforms [9, 43]. Thus, the presence of micronuclei is indicative of DNA damage at the chromosome level resulting in partial chromosome deletion, addition or rearrangement (clastogenicity) or whole chromosome gain/loss (aneugenicity). I have applied the micronucleus assay to measure chromosome damage in response to folic acid deficient and supplemented diets in two mouse studies.

The weaning diet study was used to determine the clastogenic/aneugenic effects of folic acid deficient and supplemented diets over 12 weeks followed by 3 weeks on a control diet, and addresses the potentially transient effects of diet by comparing micronucleus frequency in immature (RET) versus mature (NCE) RBC. I observed a 24% increase in MN-NCE in mice weaned to the folic acid deficient diet, but no effect on MN-RET. RET are immature red blood cells (lifespan of approximately 15 hours) that differentiate into NCE, which are mature RBC [65, 76]. In contrast, NCE have estimated life spans of 30-40 days [77, 78]. The micronucleus frequency in the NCE reflects NCE produced over the previous month, in addition to those produced in the last 3 weeks. The 3 week period during which mice were fed the control diet would have a much smaller impact on the measured micronucleus frequency in the NCE cellular population. Thus, the experimental design allowed us to measure the transient effects of dietary changes in RET, as well as measure the persistent effects of the diet on the NCE population. The absence of an increase in micronuclei in RET after three weeks on the control diet indicates that the DNA damage was diet-dependent and transient. This finding is

supported by other studies demonstrating that folic acid supplementation in humans rescues the chromosomal damage induced by deficiency [9, 50]. I hypothesized that micronuclei result from uracil incorporation into DNA or perturbations in DNA methylation from the deficient diet. However, there were no differences in either of these measures in the weaning study suggesting that they are either not involved in the process of micronucleus formation, or alternatively that these DNA modifications are also transient. I speculate that it is the latter, as bone marrow cells undergo a significant rate of cell turnover and can therefore quickly clear cells with these characteristics. Together our data demonstrate that folic acid deficiency, but not supplementation, leads to transient chromosome damage in red blood cells, and that the re-introduction of folic acid reverses the effect.

The *Pig-a* assay is used to measure DNA sequence mutation in RBC based on GPI anchor deficiency [44]. It has been proposed that changes in DNA methylation may affect *Pig-a* mutation frequency. Since cellular methylation reactions are dependent on methyl donors from folate, this study was used to examine this. No changes in RET or NCE *Pig-a* mutation frequencies were observed in male mice weaned onto the deficient, control or supplemented diets. Therefore I propose that low levels of alterations in methylation do not affect *Pig-a* expression. However, since I did not see changes in global DNA methylation in this study, it would be important to look at *Pig-a* mutation as an endpoint in mice that were not fed the control diet for 3 weeks prior to sacrifice to confirm this hypothesis.

The multiple generation study was conducted to determine the cumulative effects of a deficient or supplemented folic acid diet over multiple generations and for a

prolonged period of time on DNA damage. There was a significant 260% and 174% increase in MN-RET and MN-NCE in the deficient line after three generations and 32 week exposure. Given that exposure to a relatively high dose of the mutagen ENU (used as a positive control in this study) produced an 11-fold increase in micronucleus frequency, the 2-fold increase caused by folate deficiency is quite substantial [79]. Global DNA methylation did not differ between the dietary groups. However, there was an apparent trend towards increased DNA uracil content in bone marrow cells with declining dietary folic acid (40% higher incorporation of uracil in the DNA of deficient lines relative to supplemented) and a significant correlation between DNA uracil content and micronuclei, supporting the hypothesis that increased uracil content in nuclear DNA leads to chromosome damage.

There are two possible explanations for the more robust response observed in the multi-generational deficient mice relative to those mice placed on deficient diets at weaning for 12 weeks. First, F3 male mice were fed the folic acid deficient diet for a prolonged period (32 weeks) prior to euthanasia, which may have led to an accumulation of micronuclei. Alternatively, folic acid deficiency over three generations may have resulted in a transgenerational cumulative effect on chromosome damage. If the deficient diet results in chromosome changes or hypomethylation that is transmitted through germ cells, genetic instability could manifest in the next generation and be propagated over multiple generations [80]. Although there were no significant differences in global DNA methylation, direct quantification of DNA sequence-specific methylation changes in germ cells within and across generations and diets could provide mechanistic insight into the observed response. Given this interesting finding, future studies on the

transgenerational effects of folic acid deficiency should wean the final generation of mice to the control diet. Analysis of micronucleus frequency in these mice could delineate the immediate effects of the diet from the inherited transgenerational effects.

Characterization of DNA damage, chromatin structure and DNA methylation in parental gametes among the dietary groups could also shed light on this outcome. One final consideration is that three generations of folic acid deficiency may have selected for offspring that are genetically tolerant of chromosomal instability or exhibit enhanced DNA repair, which could be affecting the observed results. For example, offspring selected for enhanced uracil repair would have decreased uracil content and reduced chromosome damage. A significant difference in absolute DNA uracil content between the two studies suggests that indeed differences in age or exposure paradigm impacts uracil incorporation. Gene expression analysis will aid in determining whether this is the case.

As found in the weaning study, there was no change in micronucleus frequency in mice fed the supplemented diet for three generations and 32 weeks. There was a trend for reduction in bone marrow DNA uracil content compared to the control diet ($P=0.11$), suggesting that there may be a lower limit to the relationship between uracil-mediated genome instability and micronucleus formation. Folic acid supplementation did not affect global DNA methylation measures.

It has been suggested that the amount of folic acid used in fortified foods should be doubled in order to ensure that women of childbearing age attain optimal folic acid intake to prevent neural tube defects [81]. However, potential adverse effects of high folic acid intake are unclear and work is needed to establish whether negative health outcomes

are related to high folic acid intake in the general population. I demonstrate no effect of folic acid supplementation on the induction of micronuclei in RBC in male mice, even after having been fed the supplemented diet for 32 weeks and over multiple generations. The data indicate that supplemental folic acid at the level used in this study, which is consistent with combined folic acid consumption from fortified foods and vitamin supplements, was not detrimental, nor did it provide additional benefit, such as a decrease in micronucleus frequency, compared to the control diet. Thus, chronic exposure to excess folic acid at this level does not affect chromosome damage in RBC, an important finding considering the high folate status of a significant proportion of the Canadian population [16].

In the present study I have specifically quantified chromosomal effects of folate supplementation in Balb/c male mice, which are deficient in DNA-PKs and hence compromised in the repair of DNA double strand breaks [82]. It would be cautious to evaluate these effects in other compromised models, such as inbred mice deficient in folate metabolism or uracil repair. Methylated CpG dinucleotides can also be spontaneously deaminated to thymidine [83]. Therefore, an analysis of DNA sequence mutation rates in response to folic acid deficiency or supplementation should be performed. Finally, adverse effects of folic acid supplementation, such as delayed embryonic development in mice or increased progression of cancer in individuals with pre-neoplastic lesions in the colon, have been demonstrated and should therefore continue to be a focus of future research [18, 84].

2.6 Conclusions

In conclusion, folic acid deficiency clearly leads to chromosome damage, which is consistent with previous reports. Mandatory fortification of cereal products with folic acid in North America has essentially eliminated folate deficiency and therefore, may protect this population from chromosome damage and related pathologies such as cancer that would result from folate deficiency. Our data do not support an association of high folic acid intake with genome instability, as indicated by RBC micronucleus frequency. Our findings suggest that excess folic acid at this level is likely not detrimental nor does it provide additional protection against chromosome damage in comparison to adequate folic acid intake.

Chapter 3:
Investigating the effects of dietary folic acid on
sperm count, DNA damage and mutation in
Balb/c mice

This data has been published in Swayne *et al.* (2012): Swayne, B.G., Kawata, A., Behan, N.A., Williams, A., Wade, M., MacFarlane, A.J., Yauk, C.L. (2012) Investigating the effects of dietary folic acid on sperm count, DNA damage and mutation in Balb/c mice.

Mutation Research, 737:1-7.

3.0 Chapter 3: Investigating the effects of dietary folic acid on sperm count, DNA damage and mutation in Balb/c mice

3.1 Abstract

To date, fewer than 50 mutagens have been studied for their ability to cause heritable mutations. The majority of those studied are classical mutagens like radiation and anti-cancer drugs. Very little is known about the dietary variables influencing germline mutation rates. Folate is essential for DNA synthesis and methylation and can impact chromatin structure. I therefore determined the effects of folic acid-deficient (0 mg/kg), control (2 mg/kg) and supplemented (6 mg/kg) diets in early development and during lactation or post-weaning on mutation rates and chromatin quality in sperm of adult male Balb/c mice. The sperm chromatin structure assay and mutation frequencies at expanded simple tandem repeats (ESTRs) were used to evaluate germline DNA integrity. Treatment of a subset of mice fed the control diet with the mutagen ethylnitrosourea (ENU) at 8 weeks of age was included as a positive control. ENU treated mice exhibited decreased cauda sperm counts, increased DNA fragmentation and increased ESTR mutation frequencies relative to non-ENU treated mice fed the control diet. Male mice weaned to the folic acid deficient diet had decreased cauda sperm numbers, increased DNA fragmentation index, and increased ESTR mutation frequency. Folic acid deficiency in early development did not lead to changes in sperm counts or chromatin integrity in adult mice. Folic acid supplementation in early development or post-weaning did not affect germ cell measures. Therefore, adequate folic acid intake in adulthood is important for preventing chromatin damage and mutation in the male germline. Folic acid

supplementation at the level achieved in this study does not improve nor is it detrimental to male germline chromatin integrity.

3.2 Introduction

Germline mutation may lead to various detrimental outcomes including embryonic lethality, inherited genetic disease or transgenerational genetic instability [85]. Since the germline has a very low spontaneous mutation frequency, germ cell mutation assays generally require large numbers of animals and high chemical doses to establish an agent as mutagenic [29]. As a result, much less is known about the environmental variables that influence germ cell mutation rates compared to somatic cells. To date, fewer than 50 mutagens have been studied for their ability to cause heritable mutations [28]. Existing evidence supports the germline mutagenicity of various agents including radiation, mutagenic anti-cancer drugs, and specific environmental mixtures (tobacco smoke, urban air pollution) [39, 40, 86-91]. However, relatively little is known about dietary variables that affect germ cell mutation rates.

Folate, an essential B vitamin, is required for the *de novo* synthesis of purines, thymidylate and methionine [92]. As such, folate is required for the maintenance of DNA synthesis and methylation, and consequently chromatin structure and gene expression (Figure 3-1). In somatic cells, folate deficiency can lead to increased uracil incorporation into DNA, DNA double strand breaks, genome instability and DNA hypomethylation [1, 9-12]. However, few studies have examined the effects of folic acid deficiency on germ cells.

Figure 3-1: Folate-mediated one-carbon metabolism. Folate is required for the synthesis of purines, thymidylate and methionine. Methionine can be converted to AdoMet, the major methyl donor used for cellular methylation reactions. AdoHcy, S-adenosylhomocysteine; AdoMet, S-adenosylmethionine; DHF, dihydrofolate; DHFR, dihydrofolate reductase; FTHFS, formyltetrahydrofolate synthetase; MTHFC, methenyltetrahydrofolate cyclohydrolase; MTHFD, methylenetetrahydrofolate dehydrogenase; MTHFR, methylenetetrahydrofolate reductase; SHMT1, cytoplasmic serine hydroxymethyltransferase; THF, tetrahydrofolate, TS, thymidylate synthase.

Folate deficiency is associated with indicators of reduced male fertility. For example, low folate in seminal plasma is correlated with decreased sperm counts and increased sperm DNA damage in humans [30, 31]. An inverse relationship between total daily folate intake and the frequency of aneuploid sperm in humans has also been observed [32]. In mice, treatment with methotrexate, an antifolate chemotherapeutic, leads to decreased sperm counts and increased sperm DNA damage [33, 93]. These studies demonstrate that folate impacts sperm health and DNA integrity, suggesting that folate deficiency may be mutagenic to germ cell DNA.

In 1998 Canada and the US mandated fortification of white flour with folic acid to reduce the incidence of neural tube defects [5]. Folic acid fortification has successfully eliminated folate deficiency in Canada; however, the folate status of a significant proportion of the general population is indicative of folic acid intakes at or above the tolerable upper intake level [16, 94]. High folic acid intake can overwhelm enterocyte metabolism and result in the presence of unmetabolized folic acid in circulation [55, 56]. Circulating folic acid must be metabolized by the tissues and the presence of unmetabolized folic acid could result in the accumulation of cellular dihydrofolate, an inhibitor of methylenetetrahydrofolate reductase [57] and thymidylate synthase [58]. Inhibition of these enzymes has been proposed to decrease methionine and thymidylate synthesis [2]. Consequently, high folic acid intake may, paradoxically, result in a functional folate deficiency, resulting in increased mutation rates and genome instability.

In the present study I explore the effects of folic acid deficiency and supplementation on male germ cell integrity. I evaluated three measures of sperm health including sperm count, DNA damage using the sperm chromatin structure assay (SCSA),

and DNA sequence mutations at tandemly repeated sequences. The effects of dietary folic acid *in utero* and during lactation (early development) or from weaning for 15 weeks were investigated. Three dietary groups representing folic acid deficiency (0 mg/kg), the recommended daily intake of folic acid (control, 2 mg/kg), and folic acid supplementation (6 mg/kg) at three times the daily recommended intake and approximating the consumption of a 1 mg/day folic acid supplement and folic acid from fortified foods were used.

3.3 Materials and methods

3.3.1 Mice

All mice were cared for in accordance with the Guidelines of the Canadian Council on Animal Care, described in the CACC Guide to the Care and Use of Experimental Animals [62]. The study was approved by the Health Canada Animal Care Committee.

Diets: The control diet was AIN-93G [63], which contains 2 mg folic acid/kg diet (Dyets, Inc, Bethlehem, PA). The folic acid deficient diet was modified AIN-93G containing 0 mg folic acid/kg diet (Dyets, Inc.) and the folic acid supplemented diet was modified AIN-93G containing 6 mg folic acid/kg diet (Dyets, Inc.).

Colony Founders: 52 female and 26 male Balb/c mice (7 weeks old) were purchased from Jackson Labs (Bar Harbor, ME) to establish a breeding colony and to give rise to the F0 generation. At 8 weeks of age breeding trios were set-up with 2 females and 1 male, all were fed a fixed formula, non-purified diet (Teklad Diets, Madison, WI) during the breeding and post-natal periods.

F0: At 21 days of age, groups of female mice were weaned to one of the three experimental diets, deficient (n=32), control (n=32) or supplemented (n=32) (Figure 3-2). At 7 weeks of age, breeding trios were set-up with aged-matched male mice that had been fed the control diet, and fed the diet to which the female had been weaned. Female mice were fed their respective diets during pregnancy, postpartum and lactation.

F1: At 21 days of age, 12 male mice from separate litters were selected from the deficient and the supplemented maternal diet groups (12 pups from each group) and were weaned to the control folic acid diet (2 mg/kg). Twenty four male mice from the control maternal diet group (two per independent litter) were weaned to the control diet. Male mice from dams fed the control diet were also weaned to either the deficient or supplemented diets (n=12 for each diet from 12 independent litters). Male mice were fed their respective diets for 15 weeks and killed at 18 weeks of age (Figure 3-2). For breeding purposes (F2 and F3 generations were produced as part of another study) all mice were fed the control diet for 3 weeks prior to sacrifice to ensure that females, with which the males were co-housed, were folate replete.

As a positive control for DNA mutation, half of the male mice from the control diet (n=12) at 8 weeks of age were given a single intraperitoneal injection of 75 mg/kg ethylnitrosourea (ENU, Sigma-Aldrich) dissolved in M/15 phosphate buffer, pH 6.0, and sacrificed at 18 weeks of age.

Figure 3-2: Pedigree depicting the dietary groups analyzed in the present study. Diets contained 0 mg/kg folic acid in the deficient diet, 2 mg/kg folic acid in the control diet and 6 mg/kg folic acid in the supplemented diet. (*12 mice from this group were treated with ENU as a positive control for DNA damage and mutation.)

3.3.2 Red Blood Cell folate concentrations

The *Lactobacillus casei* microbiological assay was used to measure RBC folate as previously described [64]. Folate content was normalized to total protein, which was determined using the Lowry assay [64].

3.3.3 Cauda epididymal sperm counts

One cauda from each mouse was used for sperm collection and counting. Each cauda was placed into 1 mL of TNE buffer (0.01 M Tris-HCl, 0.15 M NaCl and 1 mM EDTA, pH 7.4) on ice and minced with iris scissors until no obvious piece of tissue was visible by eye. The sample was then filtered through a 70 µm filter (DAKO Diagnostics Canada Inc., Mississauga, Canada) to remove any residual pieces of tissue. Ten µL of sample was diluted 1:10 in TNE buffer and counted by two independent individuals using a haemocytometer whose results were averaged to give the estimate of cauda sperm content.

3.3.4 Sperm Chromatin Structure Assay

Sperm chromatin integrity was evaluated using the SCSA as previously described [46, 95]. On the day of analysis, 200 µl of sperm samples with a concentration of $2.5 - 3 \times 10^6$ cells/mL in TNE buffer were quickly thawed at 37 °C. Immediately, sperm samples were gently mixed with 400 µl of acid-detergent solution (0.1% Triton X-100, 0.15 M NaCl and 0.08 N HCl, pH 1.2) on ice for 30 seconds and then stained with 1.2 mL of 6 µg/ml acridine orange staining solution in phosphate-citrate buffer (0.037 M citric acid, 0.126 M Na₂HPO₄, 1 mM EDTA disodium, 0.15 mM NaCl, pH 6.0). The stained samples

analysed using a FACSCalibur flow cytometer (BD Biosciences, Mississauga, Canada) immediately and data acquisition started exactly 3 min after the addition of acid-detergent solution. Duplicate aliquots were analysed for each sample and a total of 10 000 events were collected for each measurement at a flow rate of 200–300 cells/sec. A pool of normal mouse cauda sperm was used for negative and positive controls and these were analysed every 10th sample to ensure stable cytometer parameters. Positive controls were obtained by boiling an aliquot of the pooled sperm for 3 minutes. Raw data were analyzed using FCS express 4 software (De Novo Software, Los Angeles, CA). Results were expressed as DNA fragmentation index (DFI). DFI is calculated as the proportion of red fluorescence to the total fluorescence for each sperm. In a sample of normal sperm, DFI values will cluster tightly whereas samples from animals with increased levels of sperm DNA damage will have a subpopulation of sperm with much higher DFI values. I report the proportion of cells with DFI values higher than the range of tight DFI values observed in the negative control samples (% DFI).

3.3.5 Isolation of DNA for mutation analysis

Sperm DNA was prepared using the methods described by Yauk *et al.* [48]. All DNA isolations were carried out in a laminar flow hood to minimize the risk of PCR template contamination. Briefly, sperm cells were isolated from one cauda epididymus per mouse and somatic cells were lysed with 0.15% sodium dodecyl sulfate. Sperm cells were re-suspended in 1 mL 0.2 X sodium-chloride-sodium citrate, 1M 2-mercaptoethanol, 1% sodium dodecyl sulfate, and digested with Proteinase K overnight at 37°C. DNA was extracted using a standard phenol:chloroform technique followed by

ethanol precipitation, and dissolved in distilled water. One μg of DNA for each mouse was digested overnight at 37°C with the restriction enzyme *MseI* (New England Bio Labs, Ipswich, MA). All DNA dilutions used for PCR were in $5\text{ ng}/\mu\text{L}$ herring sperm DNA in 5 mM Tris-HCL, pH 8.

3.3.6 Single molecule PCR for detection of mutants

Template DNA was diluted to concentrations resulting in $\sim 40\text{-}60\%$ of the PCR amplifications producing observable products. The *Ms6-hm* allele was amplified using HM1 forward (5' AGA GTT TCT AGT TGC TGT GA-3') and HM1 reverse primers (5'-GAG AGT CAG TTC TAA GGC-3') and the Roche High Fidelity PCR System, dNTPack (Roche Diagnostics, Laval, QC) on a PTC-225 DNA Engine Tetrad Thermocycler unit (MJ Research now BioRad, Mississauga, ON). PCR products were electrophoresed on 0.8% agarose gels and visualized by Southern blotting and hybridization with αP^{32} radiolabelled *Ms6-hm* probe. Wells containing observable PCR products were then electrophoresed on 40 cm long, 0.8% agarose gels at 120V in cooled chambers for $\sim 44\text{-}48$ hours. Gels were visualized by Southern blotting and hybridized with *Ms6-hm* probe. Images of the blots were normalized to achieve 10 cm spacing between the 3 and 6 kbp bands of an in-lane DNA standard (50 ng per well of 1 kbp ladder, Invitrogen). Gels were independently scored by two individuals, each blinded to the sample treatment and results observed by the other individual, by measuring the distance between each *Ms6-hm* band and the in-lane DNA standard. The average allele size per gel was calculated and bands were considered to be a mutant if they migrated

either 1 mm more or less than the average allele size. Mutation frequency was calculated as the ratio of mutant bands to total bands.

3.3.7 Statistical Analysis

Differences among the diet groups for RBC folate, cauda sperm numbers and % DFI were identified using a two-sample bootstrap test (t-Pivot method) assuming unequal variances [69, 70] in R [71]. For the ESTR assay, a Poisson distribution was used to estimate the number of progenitor PCR template molecules, determined from the number of wells used and the number of positive PCR estimated for each sample. Generalized estimating equations (GEEs) [72, 96] assuming a Poisson distribution for the error were used to estimate the mutation frequencies and selected pairwise comparisons using the geepack library [74, 97, 98] in R [71]. The control group for all measures was considered to be the animals fed the 2 mg/kg folate diets during early development and post-weaning.

3.4 Results

3.4.1 Red Blood Cell Folate Concentrations

Red blood cell (RBC) folate was decreased 55% in male offspring weaned to the deficient diet relative to control mice ($P<0.01$) (Figure 3-3). There were no effects of folic acid supplementation post-weaning or of folic acid deficient or supplemented diets in early development, on RBC folate. It should be noted that mice from the post-weaning diet groups were fed the control folic acid diet for three weeks prior to being killed for

breeding purposes as part of another study. Treatment with ENU caused a 15% increase in RBC folate ($P=0.05$) (Figure 3-3).

Figure 3-3: Red blood cell folate in male offspring from female mice fed diets containing 0 mg/kg folic acid (deficient), 2 mg/kg folic acid (control) or 6 mg/kg folic acid (supplemented) during pregnancy/postpartum and lactation (early development). Male offspring from each dam diet group were weaned to the control diet and fed the diet for 15 weeks. A subset of male offspring from dams fed the control diet were weaned to either the deficient or supplemented diet and fed these diets for 15 weeks. Error bars represent the standard error. (* $P < 0.05$ for positive control group [ENU] compared to control diet group, ** $P < 0.01$ for weaning deficient diet group compared to control diet group.)

3.4.2 Cauda Epididymal Sperm Counts

Male offspring weaned to the deficient diet had decreased cauda sperm number relative to control mice ($P=0.03$) (Table 3-1). Exposure to the post-weaning supplemented diet did not lead to changes in cauda sperm number. Neither the early developmental deficient or supplemented diets influenced cauda sperm number (Table 3-1). Treatment with ENU resulted in a 42% decrease in sperm number ($P=0.01$) (Table 3-1).

3.4.3 Sperm Chromatin Structure Assay

The percentage of sperm with abnormal DFI (% DFI) was higher for male offspring weaned to the deficient diet compared to control mice ($P=0.04$) (Table 3-1). No change in the % DFI was observed in males weaned to the supplemented diet, or in mice fed the deficient or supplemented diets in early development in comparison to control mice. Treatment with ENU resulted in a 2-fold increase in DFI ($P=0.02$).

Table 3-1: Effects of post-weaning and early developmental dietary folic acid deficiency and supplementation on sperm count and sperm chromatin damage in male mice.

	Sperm Counts		Sperm chromatin Structure Assay	
	# Sperm per Cauda ($\times 10^6$)	<i>P</i>	% DFI	<i>P</i>
Post-weaning				
Deficient	9.3 $\pm$ 1.2	0.03	5.0 $\pm$ 0.9	0.04
Supplemented	14.0 $\pm$ 1.5	0.58	2.7 $\pm$ 0.4	0.46
Control	13.0 $\pm$ 1.1		2.6 $\pm$ 0.1	
Early development				
Deficient	11.6 $\pm$ 1.4	0.47	3.7 $\pm$ 0.5	0.32
Supplemented	10.9 $\pm$ 0.8	0.15	3.1 $\pm$ 0.7	0.27
Positive Control (ENU)	7.4 $\pm$ 1.2	0.01	5.5 $\pm$ 1.0	0.02

All values are expressed as mean $\pm$ SEM. All *P* are for comparisons with control diet. Bold text indicates statistical significance.

3.4.4 Tandem Repeat Mutations

Sperm ESTR mutation frequency was increased 2-fold in male offspring weaned to the folic acid deficient diet compared to control mice ($P=0.03$) (Figure 3-4). There was no change in ESTR mutation frequency for male offspring weaned to the supplemented diet. The early developmental deficient and supplemented diets did not lead to changes in ESTR mutation frequency (Figure 3-4). A two-fold increase in ESTR mutation frequency was observed for the ENU-treated positive control group ($P=0.01$) (Figure 3-4).

Figure 3-4: Effect of folate status on tandem repeat mutation frequencies in the sperm of male offspring. Error bars represent the standard error. (* $P < 0.05$ compared to control diet group).

3.5 Discussion

This study was designed to examine the effect of dietary folic acid on sperm count, chromatin structure and DNA mutation. Mice were fed folic acid deficient or supplemented diets either in early development (*in utero* and during lactation) or post-weaning for 15 weeks. The control diet approximates the recommended dietary allowance of folic acid for adults, which is 0.4 mg per day. The supplemented diet contained 3-fold folic acid of the control diet, an environmentally relevant amount that adults achieve through the consumption of both fortified foods and supplements [61, 99].

Sperm counts are a crude measure of fertility. Decreased sperm counts generally indicate impaired spermatogenesis or increased elimination of germ cells during spermatogenesis [33, 100]. Males weaned to the deficient diet had decreased cauda sperm number, similar to observed associations between folate status or intake and sperm number in men [31]. Mice lacking methylenetetrahydrofolate reductase, a key enzyme in folate metabolism, also had reduced sperm counts [101]. Folic acid deficiency could reduce sperm counts in two ways. Folic acid deficiency decreases the *de novo* synthesis of purines and thymidylate, which can lead to the misinsertion of dNTPs and subsequent genomic instability [102, 103]. The methyl groups from folate are also required for the production of methionine, which is converted to S-adenosylmethionine, the major cellular methyl donor [49]. DNA and histone methylation may also be affected by folic acid deficiency, which could lead to changes in chromatin packaging and gene expression. Therefore DNA damage and/or altered DNA and histone methylation could lead to impaired spermatogenesis and decreased sperm counts.

The sperm chromatin structure assay is an independent predictor of successful pregnancy [47]. The assay measures the susceptibility of sperm DNA to acid-induced denaturation and produces a measure called the DNA fragmentation index [46]. An increased DFI indicates an increase in DNA strand breaks or protamine defects [47]. I found an increased percentage of sperm with abnormal DFI in male mice weaned to the deficient diet. Chromatin breaks are most likely to occur and persist during the final 3 weeks of spermatogenesis, as the cells are primarily DNA repair deficient during these latter stages. Therefore, it is of note that despite being fed a folate-replete diet for three weeks before necropsy, the deficient mice still had a significant increase in the DFI. Since folic acid deficiency leads to uracil incorporation into DNA and hence, increased DNA strand breaks, I speculate that this mechanism could be contributing to increased DFI. Direct measurement of sperm DNA uracil incorporation and associated strand breaks will clarify this issue. An increase in DFI can also indicate protamine defects [47]. During spermatogenesis, sperm DNA is condensed and the majority of histones are replaced with protamines [104]. The histones that are retained in sperm DNA are associated with the promoters of genes involved in embryo development (imprinted genes, *HOX* genes, etc.) [105]. These promoters have been found to be hypomethylated [105]. Folic acid deficiency inhibits the production of methionine and subsequently S-adenosylmethionine, the universal methyl donor, and can cause DNA hypomethylation [12]. Therefore, folic acid deficiency could affect methylation and histone replacement in sperm DNA. Our data, and data relating low folate in seminal plasma with increased sperm chromatin damage in men [30], illustrate that folic acid deficiency can lead to chromatin damage in sperm, which can be prevented with adequate folic acid intake.

DNA damage in sperm can lead to impaired fertility or adverse effects on early embryonic development [85]. DNA damage that becomes fixed as mutations in sperm DNA may be inherited and result in a variety of genetic disorders. Because germ cell mutations are so rare (i.e., $\sim 1.1 \times 10^{-8}$ per nucleotide per haploid genome [106]), recent efforts have focussed on quantifying mutation rates in highly variable non-coding regions of the genome [106]. Germ cell mutation frequencies can be measured at mouse ESTR loci. ESTRs are short 4-10bp repeat units that are tandemly repeated to form long homogeneous arrays up to 20kb in length [34, 35]. They have high rates of mutation in somatic and germ cells, allowing for the determination of mutation frequencies at environmentally relevant doses using small sample sizes [36]. Although ESTR sequences are found in non-coding regions of DNA, the doubling dose for ESTR mutations is similar to that of coding DNA [107], and these loci show a dose-response relationship with both radiation and chemical mutagens [107, 108]. Thus, ESTRs provide an efficient means to survey the ability of agents to cause mutations and genetic instability in the germline of mice.

In the present study I observed a two-fold increase in ESTR mutation frequency for mice weaned to the deficient diet. I note that exposure to 75 mg/kg of the powerful germ cell mutagen ENU caused a virtually identical increase in ESTR mutation frequency. Thus, the increased mutation frequency observed by folate deficiency is substantial. ESTRs are thought to mutate through an indirect mechanism involving the formation of secondary structures that result in the gain or loss of repeat units during DNA repair or replication processes occurring in pre-meiotic stages of spermatogenesis [34, 35]. Thus, the final 4 weeks prior to sample collection for ESTR analysis is not

anticipated to affect the mutation outcome. The post-weaning experimental design effectively captures sperm derived from spermatogonial stem cells exposed to 12 weeks of folic acid deficiency, and a subsequent 3 weeks of deficiency for germ cells committed to spermatogenesis, including 2 weeks as pre-meiotic spermatocytes.

There are three potential mechanisms that could lead to the increase in ESTR mutation frequency in the weaning folic acid deficient group. The incorporation of uracil into DNA, as a result of folic acid deficiency, is repaired by uracil glycosylase [7]. Futile cycles of uracil incorporation and repair can introduce double strand breaks, which may initiate a cell cycle checkpoint resulting in cell cycle arrest for DNA repair or programmed cell death. This can lead to global DNA polymerase pausing and subsequent formation of secondary structures at ESTR loci [34]. Folic acid deficiency also impairs *de novo* purine synthesis, which can lead to the misinsertion of dNTPs. Repair of these misinsertions may also initiate cell cycle arrest for repair and lead to polymerase pausing [34]. Another possibility is that folic acid deficiency may affect sperm DNA methylation, which may indirectly impact ESTR mutation frequency. For example, 5-azacytidine, an agent that causes global DNA hypomethylation, causes an increase in ESTR mutation frequency in embryonic fibroblast cells in culture [35]. It has been hypothesized that chemical exposures that alter DNA methylation cause changes in chromatin conformation and affect the ability of secondary structures to be repaired at ESTR loci [35]. Since folic acid deficiency is known to cause DNA mutation, damage and global DNA hypomethylation, all of these pathways could lead to increased ESTR mutation in the germline. In our follow up studies, I will investigate the contribution of uracil incorporation and DNA methylation to ESTR mutation induction by quantifying these

measures directly in the sperm DNA of folic acid deficient mice. In addition, ESTR mutation frequencies in the descendants of these mice will be evaluated to determine if folic acid deficiency leads to transgenerational genetic instability. Studying the offspring will also provide insight into whether altered DNA methylation causes transgenerational or other effects in germ cells.

Males that were folic acid deficient during early development did not exhibit statistically significant increases in sperm chromatin damage or DNA mutation. The folic acid deficient diet was fed to female mice for four weeks prior to breeding, as well as throughout pregnancy and lactation. Male offspring were then fed the control diet from weaning for 15 weeks. Thus, these male offspring were folic acid deficient during the initial stages of germ cell development before being weaned to the control diet. Since these mice were only exposed to the folic acid deficient diet from embryo to weaning (less time than the post-weaning mice), the data suggest that early developmental exposure to deficiency has no effect or the period of exposure was not long enough to cause germ cell effects or the effect was transient and recovered during the post-weaning period.

In contrast to the significant impacts of folate deficiency, folic acid supplementation did not affect sperm counts, sperm chromatin damage or ESTR mutation frequencies. Thus, exposure to folic acid supplementation at the level achieved in this study during early development or post-weaning is not detrimental to the male germline. I am currently measuring ESTR mutation frequencies in mice from each of the diet groups that were exposed to ENU. The results will allow us to determine whether early life or

post-weaning dietary folic acid impacts chemically-induced mutation frequency in the germline.

Even though mice were fed the control diet for 3 weeks prior to sampling, RBC folate was decreased by 55% in male mice weaned to the deficient diet. Male mice weaned to the supplemented diet did not have increased RBC folate, likely as a result of being fed the folate-replete diet. ENU treatment led to a 15% increase in RBC folate in male mice fed the control diet suggesting that ENU treatment may perturb folate metabolism. The results must be verified in future studies at which point the mechanism can be pursued.

3.6 Conclusions

I demonstrate that folic acid deficiency in adult male mice causes decreases in sperm numbers, and increases in germline chromatin damage and DNA mutation. Supplementation with folic acid in early development or post-weaning does not cause changes in the germ cell measures examined. Increased DNA strand breaks or hypomethylation are both potential mechanisms underlying the observed effects. Our study highlights the importance of adequate folic acid intake for male fertility and for the prevention of DNA mutation in the germline.

Chapter 4:

***In utero* supplementation with folic acid may
protect the germline of male offspring from ENU-
induced damage**

4.0 Chapter 4: *In utero* supplementation with folic acid may protect the germline of male offspring from ENU-induced damage

4.1 Abstract

Infertility rates are on the rise in Canada, and the prevalence of birth defects is between 2-3%. Many of the causes behind this are unknown, however, environmental exposures are difficult to characterize and could be contributing factors. In this study, I evaluate whether dietary factors can mitigate the effects of environmental exposures on germ cells. I examined the interaction between dietary folic acid intake (early developmental or post-weaning) and ethylnitrosourea (ENU) -induced mutagenesis in the male germline. Male Balb/c mice were fed folic acid deficient (0 mg/kg), control (2 mg/kg) or supplemented (6 mg/kg) diets during early development or post-weaning. Germ cell integrity was evaluated using sperm counts, the sperm chromatin structure assay, and tandem repeat mutation frequencies. I show that ENU treatment leads to a ~50% decrease in cauda sperm numbers, a 3% increase in sperm chromatin damage, and a 2-fold increase in sperm tandem repeat mutation frequency. Dietary folic acid intake did not promote or prevent ENU-induced changes in sperm count or chromatin damage. However, tandem repeat mutation frequencies in adults supplemented with folic acid in early development were not significantly increased following ENU treatment. Thus, folic acid supplementation during critical stages of gametogenesis may protect the germline of male mice from genotoxin-induced mutation.

4.2 Introduction

Every day we are exposed to potentially toxic chemicals in the air we breathe, the water we drink and the food we eat. Some of these toxicants may have the ability to affect germ cell quality, which could lead to decreased fertility and decreased chances of siring a healthy offspring. The number of couples seeking the use of assisted reproductive technologies to become pregnant has increased in recent years suggesting that the prevalence of infertility is on the rise [26]. Bushnik *et al.* (2012) estimated the infertility rate in Canada to be between 11.5-15.7% in 2009-2010 [26]. This is an increase over previous estimations of infertility, which were 5.4 % in 1984 and 8.5% in 1992 [26]. There are many different factors that have been shown to affect fertility, including increased female age, obesity, smoking and alcohol use. However, pin-pointing the exact causes of infertility can be difficult as we are also constantly exposed to a wide range of potentially hazardous chemical agents that may impact fertility.

It has been argued that certain environmental exposures can cause inherited mutation, which can lead to detrimental outcomes including embryonic lethality and genetic disease in offspring [85]. A report from the Canadian Perinatal Surveillance System in 2002 found that 2-3% of babies are born with a birth defect, and of these defects, 40-60% have unexplained causes [27]. The main objective behind the establishment of the Canadian Perinatal Surveillance System is to identify potential teratogens [27]. They define a teratogen as “a factor that has an adverse effect on an embryo or a fetus between fertilization and birth [27]”. Teratogenicity is different from mutagenicity, but given the low rate of germ cell mutation in the human population, it is equally challenging to identify factors that contribute to inherited mutations. Therefore, it

is important to identify teratogenic or mutagenic factors that mitigate their effects, a challenging problem as we are exposed to combinations and mixtures of mutagens daily.

The focus of this study was to determine if dietary factors, specifically folic acid, can be used to mitigate the effects of environmental agents on sperm DNA quality in the form of chromatin damage and DNA mutation. I hypothesized that supplementation with folic acid during early development or post-weaning may protect offspring against induced mutation later in life. ENU was used as a model agent as it is a well-established germ cell mutagen [109-111]. ENU is a monofunctional alkylating agent that adds ethyl groups to the N and O positions of DNA bases resulting in the formation of DNA adducts [108, 112-114]. These adducts can lead to base substitution mutations or can be repaired leading to single strand breaks [108, 112-114].

The present study utilizes expanded simple tandem repeat (ESTR) DNA analysis (described in Chapter 3) to study sperm mutations. ENU is a well-established mutagen that causes ESTR mutations. For example, Dubrova *et al.* exposed male Balb/c and CBA/Ca mice to 150 mg/kg ENU 10 weeks prior to mating [112]. Treatment with ENU resulted in a ~4 fold increase in ESTR mutation frequency in somatic cells in both mouse strains [112]. In addition to causing increases in ESTR mutation frequencies, ENU has also been shown to affect DNA methylation *in vitro* [35]. Yauk *et al.* examined global DNA methylation in mouse embryonic fibroblast cells following treatment with ENU [35]. Using the cytosine extension assay, they found that DNA in cells treated with ENU was hypomethylated [35]. These studies demonstrate that ENU causes increased germline mutation frequencies at ESTR loci and leads to global DNA hypomethylation.

Folic acid deficiency causes increased DNA strand breaks and DNA hypomethylation in somatic cells [1, 9-12]. I have recently shown that folic acid deficiency also leads to an increase in sperm ESTR mutation frequency (see Chapter 3, 3.4.4). I hypothesized that the 2-fold increase in ESTR mutation frequency that I observed could be caused by the formation of secondary structures at ESTR loci in response to global DNA polymerase pausing to repair the misinsertion of DNA bases [7, 34, 35]. Another potential mechanism is that alterations in DNA methylation could indirectly lead to increased ESTR mutation frequency [35].

Since both ENU and folic acid deficiency have been shown to increase sperm ESTR mutation frequency and lead to changes in DNA methylation, the goal of this study was to determine if folic acid supplementation can be used to protect the genome from induced mutations from external agents. Two main developmental stages of exposure were examined (Figure 4-1): maternal folic acid intake during pregnancy/postpartum and lactation (early developmental exposure) and post-weaning folic acid intake. Three folic acid diets were used: 0 mg/kg folic acid (deficient), 2 mg/kg (control) and 6 mg/kg (supplemented). Sperm count, chromatin structure and ESTR mutation frequencies were measured to evaluate germ cell health following ENU exposure.

4.3 Methods

4.3.1 Mice

All mice were cared for in accordance with the Guidelines of the Canadian Council on Animal Care, described in the CACC Guide to the Care and Use of

Experimental Animals [62]. The study was approved by the Health Canada Animal Care Committee.

Diets: The control diet was AIN-93G [63], which contains 2 mg folic acid/kg diet (Dyets, Inc, Bethlehem, PA). The folic acid deficient diet was modified AIN-93G containing 0 mg folic acid/kg diet (Dyets, Inc.) and the folic acid supplemented diet was modified AIN-93G containing 6 mg folic acid/kg diet (Dyets, Inc.).

Colony Founders: 52 female and 26 male Balb/c mice (7 weeks old) were purchased from Jackson Labs (Bar Harbor, ME) to establish a breeding colony and to give rise to the F0 generation. At 8 weeks of age breeding trios were set-up with 2 female mice and 1 male mouse. All colony founder mice were fed a fixed formula, non-purified diet (Teklad Diets, Madison, WI) during the breeding and post-natal periods.

F0: At 21 days of age, groups of female mice were weaned to one of the three experimental diets, deficient (n=32), control (n=32) or supplemented (n=32) (Figure 4-1). At 7 weeks of age, breeding trios were set-up with aged-matched male mice, and fed the diet to which the female had been weaned. Female mice were fed their respective diets during pregnancy, postpartum and lactation.

F1: At 21 days of age, 24 male mice from the deficient and 24 male mice from the supplemented maternal diet groups were weaned to the control folic acid diet (2 mg/kg). 24 male mice from the control maternal diet group were weaned to the control diet. Male mice from dams fed the control diet were also weaned to either the deficient or supplemented diets (n=24 for each diet). Male mice were fed their respective diets for 15 weeks and killed at 18 weeks of age (Figure 4-1). All mice were fed the control diet for 3 weeks prior to sacrifice for breeding purposes.

At 8 weeks of age half of the male mice from each of the diet groups (n=12) were given a single intraperitoneal injection of 75 mg/kg ENU (Sigma-Aldrich) dissolved in M/15 phosphate buffer, pH 6.0. The other half of the mice from each diet group were given a single intraperitoneal injection of the vehicle (M/15 phosphate buffer, pH 6.0).

Figure 4-1: Pedigree depicting exposures and diets for analysis of ESTR mutation frequencies in mouse sperm. The F0 mice were fed the diets from weaning and throughout breeding and lactation, while the F1 mice were fed the diets from weaning (post-natal day 21) to 18 weeks of age. At 8 weeks of age, half of the male mice from each diet group were injected with 75 mg/kg ENU and the other half with the vehicle (M/15 phosphate buffer, pH 6.0). Mice were sacrificed 10 weeks post-ENU exposure, at 18 weeks of age. Numbers inside the pedigree symbols represent the 0 mg/kg folic acid (deficient) diet, 2 mg/kg folic acid (control) diet and 6 mg/kg folic acid (supplemented) diet.

4.3.2 Red Blood Cell folate

RBC folate was measured using the *Lactobacillus casei* as described in section 3.3.2.

4.3.3 Cauda epididymal sperm counts

Cauda epididymal sperm counts were measured using a haemocytometer as described previously in section 3.3.3.

4.3.4 Sperm Chromatin Structure Assay

Sperm chromatin integrity was evaluated using the flow cytometry based SCSA as previously described in section 3.3.4.

4.3.5 Single molecule PCR for detection of mutants

Sperm DNA was isolated using the method described in section 2.3. Single molecule PCR was used to detect ESTR mutants as described in section 3.3.5-6.

4.3.6 Statistical Analysis

Differences among the diet groups for RBC folate, cauda sperm numbers, % DFI and standard deviation (SD) of DFI were identified using a two-sample bootstrap test (t-Pivot method) assuming unequal variances [69, 70] in R [71]. For the ESTR assay, a Poisson distribution was used to estimate the number of progenitor PCR template molecules, determined from the number of wells used and the number of positive PCR estimated for each sample. Generalized estimating equations (GEEs) [72, 96] assuming a

Poisson distribution for the error were used to estimate the mutation frequencies and selected pairwise comparisons using the geepack library [74, 97, 98] in R [71]. Two-way ANOVA and Post-hoc analyses were conducted for RBC folate, cauda sperm numbers, % DFI and ESTR mutations to determine whether there was an overall main effect of ENU treatment.

4.4 Results

4.4.1 Red Blood Cell folate

In chapter 3 (3.4.1) and Figure 4-2 I show a decrease in RBC folate in male mice fed the folic acid deficient diet from post-weaning to maturity. Supplementation did not lead to changes in RBC folate in chapter 3 and Figure 4-2, likely because mice were fed the control diet for 3 weeks prior to sacrifice. In this study, a two-way ANOVA revealed that there was no overall effect of ENU treatment on RBC folate as the interaction term was significant ($P=0.01$). ENU treatment increased RBC folate in male mice from the control, and post-weaning diet groups compared to vehicle treated mice ($P=0.05$ for control, $P=0.02$ for deficient, $P<0.01$ for supplemented) (Figure 4-2). ENU treatment did not lead to changes in RBC folate in either of the early developmental dietary groups compared to vehicle treated males (Figure 4-2).

Figure 4-2: Red blood cell folate in male offspring from female mice fed diets containing 0 mg/kg folic acid (deficient), 2 mg/kg folic acid (control) or 6 mg/kg folic acid (supplemented) during pregnancy/postpartum and lactation (early developmental), or from weaning to maturity (post weaning) (Figure 4-1). Male mice from each diet group were treated with either vehicle (open bars) or ENU (closed bars). Error bars represent the standard error. * indicates $P < 0.05$, $n=10/\text{group}$.

4.4.2 Sperm counts

I previously showed that male mice fed the deficient diet from post-weaning to maturity had decreased cauda sperm numbers compared to control fed mice (see Chapter 3, 3.4.2 and Fig. 4-3). Here I show a main effect for treatment with ENU that resulted in decreased cauda sperm number ($P<0.01$) (Figure 4-3). This was found to be statistically significant for four of the five groups: the control, both post-weaning groups and the early developmental supplemented group. The greatest decrease in sperm count (60%) was shown for ENU treated male mice from the post-weaning supplemented diet group compared to vehicle treated mice ($P<0.01$) (Figure 4-3). There were no differences in sperm numbers between the ENU treated male mice from the various diet groups, suggesting that the diets had no effect on ENU-induced decreases in sperm counts (Figure 4-3).

Figure 4-3: Effects of ENU treatment on cauda sperm counts from male offspring from the post-weaning and early developmental folic acid diets. Male mice from each diet group were treated with either vehicle (open bars) or ENU (closed bars). Error bars represent the standard error. (* $P < 0.05$, $n=10/\text{group}$) (main effect of ENU, $P<0.01$).

4.4.3 Sperm Chromatin Structure Assay

In chapter 3 I observed an increase in the percentage of sperm with abnormal DFI for male mice fed the post-weaning deficient diet (see Chapter 3, 3.4.3 and Fig. 4-4). Here I show an overall main effect of ENU on the proportion of sperm with abnormal DNA fragmentation index ($P < 0.01$). DNA fragmentation increased by 3% in the sperm of male mice treated with ENU compared to vehicle treated male mice from the control diet group ($P = 0.02$) (Figure 4-4A) and DNA fragmentation was higher in the ENU treated animals from all of the dietary groups. Standard deviation of DFI was significantly increased in 4 of the 5 diet groups ($P < 0.01$) (Figure 4-4B). Diet had no effect on DFI measures in ENU treated mice (Figure 4-4).

Figure 4-4: Effects of ENU treatment in males from the five dietary groups on (A) % sperm with abnormal DNA fragmentation index, and (B) standard deviation of DFI in sperm. Male mice from each diet group were treated with either vehicle (open bars) or ENU (closed bars). Error bars represent the standard error. (* $P < 0.05$, $n = 10/\text{group}$) (main effect of ENU, $P < 0.01$).

4.4.4 Tandem Repeat Mutations

Overall dietary effects are summarized in Chapter 3. Folic acid deficiency resulted in a significant increase in tandem repeat mutation, whereas folic acid supplementation had no effect (Chapter 3, 3.4.4). In this study, there was an overall main effect of ENU treatment on ESTR mutation frequency ($P < 0.01$). A significant increase in sperm ESTR mutation frequency was observed for ENU-treated male mice compared to diet-matched vehicle treated mice for the control diet group ($P = 0.02$), the post-weaning supplemented diet group ($P < 0.01$) and the early developmental deficient diet group ($P < 0.01$) (Figure 4-5). ENU treatment induced a non-significant ~50% increase in sperm ESTR mutation frequency in mice fed the post-weaning deficient diet; however, elevated baseline mutation rates in the vehicle-treated animals due to the dietary effects reduced the probability of detecting differences in this group. Of interest, the mice placed on supplemented diets during early development had no increase in mutation frequency following ENU treatment.

Figure 4-5: Effects of ENU treatment on tandem repeat mutation frequencies in the sperm of male offspring with different post-weaning and early developmental dietary folic acid intakes. Male mice from each diet group were treated with either vehicle (open bars) or ENU (closed bars). Error bars represent the standard error. (* $P < 0.05$, $n=5/\text{group}$) (main effect of ENU, $P<0.01$).

4.5 Discussion

Given the increasing number of couples that suffer from infertility and the relatively high incidence of birth defects, I sought to determine whether supplementation with folic acid might mitigate some of the effects of exposure to environmental mutagens. In this study, I evaluate the interaction between dietary folic acid intake during critical developmental windows and ENU-induced mutagenesis on sperm count (a measure of fertility), sperm chromatin structure (a measure of sperm DNA damage) and tandem repeat mutation in sperm (a measure of heritable mutation).

Previously I showed that post-weaning folic acid deficiency leads to decreased cauda sperm number, increased chromatin damage and a 2-fold increase in sperm ESTR mutation frequency (see Chapter 3). In the present study, I find evidence that folic acid supplementation during key early developmental time points may provide some protection from genotoxicity later in life. Women of reproductive age in Canada are encouraged to take folic acid supplements containing 0.4 mg of folic acid daily in order to protect against fetal neural tube defects. The 6 mg/kg supplemented diet contains 3-fold folic acid relative to the control diet (2 mg/kg). This is an environmentally relevant level of supplementation as it is equivalent to the folic acid intake achieved by an adult consuming fortified foods and vitamin supplements containing 1.0 mg of folic acid. I show that mice exposed to early developmental folic acid supplementation are protected from the induction of sperm ESTR mutation frequency when exposed to ENU. This suggests that supplemental dietary folic acid during pregnancy protected the male offspring's germline from ENU-induced mutagenesis as an adult. The beneficial effect of

supplementation did not extend to ENU-induced DNA damage at the chromatin level or on sperm counts.

Sperm counts were decreased by approximately 50% in male mice treated with ENU, indicating that ENU impairs spermatogenesis and leads to decreased fertility across all of the dietary groups. Decreased sperm count post-ENU treatment has been demonstrated previously [110]. Russell *et al.* showed that male mice treated with 250 mg/kg ENU went through a period of sterility 3 weeks post-ENU treatment as a result of increased spermatogonial cell killing [110].

The sperm chromatin structure assay was used to evaluate sperm chromatin integrity. The percentage of sperm with abnormal DNA fragmentation index (indicating increased DNA strand breaks or protamine defects) increased by approximately 3% in male mice treated with ENU. Increased DNA damage by ENU treatment was supported by the increase in the standard deviation of DFI across most diets, indicating greater variability in % DFI in mice treated with ENU. One explanation for the very small impact of ENU treatment on %DFI is that ENU-treated spermatogonia with high DFI values may undergo cell killing (i.e., programmed cell death), and would therefore not be scored in our system. Another explanation is that SCSA is not as sensitive to sperm chromatin changes in mouse sperm as it is in human sperm. Mouse sperm is more compact and condensed than human sperm, as a result of a higher protamine content, making it much harder to induce and measure changes in chromatin structure using the SCSA assay [115]. This is in agreement with other studies showing that radiation and butadiene result in large decreases in sperm concentration, and marginally significant, or small increases in %DFI [116, 117].

Treatment with ENU caused a 2-fold increase in sperm ESTR mutation frequency in the control diet group. This result was expected as ENU has been shown to cause increased sperm ESTR mutation previously [112].

Although treatment with ENU did not cause an overall main effect on RBC folate, ENU treatment appeared to increase RBC folate concentrations in male mice from the control diet group and the post-weaning folic acid deficient and supplemented diet groups. No change in RBC folate was observed in ENU treated male mice from the early developmental diet groups. This suggests that ENU causes folate to be sequestered in the tissues and could be due to ENU inducing folate transport into the tissues or from increased folate uptake from the diet in the intestine. These mechanisms could be occurring from a damage response from ENU treatment.

Post-weaning folic acid intake did not affect sperm count, DFI or tandem repeat mutation frequencies in ENU treated mice. This suggests that folic acid deficient and supplemented diets at the time of ENU treatment, do not affect ENU-induced effects in male germ cells.

The early developmental deficient diet did not affect the germ cell measures in ENU treated mice. This means that folic acid deficiency during development does not further decrease the sperm counts, increase sperm chromatin damage or increase sperm ESTR mutation caused by ENU treatment. Supplementation with folic acid in early development did not provide any potential protection from decreasing sperm counts in the ENU-treated males, indicating that supplementation with folic acid does not prevent, or diminish, induced spermatogonial killing. In contrast to the above data demonstrating no effect of dietary folate on sperm metrics in mice exposed to ENU, I found evidence for

protective effects of folic acid supplementation during early developmental windows on tandem repeat mutation. Treatment with ENU caused a 2-fold increase in sperm ESTR mutation frequency in the control diet group. However, I did not observe this increase in sperm ESTR mutation frequency in male mice fed the supplemented diet during early development following ENU-treatment relative to ENU-treated males on the control diet. During the early developmental dietary period, folic acid supplementation occurred when primordial germ cells are migrating and under-going DNA de-methylation just prior to sex determination, where re-methylation in a sex-specific manner occurs [118]. It is during this period that epigenetic reprogramming has been shown to be susceptible to environmental factors [119]. For example, Anway *et al.* exposed pregnant female rats during the period of gonadal sex determination to the endocrine disruptors vinclozolin and methoxychlor [120]. Treatment with these agents lead to reduced spermatogenic capacity and altered sperm DNA methylation patterns in male offspring (F1), as well as in the F2-F4 generations [120]. Since the frequency of males with reduced spermatogenic capacity was similar in all generations (>90%), it suggests that epigenetic inheritance is leading to the observed effect [120]. If folic acid supplementation during primordial germ cell development causes altered DNA or histone methylation, this may prevent ENU-induced ESTR mutations. ESTR mutation is known to occur via an indirect process that may be associated with epigenetic mechanisms [35, 39]. Thus, our results support the hypothesis that dietary variables that can influence epigenetics may mediate some of the effects of exposure to germ cell tandem repeat mutagens.

Folic acid supplementation in early development appeared to mitigate the effects of ENU-mutagenesis in adult male mice. Future studies investigating how early

developmental folic acid supplementation protects the germline of male offspring from ENU-induced mutagenesis would help to validate this hypothesis. A good place to start would be looking at sperm DNA methylation profiles in male mice exposed to the supplemented diet in early development compared to ENU treated mice. This theory could also be confirmed by looking at other endpoints. For example, measuring micronucleus induction in ENU-treated male mice fed the supplemented folic acid diet in early development.

Chapter 5:

Discussion

5.0 Chapter 5: Discussion

5.1 Summary of results

The main goal of this thesis was to examine the effects of dietary folic acid on spontaneous and induced DNA damage and mutation. In Chapter 2 I investigated the effects of folic acid deficient and supplemented diets on somatic cell DNA damage, in the form of micronuclei. I showed that the folic acid deficient diet caused a statistically significant increase in MN frequency in red blood cells of Balb/c mice, and that increased uracil content in DNA was likely the mechanism behind this damage. The supplemented diet did not affect MN frequency, and therefore diets high in folic acid do not appear to provide protection from, or promote, chromosome damage in red blood cells. The *Pig-a* assay revealed that neither folic acid deficient nor supplemented diets affect DNA sequence mutations.

Chapter 3 of this thesis measured the relationship between dietary folic acid and germ cell effects including: sperm count (as a measure of fertility), sperm chromatin damage (in the form of DNA strand breaks or protamine defects), and sperm tandem repeat mutations to measure heritable mutations. I found that post-weaning folic acid deficient diets resulted in reduced sperm numbers, increased sperm chromatin damage and increased tandem repeat mutation. Folic acid deficiency during early development did not affect these germ cell measures, demonstrating that it is the folate status of the adult male mouse that dictates germ cell health. The post-weaning and early developmental folic acid supplemented diets did not affect sperm count, chromatin damage or tandem repeat mutation. This indicates that supplementation at the level achieved in this study does not affect germ cell integrity.

In the fourth chapter I examined the interaction between dietary folic acid and ENU-treatment on germ cell health to determine if folic acid supplementation moderates the effects of ENU. ENU treatment alone caused decreased sperm counts, increased sperm chromatin damage and increased tandem repeat mutation frequencies. Dietary folic acid did not promote or mitigate the effects of ENU on sperm number or sperm chromatin damage. However, early developmental supplementation with folic acid resulted in the abolishment of a measurable increase in tandem repeat mutation frequency in male mice exposed to ENU as adults; mutation frequency in this group was identical to non-ENU treated control diet mice. This suggests that folic acid supplementation during early development may protect the germline of male mice from ENU-induced mutagenesis as an adult.

5.2 Relevance of findings to human health

Based on our results and the results of others, it is clearly important for individuals in the population to consume adequate amounts of folic acid to prevent somatic DNA damage caused by folic acid deficiency, and to help prevent the onset of cancer. However, another major concern for public health is the potentially mutagenic and clastogenic effects of diets with excessively high levels of folic acid. I show that a biologically relevant supplementation, which would be achieved by taking a 1 mg supplement daily and ingesting fortified foods, does not cause somatic DNA mutation in mice. This finding alleviates concerns over the fact that the majority of the Canadian population consumes high amounts of folic acid and has high red blood cell folate concentrations.

Women of reproductive age in Canada are encouraged to supplement with folic acid to prevent a NTD affected birth. The results of this thesis strongly suggest that men of reproductive age should also make sure they are consuming enough folic acid to prevent decreased fertility and sperm DNA damage and mutation. I also demonstrate that the level of supplementation used in this study did not adversely affect germ cell health. Based on the results from Chapter 3 and published literature, it would be advisable for men to supplement with folic acid prior to conception.

The final important message from this thesis is that supplementation with folic acid during early developmental time points (*in utero* and throughout lactation) may protect the germline of male mice from environmental damage incurred as an adult. This is a surprising and promising result that will need to be validated by extensive additional work, especially since other studies have demonstrated that high folic acid intake may have detrimental effects [2, 19]. Since pregnant women and women of reproductive age in Canada are already encouraged to supplement with folic acid to prevent a neural tube defect affected birth, this is another potential positive effect of the Canadian legislation to fortify diets with folic acid.

5.3 Limitations/Improvements that could be made in future studies

Throughout this project there are several places where technologies or experimental design could be refined to provide a better idea of the mechanisms of effects. In chapter 2 I showed that folic acid deficiency caused a 1.2 fold increase in MN frequency in mature red blood cells of male mice fed the folic acid deficient diet post-weaning for 12 weeks, followed by 3 weeks of consuming the control diet. No increase

was observed in immature red blood cells (15 hour lifespan) because the MN frequencies were not collected at a time when the folate concentrations would have been at their lowest. Therefore, the mature red blood cells (which represent red blood cells formed over the previous 30-40 days) would include red blood cells formed during exposure to both the deficient and control diets [65, 76]. Uracil content in nuclear DNA was not increased in these mice, which made it difficult to explain the exact mechanism leading to MN formation. RBC folate was also not likely at its peak in mice from the supplemented diet group. These mice were fed the control diet for three weeks prior to sampling during the breeding period and folate levels had likely decreased at the time of sacrifice. Since the multiple generation study did not show changes in micronucleus formation in mice fed the supplemented diet, I believe that supplementation does not lead to MN formation or protect against it. As MN formation and uracil incorporation are transient effects, in the future mice should be fed their diets right up until the time of sampling, or blood samples should be taken throughout the experiment.

In Chapter 2 the *Pig-a* assay was used to measure sequence mutation in male mice weaned to the folic acid deficient and supplemented diets. I did not observe any changes in *Pig-a* mutation frequency in these mice, indicating that folic acid deficiency/supplementation do not affect DNA sequence mutation. The current protocol for the *Pig-a* assay has been changed to increase the number of cells counted. This means that when our samples were analysed, the assay was not counting enough cells and therefore our numbers are not high enough. It would be worth repeating this experiment with the new protocol to verify our results. Also, *Pig-a* is a new technique and it would have been preferable to use a more established DNA sequence mutation screening tool,

such as the MutaMouse™. The MutaMouse™ carries a *lacZ* transgene on chromosome 3, and mutations in this transgene can be measured by packaging into phages, infecting *E. coli*, and plating the bacteria on selective media [121]. Plaque formation can then be counted to determine mutant frequency [121]. This assay has been widely used to evaluate sequence mutation and may have been a better choice for evaluating this endpoint.

I did not observe changes in global DNA methylation in chapter 2 using the cytosine extension assay or the methylamp global DNA methylation quantification kit. The lack of measurable effects could be because these assays lack the sensitivity required to detect changes in DNA methylation induced by folic acid deficiency. In the cytosine extension assay, a methyl-sensitive restriction enzyme is used to digest genomic DNA. Restricted DNA ends are then labelled with radioactive nucleotides and a scintillation counter is used to measure the radioactivity of the sample. I found that the cytosine extension assay produced highly variable results; this could be a result of incomplete digestion or problems with incorporation of the radioactive nucleotide [122]. The methylamp kit is an ELISA-based assay that depends on the ability of a 5-methylcytosine antibody to bind to genomic DNA, followed by colorimetric quantification. The colorimetric steps are extremely time sensitive, and results can change depending on the length of time between adding the developing solution and stop solution. This assay also showed large variability within the same sample. In the future, a more sensitive assay should be used to measure changes in DNA methylation. For example, LINE/SINE methylation may be a better predictor of global DNA methylation as one-third of the human genome is made up of these elements, and they are silenced by DNA methylation

[123]. MeDIP-SEQ, which involves immunoprecipitation using an antibody to methylcytosine followed by high-throughput sequencing, could also be used to evaluate global and gene-specific DNA methylation patterns [124, 125]. Another method that has been used previously to measure global DNA methylation in mice fed folic acid deficient diets is liquid chromatography-mass spectrometry [12]. All of these methods are more sensitive and may be able to detect small changes in DNA methylation caused by folic acid deficiency.

It would also be helpful to find another way of measuring germline mutation frequencies. The SM-PCR method is technically difficult, time consuming and labour intensive to perform. In addition, the protocol requires electrophoresis on agarose gels at high voltage for 48 hours, followed by Southern blotting and probing. From SM-PCR to sample analysis there are many potential places for technical errors to occur, making the technique highly problematic. However, ESTRs provide the only means today to measure DNA sequence mutations in germ cells in small sample sizes for environmentally relevant doses of mutagenic agents. The field would benefit greatly from an improved technique that is faster, less troublesome, and less labour intensive.

5.4 Future directions

The results of this thesis raise many interesting questions that should be the subject of future work. Below I outline some important experiments that I feel would provide additional insight into the mechanisms behind the responses observed, and the biological relevance of the findings.

In chapter 2 I proposed two explanations for the 3-fold increase in MN frequency observed in the F3-deficient mice from the multiple generation study: (a) accumulation of micronuclei over 32 weeks on the deficient diet, and (b) a transgenerational cumulative effect of 3-generations of the deficient diet. A follow-up study to determine which of these is responsible for the highly elevated MN frequency observed in this group should be conducted. One specific experiment that should be conducted is to feed male mice the folic acid deficient diet for 32 weeks and measure MN frequency every 8 weeks. The accumulation of MN in red blood cells could thus be measured over time to see if it is the length of time on the folic acid deficient diet contributes to increasing MN frequencies.

To explore the potential transgenerational repercussions of the deficient diet, tandem repeat mutation frequencies in the sperm of F2 male mice (descendants of the F1 male mice from chapter 3) should be investigated. These F2 male mice have not been directly exposed to the folic acid deficient diet, and therefore, characterization of MN or mutations in these mice would help to determine if folic acid deficiency leads to transgenerational effects. Previous work has shown that exposure to agents that cause ESTR mutations also leads to persistent instability up to the F2 generation [112, 126]. This transgenerational instability is hypothesised to result from epigenetic effects and thus, folic acid levels could be envisioned to affect persistent genetic instability [112]. The manifestation of transgenerational genetic instability in the descendants of folic acid deficient mice would support the hypothesis that epigenetic changes involving DNA methylation play a role in this outcome. It is also important to evaluate sperm DNA methylation in both the F1 and F2 male mice to determine if folic acid deficiency causes changes in sperm DNA methylation that potentially contribute to germ cell effects.

It is unclear how folic acid supplementation during early development may have prevented an increase in tandem repeat mutation frequency following ENU exposure (Chapter 4). This is one of the most fascinating findings of this study and warrants extensive further work. First, the study should be reproduced. Analysis using other DNA sequence elements would also shed light into whether this effect was related exclusively to the ESTR loci examined in this study. If these results are reproducible, further work should investigate the mechanism behind this outcome. One thing to explore would be the DNA methylation levels and location in the sperm of male mice exposed to the folic acid supplemented diet during early development (both vehicle and ENU treated mice). Testicular gene expression in these mice could identify which pathways are involved in potentially mitigating induced mutation in mice exposed to ENU. Looking at the effect of early developmental folic acid supplementation on other endpoints in ENU-treated male mice may also help to determine if supplementation has other protective effects.

5.5 Conclusions

In conclusion, this thesis demonstrates that folic acid deficiency is detrimental to both somatic and germ DNA integrity, and could potentially lead to adverse health effects including cancer, infertility and heritable mutation. Supplementation with folic acid did not negatively affect somatic or germ cell DNA, suggesting that the current level of supplementation achieved in the human population is not detrimental to the genome. Finally, preliminary data suggest that maternal supplementation with folic acid during pregnancy and lactation may prevent the effects of environmental mutagens on germline DNA sequence mutations.

8.0 References

1. Beetstra, S., et al., *Folic acid deficiency increases chromosomal instability, chromosome 21 aneuploidy and sensitivity to radiation-induced micronuclei*. Mutat Res, 2005. **578**(1-2): p. 317-26.
2. Sauer, J., J.B. Mason, and S.W. Choi, *Too much folate: a risk factor for cancer and cardiovascular disease?* Curr Opin Clin Nutr Metab Care, 2009. **12**(1): p. 30-6.
3. Pitkin, R.M., *Folate and neural tube defects*. Am J Clin Nutr, 2007. **85**(1): p. 285S-288S.
4. Wald, N., J. Sneddon, J. Densem, C. Frost and R. Stone, *Prevention of neural tube defects: results of the Medical Research Council Vitamin Study*. MRC Vitamin Study Research Group. Lancet, 1991. **338**(8760): p. 131-7.
5. De Wals, P., et al., *Reduction in neural-tube defects after folic acid fortification in Canada*. N Engl J Med, 2007. **357**(2): p. 135-42.
6. CanadaGazette:partII, *Regulatory impact analysis statement, SOR/98-550*, 1998. p. 3029-33.
7. Duthie, S.J., *Folate and cancer: how DNA damage, repair and methylation impact on colon carcinogenesis*. J Inherit Metab Dis, 2011. **34**(1):109-9.
8. Field, M.S., D.D. Anderson, and P.J. Stover, *Mthfs is an Essential Gene in Mice and a Component of the Purinosome*. Front Genet, 2011. **2**: p. 36.
9. Blount, B.C., et al., *Folate deficiency causes uracil misincorporation into human DNA and chromosome breakage: implications for cancer and neuronal damage*. Proc Natl Acad Sci U S A, 1997. **94**(7): p. 3290-5.
10. Duthie, S.J. and A. Hawdon, *DNA instability (strand breakage, uracil misincorporation, and defective repair) is increased by folic acid depletion in human lymphocytes in vitro*. Faseb J, 1998. **12**(14): p. 1491-7.
11. Bistulfi, G., et al., *Mild folate deficiency induces genetic and epigenetic instability and phenotype changes in prostate cancer cells*. BMC Biol, 2010. **8**: p. 6.
12. Linhart, H.G., et al., *Folate deficiency induces genomic uracil misincorporation and hypomethylation but does not increase DNA point mutations*. Gastroenterology, 2009. **136**(1): p. 227-235 e3.
13. Collins, A.R., D.T. Black, and C.A. Waldren, *Aberrant DNA repair and enhanced mutagenesis following mutagen treatment of Chinese hamster Ade-C cells in a state of purine deprivation*. Mutat Res, 1988. **193**(2): p. 145-55.
14. Schernhammer, E.S., et al., *Dietary folate, alcohol and B vitamins in relation to LINE-1 hypomethylation in colon cancer*. Gut, 2010. **59**(6): p. 794-9.
15. Kulis, M. and M. Esteller, *DNA methylation and cancer*. Adv Genet, 2010. **70**: p. 27-56.
16. Colapinto, C.K., D.L. O'Connor, and M.S. Tremblay, *Folate status of the population in the Canadian Health Measures Survey*. CMAJ, 2011. **183**(2): p. E100-6.
17. Wang, X., et al., *Efficacy of folic acid supplementation in stroke prevention: a meta-analysis*. Lancet, 2007. **369**(9576): p. 1876-82.
18. Pickell, L., et al., *High intake of folic acid disrupts embryonic development in mice*. Birth Defects Res A Clin Mol Teratol, 2011. **91**(1): p. 8-19.

19. Pickell, L., et al., *High intake of folic acid disrupts embryonic development in mice*. Birth Defects Res A Clin Mol Teratol, 2011. **91**(1):8-19.
20. Klaunig, J.E., L.M. Kamendulis, and B.A. Hocevar, *Oxidative stress and oxidative damage in carcinogenesis*. Toxicol Pathol, 2010. **38**(1): p. 96-109.
21. Joshi, R., et al., *Free radical scavenging behavior of folic acid: evidence for possible antioxidant activity*. Free Radic Biol Med, 2001. **30**(12): p. 1390-9.
22. Vogelstein, B. and K.W. Kinzler, *Cancer genes and the pathways they control*. Nat Med, 2004. **10**(8): p. 789-99.
23. MacGregor, J.T., et al., *Cytogenetic damage induced by folate deficiency in mice is enhanced by caffeine*. Proc Natl Acad Sci U S A, 1990. **87**(24): p. 9962-5.
24. McDorman, E.W., B.W. Collins, and J.W. Allen, *Dietary folate deficiency enhances induction of micronuclei by arsenic in mice*. Environ Mol Mutagen, 2002. **40**(1): p. 71-7.
25. Knock, E., et al., *Low dietary folate initiates intestinal tumors in mice, with altered expression of G2-M checkpoint regulators polo-like kinase 1 and cell division cycle 25c*. Cancer Res, 2006. **66**(21): p. 10349-56.
26. Bushnik, T., et al., *Estimating the prevalence of infertility in Canada*. Hum Reprod, 2012. **27**(3): p. 738-46.
27. Kohut, R. and I.D. Rusen, *Congenital anomalies in Canada*. A perinatal report, 2002.
28. Demarini, D., *Declaring the existence of human germ cell mutagens*. Environ Mol Mutagen, 2012. **53**(3):166-72.
29. Singer, T.M., et al., *Detection of induced male germline mutation: correlations and comparisons between traditional germline mutation assays, transgenic rodent assays and expanded simple tandem repeat instability assays*. Mutat Res, 2006. **598**(1-2): p. 164-93.
30. Boxmeer, J.C., et al., *Low folate in seminal plasma is associated with increased sperm DNA damage*. Fertil Steril, 2009. **92**(2): p. 548-56.
31. Wallock, L.M., et al., *Low seminal plasma folate concentrations are associated with low sperm density and count in male smokers and nonsmokers*. Fertil Steril, 2001. **75**(2): p. 252-9.
32. Young, S.S., et al., *The association of folate, zinc and antioxidant intake with sperm aneuploidy in healthy non-smoking men*. Hum Reprod, 2008. **23**(5): p. 1014-22.
33. Padmanabhan, S., et al., *Cytotoxic and genotoxic effects of methotrexate in germ cells of male Swiss mice*. Mutat Res, 2008. **655**(1-2): p. 59-67.
34. Yauk, C.L., *Advances in the application of germline tandem repeat instability for in situ monitoring*. Mutat Res, 2004. **566**(2): p. 169-82.
35. Yauk, C.L., et al., *Tandem repeat mutation, global DNA methylation, and regulation of DNA methyltransferases in cultured mouse embryonic fibroblast cells chronically exposed to chemicals with different modes of action*. Environ Mol Mutagen, 2008. **49**(1): p. 26-35.
36. Dubrova, Y.E., *Radiation-induced mutation at tandem repeat DNA Loci in the mouse germline: spectra and doubling doses*. Radiat Res, 2005. **163**(2): p. 200-7.

37. Dubrova, Y.E., et al., *Stage specificity, dose response, and doubling dose for mouse minisatellite germ-line mutation induced by acute radiation*. Proc Natl Acad Sci U S A, 1998. **95**(11): p. 6251-5.
38. Barber, R.C., et al., *The effects of in utero irradiation on mutation induction and transgenerational instability in mice*. Mutat Res, 2009. **664**(1-2): p. 6-12.
39. Yauk, C., et al., *Germ-line mutations, DNA damage, and global hypermethylation in mice exposed to particulate air pollution in an urban/industrial location*. Proc Natl Acad Sci U S A, 2008. **105**(2): p. 605-10.
40. Glen, C.D., A.G. Smith, and Y.E. Dubrova, *Single-molecule PCR analysis of germ line mutation induction by anticancer drugs in mice*. Cancer Res, 2008. **68**(10): p. 3630-6.
41. Yauk, C.L., et al., *Mainstream tobacco smoke causes paternal germ-line DNA mutation*. Cancer Res, 2007. **67**(11): p. 5103-6.
42. Dertinger, S.D., D.K. Torous, and K.R. Tometsko, *Simple and reliable enumeration of micronucleated reticulocytes with a single-laser flow cytometer*. Mutat Res, 1996. **371**(3-4): p. 283-92.
43. Fenech, M., *Cytokinesis-block micronucleus cytome assay*. Nat Protoc, 2007. **2**(5): p. 1084-104.
44. Bryce, S.M., J.C. Bemis, and S.D. Dertinger, *In vivo mutation assay based on the endogenous Pig-a locus*. Environ Mol Mutagen, 2008. **49**(4): p. 256-64.
45. Schuler, M., et al., *Need and potential value of the Pig-a in vivo mutation assay-a HESI perspective*. Environ Mol Mutagen, 2011. **52**(9): p. 685-9.
46. Evenson, D.P., K.L. Larson, and L.K. Jost, *Sperm chromatin structure assay: its clinical use for detecting sperm DNA fragmentation in male infertility and comparisons with other techniques*. J Androl, 2002. **23**(1): p. 25-43.
47. Bungum, M., L. Bungum, and A. Giwercman, *Sperm chromatin structure assay (SCSA): a tool in diagnosis and treatment of infertility*. Asian J Androl, 2011. **13**(1): p. 69-75.
48. Yauk, C.L., et al., *A novel single molecule analysis of spontaneous and radiation-induced mutation at a mouse tandem repeat locus*. Mutat Res, 2002. **500**(1-2): p. 147-56.
49. Stover, P.J., *Physiology of folate and vitamin B12 in health and disease*. Nutr Rev, 2004. **62**(6 Pt 2): p. S3-12; discussion S13.
50. Everson, R.B., et al., *Association of marginal folate depletion with increased human chromosomal damage in vivo: demonstration by analysis of micronucleated erythrocytes*. J Natl Cancer Inst, 1988. **80**(7): p. 525-9.
51. Goulian, M., B. Bleile, and B.Y. Tseng, *Methotrexate-induced misincorporation of uracil into DNA*. Proc Natl Acad Sci U S A, 1980. **77**(4): p. 1956-60.
52. MacFarlane, A.J., et al., *Shmt1 heterozygosity impairs folate-dependent thymidylate synthesis capacity and modifies risk of apc(min)-mediated intestinal cancer risk*. Cancer Res, 2011. **71**(6): p. 2098-107.
53. Reidy, J.A., *Folate- and deoxyuridine-sensitive chromatid breakage may result from DNA repair during G2*. Mutat Res, 1987. **192**(3): p. 217-9.
54. Fenech, M., et al., *Molecular mechanisms of micronucleus, nucleoplasmic bridge and nuclear bud formation in mammalian and human cells*. Mutagenesis, 2011. **26**(1): p. 125-32.

55. Kelly, P., et al., *Unmetabolized folic acid in serum: acute studies in subjects consuming fortified food and supplements*. Am J Clin Nutr, 1997. **65**(6): p. 1790-5.
56. Bailey, R.L., et al., *Unmetabolized serum folic acid and its relation to folic acid intake from diet and supplements in a nationally representative sample of adults aged > or =60 y in the United States*. Am J Clin Nutr, 2010. **92**(2): p. 383-9.
57. Matthews, R.G. and S.C. Daubner, *Modulation of methylenetetrahydrofolate reductase activity by S-adenosylmethionine and by dihydrofolate and its polyglutamate analogues*. Adv Enzyme Regul, 1982. **20**: p. 123-31.
58. Baram, J., et al., *Identification and biochemical properties of 10-formyldihydrofolate, a novel folate found in methotrexate-treated cells*. J Biol Chem, 1988. **263**(15): p. 7105-11.
59. MacFarlane, A.J., L.S. Greene-Fineston, and Y. Shi, *Vitamin B12 and homocysteine status in a folate-replete population: Results from the Canadian Health Measures Survey*. Am J Clin Nutr, 2011. **94**(4):1079-87.
60. Shakur, Y.A., et al., *Folic acid fortification above mandated levels results in a low prevalence of folate inadequacy among Canadians*. Am J Clin Nutr. **92**(4): p. 818-25.
61. Yang, Q., et al., *Folic acid source, usual intake, and folate and vitamin B-12 status in US adults: National Health and Nutrition Examination Survey (NHANES) 2003-2006*. Am J Clin Nutr, 2010. **91**(1): p. 64-72.
62. Olfert, E.D., B.M. Cross, and A.A. McWilliam, eds. *Guide to the Care and Use of Experimental Animals*. Second ed. 1993, Canadian Council on Animal Care: Ottawa.
63. Reeves, P.G., F.H. Nielsen, and G.C. Fahey, Jr., *AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet*. J Nutr, 1993. **123**(11): p. 1939-51.
64. Herbig, K., et al., *Cytoplasmic serine hydroxymethyltransferase mediates competition between folate-dependent deoxyribonucleotide and S-adenosylmethionine biosyntheses*. J Biol Chem, 2002. **277**(41): p. 38381-9.
65. Dertinger, S.D., et al., *Three-color labeling method for flow cytometric measurement of cytogenetic damage in rodent and human blood*. Environ Mol Mutagen, 2004. **44**(5): p. 427-35.
66. Phonethepswath, S., et al., *Pig-a mutation: kinetics in rat erythrocytes following exposure to five prototypical mutagens*. Toxicol Sci, 2010. **114**(1): p. 59-70.
67. MacFarlane, A.J., et al., *Cytoplasmic serine hydroxymethyltransferase regulates the metabolic partitioning of methylenetetrahydrofolate but is not essential in mice*. J Biol Chem, 2008. **283**(38): p. 25846-53.
68. Pogribny, I., P. Yi, and S.J. James, *A sensitive new method for rapid detection of abnormal methylation patterns in global DNA and within CpG islands*. Biochem Biophys Res Commun, 1999. **262**(3): p. 624-8.
69. Higgins, J.J., *An introduction to modern nonparametric statistics*. 2003, Pacific Grove, CA: Brooks/Cole.
70. Efron, B. and R. Tibshirani, *An introduction to the Bootstrap*. 1993, Boca Raton, FL: Chapman & Hall/CRC.

71. RDevelopmentCoreTeam. *R: A language and environment for statistical computing*. R Foundation for Statistical Computing 2009; Available from: <http://www.R-project.org>.
72. Liang, K.Y. and S.L. Zeger, *Longitudinal data analysis using generalized linear models*. Biometrika, 1986. **73**: p. 13-22.
73. Prentice, R.L. and L.P. Zhao, *Estimating equations for parameters in means and covariances of multivariate discrete and continuous responses*. Biometrics, 1991. **47**: p. 825-839.
74. Yan, J. and J.P. Fine, *Estimating Equations for Association Structures*. Statistics in Medicine, 2004. **23**: p. 859-880.
75. Yan, J., *geepack: Yet another package for Generalized estimating equations*. R-News, 2002. **2**(3): p. 12-14.
76. Grawe, J., et al., *Evaluation of the reticulocyte micronucleus assay in patients treated with radioiodine for thyroid cancer*. Mutat Res, 2005. **583**(1): p. 12-25.
77. Hedrich, H., *The Laboratory Mouse*. 2004, Germany: Elsevier Academic Press.
78. Horký, J., J. Vácha, and V. Znojil, *Comparison of life span of erythrocytes in some inbred strains of mouse using ¹⁴C-labelled glycine*. Physiol Bohemoslov, 1978. **27**(3): p. 209-17.
79. Witt, K.L., et al., *Comparison of flow cytometry- and microscopy-based methods for measuring micronucleated reticulocyte frequencies in rodents treated with nongenotoxic and genotoxic chemicals*. Mutat Res, 2008. **649**(1-2): p. 101-13.
80. Filkowski, J.N., et al., *Hypomethylation and genome instability in the germline of exposed parents and their progeny is associated with altered miRNA expression*. Carcinogenesis, 2010. **31**(6): p. 1110-5.
81. Wilson, R.D., et al., *Pre-conceptional vitamin/folic acid supplementation 2007: the use of folic acid in combination with a multivitamin supplement for the prevention of neural tube defects and other congenital anomalies*. J Obstet Gynaecol Can, 2007. **29**(12): p. 1003-26.
82. Okayasu, R., et al., *A deficiency in DNA repair and DNA-PKcs expression in the radiosensitive BALB/c mouse*. Cancer Res, 2000. **60**(16): p. 4342-5.
83. Cooper, D.N. and M. Krawczak, *Cytosine methylation and the fate of CpG dinucleotides in vertebrate genomes*. Hum Genet, 1989. **83**(2): p. 181-8.
84. Cole, B.F., et al., *Folic acid for the prevention of colorectal adenomas: a randomized clinical trial*. JAMA, 2007. **297**(21): p. 2351-9.
85. Singer, T.M. and C.L. Yauk, *Germ cell mutagens: risk assessment challenges in the 21st century*. Environ Mol Mutagen, 2010. **51**(8-9): p. 919-28.
86. Secretan, B., et al., *A review of human carcinogens--Part E: tobacco, areca nut, alcohol, coal smoke, and salted fish*. Lancet Oncol, 2009. **10**(11): p. 1033-4.
87. Marchetti, F., et al., *Sidestream tobacco smoke is a male germ cell mutagen*. Proc Natl Acad Sci U S A, 2011. **108**(31): p. 12811-4.
88. Marchetti, F. and A.J. Wyrobek, *Mechanisms and consequences of paternally-transmitted chromosomal abnormalities*. Birth Defects Res C Embryo Today, 2005. **75**(2): p. 112-29.
89. Russell, L.B., *Effects of male germ-cell stage on the frequency, nature, and spectrum of induced specific-locus mutations in the mouse*. Genetica, 2004. **122**(1): p. 25-36.

90. Somers, C.M., *Ambient air pollution exposure and damage to male gametes: human studies and in situ 'sentinel' animal experiments*. Syst Biol Reprod Med, 2011. **57**(1-2): p. 63-71.
91. Witt, K.L. and J.B. Bishop, *Mutagenicity of anticancer drugs in mammalian germ cells*. Mutat Res, 1996. **355**(1-2): p. 209-34.
92. Fox, J.T. and P.J. Stover, *Folate-mediated one-carbon metabolism*. Vitam Horm, 2008. **79**: p. 1-44.
93. Padmanabhan, S., et al., *Methotrexate-induced cytotoxicity and genotoxicity in germ cells of mice: intervention of folic and folinic acid*. Mutat Res, 2009. **673**(1): p. 43-52.
94. MacFarlane, A.J., L.S. Greene-Finestone, and Y. Shi, *Vitamin B-12 and homocysteine status in a folate-replete population: results from the Canadian Health Measures Survey*. Am J Clin Nutr, 2011. **94**(4): p. 1079-87.
95. Evenson, D.P., Z. Darzynkiewicz, and M.R. Melamed, *Relation of mammalian sperm chromatin heterogeneity to fertility*. Science, 1980. **210**(4474): p. 1131-3.
96. Prentice, R.L. and L.P. Zhao, *Estimating equations for parameters in means and covariances of multivariate discrete and continuous responses*. Biometrics, 1991. **47**(3): p. 825-39.
97. Højsgaard, S., U. Halekoh, and J. Yan, *The R Package geepack for Generalized Estimating Equations*. Journal of Statistical Software, 2005. **15**: p. 1-11.
98. Yan, J., *geepack: Yet Another Package for Generalized Estimating Equations*. R-News, 2002. **2/3**: p. 12-14.
99. Shakur, Y.A., et al., *Folic acid fortification above mandated levels results in a low prevalence of folate inadequacy among Canadians*. Am J Clin Nutr, 2010. **92**(4): p. 818-25.
100. Abdel Aziz, A.H., et al., *A study on the reproductive toxicity of erythrosine in male mice*. Pharmacol Res, 1997. **35**(5): p. 457-62.
101. Chan, D., et al., *Strain-specific defects in testicular development and sperm epigenetic patterns in 5,10-methylenetetrahydrofolate reductase-deficient mice*. Endocrinology, 2010. **151**(7): p. 3363-73.
102. MacFarlane, A.J., et al., *Mthfd1 is an essential gene in mice and alters biomarkers of impaired one-carbon metabolism*. J Biol Chem, 2009. **284**(3): p. 1533-9.
103. Mathews, C.K., *DNA precursor metabolism and genomic stability*. FASEB J, 2006. **20**(9): p. 1300-14.
104. Carrell, D.T., B.R. Emery, and S. Hammoud, *Altered protamine expression and diminished spermatogenesis: what is the link?* Hum Reprod Update, 2007. **13**(3): p. 313-27.
105. Hammoud, S.S., et al., *Distinctive chromatin in human sperm packages genes for embryo development*. Nature, 2009. **460**(7254): p. 473-8.
106. Roach, J.C., et al., *Analysis of genetic inheritance in a family quartet by whole-genome sequencing*. Science, 2010. **328**(5978): p. 636-9.
107. Somers, C.M., et al., *Air pollution induces heritable DNA mutations*. Proc Natl Acad Sci U S A, 2002. **99**(25): p. 15904-7.

108. Vilarino-Guell, C., A.G. Smith, and Y.E. Dubrova, *Germline mutation induction at mouse repeat DNA loci by chemical mutagens*. Mutat Res, 2003. **526**(1-2): p. 63-73.
109. Liegibel, U.M. and P. Schmezer, *Detection of the two germ cell mutagens ENU and iPMS using the LacZ/transgenic mouse mutation assay*. Mutat Res, 1997. **388**(2-3): p. 213-8.
110. Russell, W.L., et al., *Specific-locus test shows ethylnitrosourea to be the most potent mutagen in the mouse*. Proc Natl Acad Sci U S A, 1979. **76**(11): p. 5818-9.
111. Shibuya, T. and K. Morimoto, *A review of the genotoxicity of 1-ethyl-1-nitrosourea*. Mutat Res, 1993. **297**(1): p. 3-38.
112. Dubrova, Y.E., et al., *Paternal exposure to ethylnitrosourea results in transgenerational genomic instability in mice*. Environ Mol Mutagen, 2008. **49**(4): p. 308-11.
113. Polyzos, A., et al., *Instability of expanded simple tandem repeats is induced in cell culture by a variety of agents: N-Nitroso-N-ethylurea, benzo(a)pyrene, etoposide and okadaic acid*. Mutat Res, 2006. **598**(1-2): p. 73-84.
114. Noveroske, J.K., J.S. Weber, and M.J. Justice, *The mutagenic action of N-ethyl-N-nitrosourea in the mouse*. Mamm Genome, 2000. **11**(7): p. 478-83.
115. Bench, G.S., et al., *DNA and total protamine masses in individual sperm from fertile mammalian subjects*. Cytometry, 1996. **23**(4): p. 263-71.
116. Pacchierotti, F., et al., *Reproductive toxicity of 1,3-butadiene in the mouse: cytogenetic analysis of chromosome aberrations in first-cleavage embryos and flow cytometric evaluation of spermatogonial cell killing*. Mutat Res, 1998. **397**(1): p. 55-66.
117. Sailer, B.L., et al., *Effects of X-irradiation on mouse testicular cells and sperm chromatin structure*. Environ Mol Mutagen, 1995. **25**(1): p. 23-30.
118. Skinner, M.K. and C. Guerrero-Bosagna, *Environmental signals and transgenerational epigenetics*. Epigenomics, 2009. **1**(1): p. 111-117.
119. Skinner, M.K., M. Manikkam, and C. Guerrero-Bosagna, *Epigenetic transgenerational actions of environmental factors in disease etiology*. Trends Endocrinol Metab, 2010. **21**(4): p. 214-22.
120. Anway, M.D., et al., *Epigenetic transgenerational actions of endocrine disruptors and male fertility*. Science, 2005. **308**(5727): p. 1466-9.
121. Swiger, R.R., *Just how does the cII selection system work in Muta Mouse?* Environ Mol Mutagen, 2001. **37**(4): p. 290-6.
122. Laird, P.W., *Principles and challenges of genomewide DNA methylation analysis*. Nat Rev Genet, 2010. **11**(3): p. 191-203.
123. Carnell, A.N. and J.I. Goodman, *The long (LINEs) and the short (SINEs) of it: altered methylation as a precursor to toxicity*. Toxicol Sci, 2003. **75**(2): p. 229-35.
124. Senner, C.E., *The role of DNA methylation in mammalian development*. Reprod Biomed Online, 2011. **22**(6): p. 529-35.
125. Saied, M.H., et al., *Genome wide analysis of acute myeloid leukemia reveal leukemia specific methylome and subtype specific hypomethylation of repeats*. PLoS One, 2012. **7**(3): p. e33213.

126. Barber, R., et al., *Elevated mutation rates in the germ line of first- and second-generation offspring of irradiated male mice*. Proc Natl Acad Sci U S A, 2002. **99**(10): p. 6877-82.