Intergenerational Effects of Folate Deficiency and Folic Acid Supplementation on Reproductive Outcomes and Epigenetic Reprogramming

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“Remember to look up at the stars and not down at your feet. Try to make sense of what you see and wonder about what makes the Universe exist. Be curious.”

Stephen Hawking

ABSTRACT

Epigenetic modifications such as DNA methylation have an essential role in developmental programs. Disruptions in gamete epigenetic reprogramming are associated with poor embryo development, adult disease, and intergenerational effects. Embryos are highly sensitive to signals from the environment. The fetal period is a key time of DNA methylation reprogramming in developing germ cells and requires an adequate supply of methyl donors that are primarily derived from the folate pathway. Folate is a B vitamin largely obtained from the diet and is crucial for supplying one-carbon donors for subsequent methylation reactions. Consequently, dietary folate deficiency (FD) and folic acid supplementation (FS) are ideal exposures for investigating the effects of environmental factors on epigenetic perturbations. Four amino-acid defined diets were utilized: a control diet (Ctrl; 2 mg/kg diet) containing the recommended daily intake of folic acid for mice, while FD was simulated with a 7-fold folic acid deficient diet (7FD; 0.3 mg/kg diet), and FS with a 10-fold (10FS; 20 mg/kg diet) or 20-fold folic acid supplemented (20FS; 40 mg/kg diet) diet. Using these diets in a mouse model, we first demonstrated that lifetime F1 FD and FS beginning in utero and spanning the entirety of male germ cell development resulted in reproductive consequences and epigenetic effects. The epigenetic effects identified were altered DNA methylation of imprinted genes of both mature F1 sperm as well as tissues of F2 progeny. Next, using a similar model, we targeted the FD and FS exposures to the prenatal and early postnatal windows of female germ cell development, which exclude the window of DNA methylation re-establishment in postnatal growing oocytes. Using a single base resolution genome-wide technique called reduced representation bisulfite sequencing, we demonstrated that FD and FS still resulted in reproductive consequences in the exposed females, which were associated with epigenetic alterations in their progeny. Genome-

wide epigenetic alterations in DNA methylation were found in genic and non-genic regions of placenta and brain tissues, with enrichment in genes of pathways involved in both embryo and nervous system development. In a third study, we contrasted the effects of the diet exposures on DNA methylation in early stage male germ cells (spermatogonia) and mature sperm and revealed that early stage germ cells harboured a much higher degree of DNA methylation perturbation. Furthermore, our mouse model indicated that environmental exposure induced DNA methylation alterations could persist for up to three generations. While the sites of altered DNA methylation in sperm did not strongly coincide across the three generations, altered sites continued to be enriched for genes associated with pathways involved in both embryo and nervous system development. Collectively, this thesis suggests that epigenetic memory exists from environmental exposures such as FD and FS, that regions relevant to embryo and nervous system development may be of increased susceptibility to DNA methylation alterations from these exposures, and that these alterations can persist up to two generations beyond the exposed group.

RÉSUMÉ

Les modifications épigénétiques telles que la méthylation de l'ADN sont essentielles pour le développement d'un organisme. La perturbation de la reprogrammation épigénétique des gamètes est associée à des conséquences négatives, notamment des changements durant le développement embryonnaire, des maladies et des effets intergénérationnels. L'embryon est très sensible aux signaux de l'environnement. En effet, la période du développement fœtal est importante pour la reprogrammation de la méthylation de l'ADN dans les gamètes. Ce processus requiert une source adéquate de donneurs de groupe méthyle qui sont principalement dérivés du métabolisme de l'acide folique, ou vitamine B9, généralement obtenue par l'alimentation. La carence en folates et la consommation excessive d'acide folique sont donc des situations idéales pour investiguer les effets de l'environnement sur l'épigénome.

Ainsi, quatre diètes au contenu en acides aminés défini ont été utilisées chez un modèle de souris de type sauvage: une diète contrôle correspondant à l'apport quotidien recommandé d'acide folique chez la souris (Ctrl; 2 mg/kg de diète), une diète appauvrie de 7 fois en acide folique (7FD; 0.3 mg/kg de diète), et deux diètes fortifiées de 10- et 20-fois par rapport au seuil de base (10FS à 20 mg/kg de diète; 20FS à 40 mg/kg de diète). Nous avons démontré que la carence en folates et la surconsommation d'acide folique pour toute la durée de vie, débutant *in utero,* causent des effets reproducteurs et épigénétiques négatifs. Ces effets incluent des perturbations de la méthylation au niveau de gènes soumis à empreinte dans le sperme du mâle F1 et dans les tissus des descendants F2.

Nous avons ensuite utilisé un modèle similaire de souris de type sauvage pour cibler les expositions de carence en folates et surconsommation d'acide folique durant la période prénatale, excluant ainsi la phase de reméthylation de l'ADN qui prend place pendant la période de

croissance de l'ovocyte. En effectuant une analyse pangénomique représentative de la méthylation de l'ADN par séquencage (technique RRBS), nous avons démontré que la carence en folates et la surconsommation d'acide folique induisent des séquelles reproductives chez la génération exposée F1, associées à des altérations épigénétiques chez les descendants F2. Des altérations de méthylation pangénomiques ont été observées dans les régions géniques et non-géniques des placentas et tissus cérébraux, plus particulièrement chez des gènes jouant un rôle dans le développement embryonnaire et celui du système nerveux.

Finalement, nous avons comparé l'effet des régimes alimentaires sur la méthylation de l'ADN dans les cellules germinales mâles au stade précoce (spermatogonies) par rapport aux spermatozoïdes matures. Nous avons observé que les spermatogonies accumulent davantage de perturbations de la méthylation de l'ADN. De plus, notre modèle a démontré que les perturbations épigénétiques au niveau de la méthylation d'ADN suite à l'exposition à des facteurs environnementaux peuvent persister jusqu'à trois générations. Bien que les sites où la méthylation est altérée ne sont pas les mêmes dans le sperme d'une génération à l'autre, ils sont continuellement enrichis en gènes impliqués dans le développement embryonnaire et celui du système nerveux.

En conclusion, nos données suggèrent que l'exposition à un facteur environnemental tel que la carence en folates ou la supplémentation excessive en acide folique peut causer des changements à la méthylation de l'ADN qui demeurent dans la mémoire épigénétique. Finalement, l'épigénome de régions importantes pour le développement du système nerveux et celui de l'embryon est peut-être plus sensible à ces facteurs environnementaux et ces changements peuvent être propagés jusqu'à deux générations suivant l'exposition.

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LIST OF ABBREVIATIONS

5methylTHF	5-methyltetrahydrofolate
5hmC	5-hydroxymethylcytosine
5mC	5-methylcytosine
ADD	ATRX-DNMT3-DNMT3L
AID	Activation-induced Cytidine Deaminase
Alx3	ALX Homeobox 3
Angpt2	Angiopoietin-2
ANOVA	Analysis of variance
APOBEC1	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like
Arhgef26	Rho guanine nucleotide exchange factor 26
Art5	ADP-Ribosyltransferase 5
Bahcc1	BAH Domain And Coiled-Coil Containing 1
BER	Base excision repair
Bp	Base pair
Ccdc24	Coiled-Coil Domain Containing 24
CGI	CpG islands
ChIP	Chromatin immunoprecipitation
Ckb	Creatine Kinase B
CL	corpora lutea
Col18a1	Collagen alpha-1(XVIII) chain
Ctrl	Control
DAVID	Database for Annotation, Visualization, and Integrated Discover

DHFR	Dihydrofolate reductase
Dlg1	Discs Large MAGUK Scaffold Protein 1
Dlk1-Gtl2	*Delta-like 1 - Gene-trap locus 2*
DMR	Differently methylated regions
DMT	Differently methylated tiles
DNA	Deoxyribonucleic acid
DNAme	DNA methylation
DNMT1	DNA Methyltransferase 1
DNMT3A	DNA Methyltransferase 3A
DNMT3B	DNA Methyltransferase 3B
DNMT3L	DNA Methyltransferase 3-like
Dpc	Days post coitum
E	Embryonic day
Efhc1	EF-Hand Domain Containing 1
ERV	Endogenous retroviruses
ESC	Embryonic stem cells
F#	Filial generation #
FACS	Fluorescence-activated cell sorting
FD	Folate deficiency
FDR	False discovery rate
FS	Folate supplemented
G1/G0	Gap phase 1 and 0
G9a	Euchromatic histone-lysine N-methyltransferase 2

gDMDs	Germline differentially methylated domains
gDMR	Germline differentially methylated regions
GVO	Germinal vesicle oocytes
H19	H19 Imprinted Maternally Expressed Transcript
H2BK20ac	Histone H2B acetylated at lysine 20
H3K27	Histone 3 lysine 27
H3K27ac	Histone 3 lysine 27 acetylated
H3K27me3	Histone 3 lysine 27 trimethylated
H3K36me3	Histone 3 lysine 36 trimethylated
H3K4	Histone 3 lysine 4
H3K4me3	Histone 3 lysine 4 trimethylated
H3K9	Histone 3 lysine 9
Hoxa10	Homeobox A10
IAP	Intracisternal a particle
ICM	Inner cell mass
ICR	Imprint control regions
IG	Intergenic
Katna1	Katanin Catalytic Subunit A1
Kcnq1ot1	KCNQ1 opposite strand/antisense transcript 1
Kmt2a	Histone-lysine N-methyltransferase 2A
LH	Luteinizing hormone
LINE	Long interspersed nuclear elements
LTR	Long terminal repeats

LUMA	LUminometric Methylation Assay
Mir153	MicroRNA 153
MTHFR	Methylenetetrahydrolate reductase
MTRR	Methionine synthease reductase
Nbea	Neurobeachin
ncRNA	Non coding RNA
Nol7	Nucleolar protein 7
NTD	Neural tube defect
Oct4/GFP	octamer-binding transcription factor 4/green fluorescent protein
PCR	Polymerase chain reaction
Peg1	Paternally Expressed Gene 1
Peg13	Paternally Expressed Gene 13
Peg3	Paternally Expressed Gene 3
PGC	Primordial germ cells
piRNAs	PIWI-interacting RNAs
Plagl	PLAG1 Like Zinc Finger 1
Plcl1	Phospholipase C Like 1 (Inactive)
PND	Postnatal day
Prm	protamines
PWWP	Pro-Trp-Trp-Pro
Rasgrf1	Ras Protein Specific Guanine Nucleotide Releasing Factor 1
RBC	Red Blood cells
RDI	Recommended daily intake

RLGS	Restriction landmark genomic scanning
RNA	Ribonucleic acid
RNA PII	Ribonucleic acid polymerase II
Rnf217	Ring Finger Protein 217
RRBS	Reduced representation bisulfite sequencing
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
Scn10a	Sodium channel protein type 10 subunit alpha
SEM	Standard error of the mean
SETDB1	SET Domain Bifurcated Histone Lysine Methyltransferase 1
SINE	Short interspersed nuclear elements
Snrpn	Small Nuclear Ribonucleoprotein Polypeptide N
Snurf	SNRPN Upstream Reading Frame
Sphkap	SPHK1 Interactor, AKAP Domain Containing
SRY	Sex-determining Region Y
Stxbp3a	Syntaxin-binding protein 3
Tbx1	T-Box Transcription Factor 1
TDG	Thymine DNA Glycosylase
Tet1	Tet Methylcytosine Dioxygenase 1
THF	Tetrahydrofolate
Ttc21b	Tetratricopeptide repeat domain 21B
Tshz3	Teashirt Zinc Finger Homeobox 3
UFA	Unmetabolized folic acid

WT	Wildtype
Zdbf2	Zinc Finger DBF-Type Containing 2

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and Camille Angle, thank you for the “brain breaks”, the laughs, the spontaneous pizza day lunches, and the company during evening and weekend experiments throughout the years.

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PREFACE TO THE THESIS

This thesis is written according to the guidelines of the McGill University Graduate and Postdoctoral Studies Office. It is written in the manuscript-based format and is comprised of six chapters, of which three are in the form that they were submitted or will be submitted for publication. The studies described herein were performed under the supervision of Dr. Jacquetta Trasler.

Gametes play unique roles in transmitting genetic information to the next generation. Epigenetic modifications provide an additional layer of information that is passed on to the early embryo to influence its development. There is increasing interest in understanding how drugs, toxins and environmental exposures perturb the epigenome of developing male and female gametes and lead to adverse health outcomes in the offspring. Folate is a B vitamin important for various biochemical reactions, including the generation of the substrate required for the most studied epigenetic modification – DNA methylation. This thesis will focus on how low and high doses of folic acid impact epigenetic programming in developing male and female germ cells and the intergenerational consequences of inheriting aberrant epigenetic modifications induced by folic acid exposures.

Chapter I is the introduction and comprises a review of the literature relevant to this thesis as well as the rationale and objectives of the thesis. Chapter II focuses on the lifetime effects of paternal FD and FS on reproductive fitness and DNA methylation in sperm and progeny and was published in the journal, Molecular Human Reproduction (23(7):461-477, 2017) and describes the effects. Chapter III concentrates on prenatal maternal exposures to FD and FS and was published in the journal, Environmental Epigenetics (23(7):461-477). Chapter IV is a manuscript in preparation and will be submitted once a few remaining experiments have

been performed, and investigates the immediate effects of FD and FS on *de novo* methylation in spermatogonia, and the heritability of altered DNA methylation across three generations. Chapter V provides a global discussion of the previous chapters, and finally Chapter VI presents the future directions and general conclusions.

CONTRIBUTIONS OF AUTHORS

The work presented in this thesis was carried out by the candidate, under the supervision of Dr. Jacquetta Trasler. The candidate participated in the design of the experiments and data analysis. The candidate wrote the first draft of the manuscripts and contributed to the revisions and submission of the manuscripts.

Chapter II was performed with the following colleagues and collaborators**:** Donovan Chan, Mahmoud Aarabi, Mylène Landry, Nathalie A. Behan, Amanda J. MacFarlane, and Jacquetta Trasler. JT and I designed the study. I coordinated the experiments, performed the breeding and maintenance of the mouse colony with contribution from ML, performed the embryo and tissue collections with contribution from ML, performed the sperm isolation and sperm counts, and I performed the bisulfite pyrosequencing experiments. NB and AJM performed the plasma and serum folate measurements. ML performed the luminometric methylation assays (LUMA). JT, DC and I prepared the figures and tables. I wrote the manuscript with the help of JT, DC, and MA. All authors edited and approved the manuscript.

Chapter III was performed with the following colleagues**:** Donovan Chan, Mylène Landry, Camille Angle, Josée Martel, and Jacquetta Trasler. JT, DC and I designed the study. I coordinated the experiments, performed the breeding and maintenance of the mouse colony with contribution from ML, performed the embryo and tissue collections with contribution from ML and JM, performed the reduced representation bilsulfite sequencing in placenta and cortex, interpreted the RRBS processed data with contributions from DC and JT, and performed the gene ontology analysis. ML performed the luminometric methylation assays (LUMA) and performed the bisulfite pyrosequencing with contributions from myself and CA. CA and JM contributed to the validation of RRBS results by pyrosequencing. DC performed the processing

of the raw RRBS sequencing data and generated the alignment of reads, identification of DMTs between groups, and annotation. JT, DC and I prepared the figures and tables. I wrote the manuscript with the help of JT and DC. All authors edited and approved the manuscript.

Chapter IV was performed with the following colleagues**:** Donovan Chan, Mylène Landry, Camille Angle, John Morris, and Jacquetta Trasler. JT, DC and I designed the study. I coordinated the experiments, performed the breeding and maintenance of the Oct4/GFP and WT mouse colonies with contribution from ML and CA, performed the tissue collections with contribution from ML and CA, processed the samples for FACS, performed the reduced representation bilsulfite sequencing, and interpreted the RRBS processed data with contributions from JM, DC and JT, and performed the gene ontology analysis. ML performed the bisulfite pyrosequencing on the sperm with contributions from myself and CA. DC performed the processing of the raw RRBS sequencing data and generated the alignment of reads, identification of DMTs between groups, and annotation. JT, DC and I prepared the figures and tables. I wrote the manuscript with the help of JT and DC. All authors edited and approved the manuscript. Oct4/GFP mice were a gift from Dr. Makoto Nagano.

CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

1) *In utero* folate deficiency and folic acid supplementation in a BALB/c model both significantly reduce adult sperm counts.
2) Paternal prenatal (F1) high (20X) folic acid supplementation results in increased post-implantation loss in the F2 generation in a BALB/c model.
3) Both paternal prenatal (F1) folate deficiency and high (20X) folic acid supplementation result in increased postnatal loss in the F2 generation in the BALB/c model
4) Prenatal paternal (F1) moderate (10X) folic acid supplementation results in increased variance in imprinted gene methylation in the placenta and brain cortex of F2 offspring in a BALB/c model
5) Maternal (F1) early life high (20X) folic acid supplementation results in increased resorption rates and decreased litter sizes in the progeny (F2) in a BALB/c model.
6) Maternal (F1) early life folate deficiency, moderate (10X) and high (20X) folic acid supplementation all result in increased abnormal embryo outcomes in the F2 generation in a BALB/c model.
7) Maternal (F1) early life folate deficient and folic acid supplemented diets are associated with subtle genome-wide DNA methylation alterations in the placenta and brain cortex of F2 offspring in genes largely associated with embryonic development and nervous system development
8) Maternal (F1) early life folate deficient and folic acid supplemented diets are associated with subtle genome-wide DNA methylation alterations which coincide with regions which methylation takes place in oocytes
9) Both hyper- and hypomethylation occurs across the genome in mature sperm following prenatal paternal folate deficiency and folic acid supplementation (10X and 20X), with the majority effect being hypomethylation.
10) The folate exposure-related DNA methylation perturbing effect was most pronounced in early spermatogonia, with less alterations found in mature sperm.
11) DNA methylation perturbations occurring in male germ cells can maintain epigenetic memory, persisting for up to three generations.

Chapter I: Introduction

1.1 Developmental Origins of Health and Disease and Their Heritability

The late 1980s saw the beginning of an interest in the effects of the intrauterine environment on future health and the onset of adult disease. This interest was founded on the idea of 'developmental plasticity', defined as "the ability of a single genotype to produce more than one alternative form of structure, physiological state or behaviour in response to environmental conditions" (Barker, 1990). Epigenetics is a mechanism through which environmental exposures during in utero development can result in adult-onset disease. Advances in the field of epigenetics have improved our understanding of epigenetics through discoveries of new epigenetic marks, characterization of their dynamics throughout development, mechanistic studies of their heritability, and the intergenerational implications of altered epigenetics when considering germ cells. Understanding how controllable environmental factors can perturb epigenetic marks and affect intergenerational health will further our understanding of intrauterine exposures in pregnancy allowing optimization of public health guidelines.

1.2 Epigenetics as a Mechanism for Intergenerational Transmission of Effects

Epigenetics is the study of mechanisms that modify gene expression without altering the genetic code itself. The genome consists of billions of DNA base-pairs which contain the genes that direct the development of an organism. A host of mechanisms exist to intricately regulate gene expression, including epigenetic factors such as covalent modifications that include DNA methylation and histone modifications. DNA methylation involves the covalent linkage of a methyl group to the 5' carbon of cytosine residues and plays a key role in controlling gene expression in embryonic development, X-chromosome inactivation, genomic imprinting, and overall transmission of genetic information without altering actual DNA sequence (Trasler, 2006). Imprinted genes exemplify epigenetic marks inherited by early embryos and have fueled

the interest in the inheritance of spontaneously acquired and altered epigenetic marks by the next generation, which could be detrimental for progeny survival.

Histones of the nucleosome core similarly undergo a range of post-translational modifications, which include the methylation of various lysine (K) residues including H3K9, H3K27, and H3K4 (Reviewed in Mozzetta et al., 2015). Epigenetic modifications can directly affect gene expression by recruiting or interfering with access of transcription factors to regulatory domains or promoter regions. Alternatively, DNA methylation and histone modifications can also indirectly affect gene expression by interacting to control local chromatin structure and affecting the binding affinity of the transcriptional machinery (Iguchi-Ariga and Schaffner, 1989; Bartolomei and Tilghman, 1997; Swales and Spears, 2005; Richards, 2006). The proper establishment of epigenetic modifications is therefore highly important as they influence gene expression and can thereby affect cell and tissue phenotypes.

Extrinsic factors that can alter epigenetic modifications are of great interest since they have the potential to disrupt important pathways. Dietary exposures, such as deficiencies or surpluses of particular nutrients or hormones, have been demonstrated to alter DNA methylation at promoters and gene expression in physiologically relevant pathways. For instance, protein restriction during pregnancy in rats has been shown to alter epigenetic regulation and expression of hepatic genes such as the glucocorticoid receptor in the offspring (Lillycrop et a., 2005). Interestingly, Lillycrop et al. found that folic acid supplementation was able to ablate the DNA methylation and gene expression effects of protein restriction. Other environmental exposures have also been demonstrated to influence DNA methylation, at imprinted and non-imprinted genes, such as in the case of blastocyst culture and manipulation (Doherty et al., 2005, Whidden

et al., 2016). It has been theorized that these epigenetic changes occur as a response to the environment, with the goal of priming the fetus to thrive in that specific type of environment.

Although environmental insults can be purely disruptive and pathological, such as in the case of toxin exposures or gestational diabetes causing late-onset cardiovascular disease in the offspring, non-pathological challenges have also been reported (Aberg et al., 2001). Such an example is described in the "thrifty" phenotype, whereby poor intrauterine environments reduced development of skeletal muscle and increased visceral fat deposition, favouring survival in poor postnatal environments (Hales and Barker, 1992, Yajnik et al., 2003). Epidemiological studies of such adaptive responses yielded interesting phenomena when phenotypes developed in a temporary environment, such as the Dutch famine. As the children of survivors did not endure life-long famine but instead were raised in resource-rich milieus, an environment-phenotype mismatch presented, which yielded increased rates of adult onset obesity, insulin resistance, and metabolic syndrome (Gluckman and Hanson, 2007).

Parent-offspring transmission of exposures can arise when epigenetic alterations occur in germ cells, for example in response to their developmental environment. Exposure-effects during windows of epigenetic programming in gametes of developing embryos are liable to be passed on, as postfertilization reprogramming of germ cell epigenetic marks is incomplete (Seisenberger et al., 2012, Lesch et al., 2014). The terms "intergenerational" and "trangenerational" are used to described these patterns of inheritance. Transgenerational effects refer to the inheritance of traits that are not a result of a direct effect or exposure to the affected organism. In this vein, a gestating embryo submitted to an environmental stimulus would be a direct exposure. As such, only altered phenotypes occurring in the second (in the case of male transmission; F2) or third (in the case of female transmission; F3) generation after a trigger can truly be described as

transgenerational inheritance, whilst altered phenotypes spanning shorter timescales are considered intergenerational (Fig 1.1).

Paternal-effect studies have offered insight on the potential for multigenerational transmission of environmental exposures, as the biological contribution to the offspring of male mice is limited to genetic and epigenetic material. Carone et al. 2010 demonstrated that paternal low-protein diet was enough to alter gene expression in offspring linked with changes in DNA methylation in the offspring tissue. There is also emerging evidence on the transmission of the prenatal environment effects on sperm. Exposures to common environmental pollutants during gestation have been reported to alter the sperm epigenome persisting up to three generations later (Herst et al., 2019). As such, focus is currently shifting towards the effects of exposures during the stages of germ cell development which coincide with key prenatal windows of epigenetic programming and the heritability of these effects.

1.3 Mouse Germ Cell Development

Male and female germ cell lineages derive from bipotential embryonic precursors called primordial germ cells (PGCs). Murine studies have detailed the development of the PGCs which arise from the epiblast-derived mesoderm at embryonic day 7.25 (E7.25), migrate to the developing hindgut endoderm at E7.5 and then continue their migration at E9.5 through the mesentery to colonize the genital ridges at E10.5-12.5 (Messerschmidt et al., 2014, Saitou et al., 2012; Tam and Snow, 1981). PGCs remain bipotent until transient sex-determining region Y (*Sry*) expression occurs from the Y chromosome soon after, at approximately E13.5, following which sex-specific development occurs (Adams and McLaren, 2002). Following this step of sex-determination of the embryo, PGC development diverges with sex-specific timing and patterns of DNA methylation acquisition occurring, eventually resulting in mature oocytes and sperm.

1.3.1 Male Mouse Germ Cell Development

Upon migration of PGCs into the gonadal ridge, male PGCs colonize the gonads and proliferate prior to sex differentiation. Subsequently, male PGCs enter mitotic arrest and are referred to as gonocytes or prospermatogonia. Prospermatogonia arrest during the G1/G0 transition of the cell cycle until spermatogenesis is initiated postnatally (Sasaki and Matsui, 2008). In the mouse postnatal testis, only a subset of prospermatogonia form functional spermatogonial stem cells (Oatley et al., 2008). Continuous production of mature sperm in the post-pubertal testis requires the renewal of spermatogonial populations by these stem cells (Oatley et al., 2008, Hermo et al., 2010). The population of undifferentiated, self-renewing prospermatogonia eventually re-enters the cell cycle and resumes mitosis, initiating spermatogenesis with waves of spermatogonia re-initiating meiotic entry at postnatal day 7 (PND7). Spermatogenesis consists of four major differentiation phases: spermatogonia (mitotic), spermatocytes (meiotic), spermatids (post-meiotic) and spermatozoa (mature sperm) and has been thoroughly outlined and reviewed (Clermont, Y., 1972). By approximately PND22, following two meiotic divisions, haploid spermatids are present, and by PND36, spermatids have undergone a complex morphological differentiation and are released into the lumen of the seminiferous epithelium whereupon they fully mature into spermatozoa before migration to, completion of maturation, and storage in the epididymides (Hess and Renato de Franca, 2008).

1.3.2 Female Mouse Germ Cell Development

Much like their male counterparts, upon their arrival in the gonadal ridge, PGCs undergo a large wave of proliferation to form the future oocytes. Further rapid mitotic divisions of these PGCs up until E13.5 produce clusters of cells classified as oogonia (Pepling, 2006). These clones will subsequently initiate meiosis only to arrest in the diplotene stage of prophase I just before

birth (Eppig et al. 2001). These germ cells, now called oocytes, persist in a resting phase, until puberty whereby select groups of primary oocytes complete meiosis and are ovulated cyclically. Waves of oocytes begin meiosis from the anterior to posterior of the ovary, beginning at E17.5 (Bullejos and Koopman, 2004). The majority of oocytes have arrested in the diplotene stage by PND5 (Borum, 1961). In their resting primary oocyte phase, the germ cells are left enveloped in a follicular epithelial cell layer and referred to as primordial follicles (Pepling and Spradling, 2001). Oocytes are arrested at the first meiotic prophase, held in meiotic arrest by the surrounding follicle cells until a surge of luteinizing hormone from the pituitary, stimulates the immature oocyte to resume meiosis (Mehlmann, 2005). The process by which the oocyte completes the first meiotic division and undergoes other cytoplasmic changes and progresses to metaphase II is called oocyte maturation. Luteinizing hormone (LH) also induces the expression of epidermal growth factor-like proteins in the follicular epithelial or granulosa cells to trigger oocyte maturation. Upon arrest in metaphase II, meiosis II is only complete upon fertilization (Smallwood and Kelsey, 2012).

1.4 Epigenetics and Germ Cell Reprogramming

1.4.1 DNA Methylation and the Machinery Involved

DNA Methylation has largely been identified to occur in the context of CpG dinucleotides in the genome. The repressive role of DNA methylation in transcription has long been recognized, although how its presence at gene promoters silences transcription is yet to be fully understood (Greenberg et al., 2019). Although rare non-CpG methylation occurs, its function in germ cells remains elusive (Smallwood et al. 2011; Ziller et al. 2011). Individual CpGs are commonly found to be hypermethylated while increased-CpG density stretches of 500-2000 base-pair-long regions associated with gene promoters called CpG islands (CGIs) are

typically hypomethylated (Meissner et al. 2008, Deaton and Bird 2011). CpG methylation is suggested to play a role in transcriptional repression by interfering with transcription factor binding at promoters. The repressive role of DNA methylation is much more evident in the context of endogenous transposable elements repression, genomic imprinting and X-inactivation (Lander et al. 2001; Hellman and Chess 2007). However, CpG methylation is also implicated in having interactions with the transcriptional machinery via enhancer methylation (Meissner 2011; Stadler et al. 2011).

DNA methylation is catalyzed by the DNA (cytsosine-5)-methyltransferases (DNMT) DNMT3A and 3B, which house two domains involved in chromatin interactions - ATRX-DNMT3-DNMT3L (ADD) and PWWP (Okano et al., 1998). Another DNA methyltransferase, DNMT1 plays a role in maintaining DNA methylation levels at the time of replication (Goll and Bestor, 2005). DNMT3L is a member of the DNMT family that does not have methyltransferase activity but cooperates with DNMT3A and 3B in *de novo* methylation. In mammals, DNA methylation is an essential developmental process which is established during gametogenesis and early embryogenesis. It is believed that DNA methylation along with other epigenetic factors such as histone tail modifications interact to give epigenetic signatures that induce different gene expression profiles to produce phenotypes in cells, tissues, and organisms. Patterns of DNA genomic methylation implemented by DNMT3A and 3B during these developmental stages in precursor cells establish the required profiles of gene expression and these patterns are maintained by DNMT1 during proliferative phases of cells.

A paralog of DNMT3B was discovered in murine male germ cells (Barua et al., 2016). Designated DNMT3C, this isoform of DNMT3B has evolved under diversifying selection of its

N-terminus in response to newly introduced transposable elements (Molaro et al., 2020). DNMT3C targets evolutionarily young transposons for *de novo* methylation and silences them, a function required to preserve male fertility in the mouse (Jain et al., 2017, Molaro et al., 2020). Although primates lack such a DNMT3C paralog, DNMT3A has been shown to have evolved under the same diversifying selection process, revealing the plasticity of the mammalian DNA methylation mechanism (Molaro et al., 2020).

Genetic mutations in epigenetic pathway enzymes are known to cause large variations in DNA methylation profiles associated with diseases in humans. Heterozygous mutations in *DNMT3A* are linked to a range of prenatal growth disorders including microcephalic dwarfism (Heyn et al. 2019). Recessive *DNMT3B* mutations have been identified to cause immunodeficiency, centromeric instability and facial anomalies syndrome (Xu et al., 1999). Meanwhile, *DNMT1* mutations are known to cause numerous neuropathies associated with cognitive deterioration (Klein et al., 2011, Winkelmann et al., 2012). Identification and characterization of disease-associated mutations in epigenetic pathway enzymes have greatly advanced the understanding of the epigenome and its role and interactions in gene regulation.

1.4.1.1 DNA Methylation Erasure in the Germ Cell

Following the fertilization of the oocyte to form the zygote, an initial wave of epigenetic reprogramming occurs early in the embryo. Immense remodelling of the paternal genome occurs before DNA replication commences and global DNA demethylation of both maternal and paternal genomes ensues before the 2-cell stage (Reik and Walter., 2001, Zhu et al., 2018). Re-methylation of the genome of the pre-implantation blastocyst soon after occurs throughout the formation of the three germ layers via gastrulation (Gao et al., 2017). Upon their emergence from

the primitive streak, PGCs hold a similar DNA methylome to the rest of the embryo only for a short time before undergoing a gamete-specific second wave of epigenetic reprogramming. Select genomic regions have been identified and documented to escape both early embryo and/or germ cell reprogramming. (Seisenberger et al., 2012).

Reprogramming includes the erasure of DNA methylation globally across genic, intergenic and repeat regions, occurring over two temporally distinct waves (Hajkova et al., 2002, Lane et al., 2003, Seisenberger et al., 2012). The initial phase of demethylation targets promoters, CGIs, introns, exons and intergenic sequences and is thus described as being global and genome-wide, commencing at about E8.0 (Seisenberger et al., 2012). By E9.5, PGCs have an overall low global DNA methylation status relative to that of the inner cell mass (Seisenberger et al., 2012, Seki et al., 2005, Guibert et al., 2012).

The secondary DNA demethylation phase in PGCs is initiated around E10.5. The second phase targets regions such as differentially methylated regions (DMRs) of imprinted genes, CGI promoters of male germ cell-specific genes needed for gamete formation and meiosis related genes, and CGIs associated with X chromosome inactivation. Demethylation of these regions is completed by E13.5 corresponding to the time when DNA methylation levels in PGCs are at their lowest levels.

DNA demethylation in mammalian germ cells occurs through both active and passive mechanisms (Popp et al. 2010, Seisenberger et al. 2012, Ohno et al. 2013, Dawlaty et al. 2013, Kagiwada et al. 2013). Passive DNA demethylation is largely responsible for global DNA demethylation and requires replication-coupled dilution of 5-methylcytosine (5mC) (Kagiwada et al. 2013). While the DNA methyltransferase responsible for DNAme maintenance (DNMT1) is expressed, the canonical somatic pathway for methylation maintenance in germ cells via NP95

is disabled. DNMT1 recruitment to the replication fork by NP95 is reduced and as such, the maintenance enzyme is excluded from the nucleus in replicating PGCs, within both phases of DNA demethylation (Seisenberger et al. 2012, Ohno et al. 2013). While DNMT1 maintenance is no longer able to function globally during DNA demethylation, sequence-specific demethylation resistance is still conferred to various regions such as DMRs and ICRs via zinc-finger protein 57 (ZFP57)–KRAB-associated protein 1 (KAP1) complex which recruit the histone methyltransferase SETDB1 along with DNMT1 and another maintenance DNA methylation factor UHRF1 (Greenberg and Bourc'his, 2019). The heterogeneity of demethylation rates and timelines of various sequences, such as imprints, highlights the presence of multiple mechanisms involved in epigenetic reprogramming.

Active demethylation requires modification of 5mC by enzymes in several pathways. This includes de-amination of 5mC into thymine via the enzymes Activation-induced Cytidine Deaminase (AID) and Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like (APOBEC1), or oxidization of 5mC into 5-hydroxymethylcytosine (5hmC) via ten-eleven translocation proteins (TET1, 2, 3). Subsequently, 5hmC is further de-aminated to thymine or oxidized, and thymine DNA glycosylase (TDG) completes the demethylation process by T/G mismatch repair via the base excision repair (BER) pathway (Kriaucionis et al. 2009, Ito et al. 2011, Morgan et al. 2004, Hajkova et al. 2010). *Tet1* and *Tdg* are expressed specifically during the time of DNA demethylation between E9.5-13.5 (Kagiwada et al. 2013). However, *Tet*1 knockout models have been discovered to be fertile and with normal genome-wide DNA demethylation, suggesting that active DNA demethylation may play a smaller role in epigenetic reprogramming (Dawlaty et al. 2013, Yamaguchi et al. 2012).

The previously prevailing idea that reprogramming completely erases epimutations and resets the epigenome has since been disproven with advances in the field of epigenetics and the discovery of environmentally influenced elements such as epialleles and metastable alleles. PGCs have been reported to retain some epigenetic memory where some genomic regions are not at all or incompletely demethylated and comparatively, still have significant methylation levels at E13.5. Such sequences include the endogenous retroviruses (ERV) long terminal repeat (LTR), LTR-ERV1 and LTR-ERVK elements, as well as intracisternal A particles (IAPs). These regions resist reprogramming and remain substantially methylated (up to 75%) across all stages of development examined. The majority of genic, intergenic, and transposon sequences are found to retain small amounts of methylation and are for the most part considered to be completely reprogrammed (Popp et al., 2010). Interestingly, a few hundred resistant CGIs not linked to IAPs as well as non-CGI promoters were identified in E13.5 male PGCs (Seisenberger et al., 2012, Guibert et al., 2012). These CGIs and non-CGI promoters were observed to have variable resistance to demethylation and maintaining these levels through to mature sperm. Investigation of these resistant regions within the two-cell embryo, inner cell mass and embryonic stem cells (ESCs) unveiled significant retained methylation levels, suggesting that epigenetic marks of certain regions may be overlooked or protected from mechanisms of active and passive demethylation. Improper establishment of DNA methylation during embryonic or PGC development at such regions protected or overlooked during reprogramming could therefore be transmitted to offspring. Transmission of aberrant DNA methylation at enhancers or regulatory regions important for development could lead to inter- or transgenerational epigenetic effects (Figure 1.1).

1.4.1.2 Male-Specific Germ Cell DNA Methylation Re-establishment

1.4.1.2.1 Acquisition of DNA Methylation in Prenatal Prospermatogonia

Upon completion of DNA methylation erasure by E13.5, male germ cells become mitotically arrested. Contrary to female PGCs, the major phase of genome-wide DNA re-methylation occurs prenatally between ~E14 to E19, a time when the DNA methyltransferases, DNMT3A, DNMT3B, and DNMT3L are all expressed at high levels in male germ cells (La Salle and Trasler 2006, Kato et al., 2007). Deficiencies in DNMT3a and 3b or DNMT3L in prenatal male germ cells result in decreased levels of DNA methylation in prospermatogonia as well as postnatal spermatogonia, cause the arrest of spermatogenesis at meiosis, and subsequent infertility in mouse models (La Salle and Trasler 2006, Kato et al., 2007, Bourc'his and Bestor, 2004, Kaneda et al., 2004).

DMRs of imprinted genes that are methylated in the male germline acquire DNA methylation between E14 and E19 (Kato et al., 2007, Lees-Murdock et al., 2003, Niles et al., 2013). DMR methylation is acquired in an allele- and sex-specific manner. For example, for the gene *H19*, the paternally inherited allele becomes completely methylated before birth, in prenatal germ cells, while the maternally inherited allele only completes its methylation in postnatal spermatogonia (Davis et al., 2000). Other genomic regions also have varied methylation timings. Most repeats achieve high methylation status by birth, while some sequences including *Sineb1*, minor satellite and major satellite sequences only attain moderate methylation (40%) (Kato et al., 2007).

While the major phase of *de novo* methylation in prenatal male germ cells occurs by E16.5, some sequences do not complete methylation until about two days after birth (PND2) (Molaro et al., 2014, Morselli et al., 2015). The first wave of *de novo* methylation takes place

between E14-E17, when much of the genome regains methylation (Molaro et al., 2014). There are four DMRs of imprinted genes that get methylated in male germ cells, the *H19-, IG-, Rasgrf1- and Zdbf2*-DMRs (Sachs et al., 2013, Lees-Murdock et al., 2003, Molaro et al., 2014, Morselli et al., 2015). Differentially methylated regions that can be traced back to the sperm and oocyte are commonly referred to as germline or gametic differentially methylated regions (gDMRs). All paternally methylated gDMRs acquire methylation during the first wave of methylation. By E16.5, the majority of retrotransposons and genic regions display intermediate to high average methylation (Molaro et al., 2014, Walsh et al., 1998, Kobayashi et al., 2013, Singh et al., 2013). However, a fraction of retrotransposons seemingly avoid this initial wave of global methylation and produce transcripts available for the amplification of secondary PIWI-interacting RNAs (piRNAs), that in turn act during the second phase of methylation. The second wave of *de novo* methylation is proposed to start at ~E17.5 and appears to be dependent on the piRNA pathway. Although the nature of this primary signal, as well as the precise mechanism and timing of methylation by piRNA-dependent sites have yet to be elucidated the second wave of *de novo* methylation is thought to be complete by ~PND2 after birth (Molaro et al., 2014, Hodges et al., 2011).

1.4.1.2.2 Acquisition of DNA Methylation in Postnatal Spermatogonia During Spermatogenesis

Whether DNA methylation established in male germ cells is affected by adult exposures has not been thoroughly investigated. There is evidence that some remodeling continues after birth suggesting that adult exposures may indeed have an impact on this remodeling. Genome-wide studies showed that global CpG methylation levels increased from ~55% at E16.5 to ~75% at PND2.5 (Molaro et al., 2014, Morselli et al., 2015). Non-repeat, intergenic regions also showed modest increases in DNA methylation levels between E16.5 and P6 (Niles et al., 2013).

A study on isolated mouse spermatogonia, spermatocytes, spermatids and sperm using the low-resolution DNA methylation profiling technique, restriction landmark genomic scanning (RLGS) showed increases and decreases in methylation at a small subset of loci between the spermatogonial and pachytene spermatocyte stages (Oakes et al., 2007). Supporting the acquisition of DNA methylation during spermatogenesis, the DNMT1 and DNMT3 enzymes show peaks of expression in spermatogonia and spermatocytes, albeit at lower levels than in prenatal prospermatogonia (La Salle and Trasler, 2006). The dynamics of DNA methylation occurring during spermatogenesis have recently begun to be elucidated through high resolution profiling of purified populations of meiotic and post-meiotic male germ cells similar to the studies that have been done in prenatal germ cells.

One essential role of *de novo* DNA methylation in postnatal male germ cell development remains in the continued silencing of retrotransposons. DNMT3L and PLD6, a phospholipase D/nuclease family protein that contributes to the generation of primary piRNAs,play important roles in this mechanism in male germ cells. Both proteins have been identified to be necessary for *de novo* methylation during meiosis, whereby KOs generated exhibited failed *de novo* methylation at and in turn, failure to silence many retrotransposons, leading to cell death in late meiotic stages (Inoue et al., 2017). As such, the postnatal window of development also yields a period of epigenetic dynamics that may be susceptible to environmental exposures which may alter DNA methylation changes, and impact future progeny.

1.4.1.3 Female-Specific Germ Cell DNA Methylation Re-establishment

Although DNA methylation is globally erased in both sexes during migration of PGCs towards the genital ridge, *de novo* re-establishment of DNA methylation in the germline occurs

in a sex-specific manner. The DNA methylome of female germ cells is largely established after birth, during oocyte growth. Following puberty, oocytes that had previously been meiotically arrested in the diplotene stage of prophase I will cyclically resume and complete the first meiotic division before ovulation (Smallwood et al., 2012).

Much of research on DNA methylation in germ cells had been focused on gDMRs of imprinted genes. Epigenetic marks in these regions escape embryonic epigenetic reprogramming following fertilization, and transmit parent-specific mono-allelic expression of genes that have been identified to be essential for mechanisms of proper embryonic development (Ferguson-Smith, 2011). Disruptions of parent-specific inheritance of imprinted gene gDMRs have been linked to several human syndromes such as Prader Willi, Angelman and Beckwith–Wiedemann (reviewed by Eroglu and Layman, 2012). Outside of gDMRs of imprinted genes, oocyte DNA methylation has been described, using whole genome approaches in both mouse and human oocytes, to be largely at CpG islands in intragenic and gene bodies. Indeed, H3K36me3, which is deposited by SETD2 in association with the RNA polII machinery, plays a critical role in promoting DNMT3B-dependent gene body DNAme in mouse embryonic stem cells. Single-base resolution maps of DNA methylomes spanning the genomes of germinal vesicle stage oocytes identified over 1000 gDMRs (Kobayashi et al., 2013). These late stage oocyte-specific differentially methylated regions were identified to resist epigenetic reprogramming in early embryogenesis implicating their importance in early embryo development. As such, the timing in which these regions acquire methylation is essential in understanding the windows of exposure which may impart intergenerational and transgenerational effects.

Characterization of the oocyte DNA methylome by whole genome approaches has identified that the majority of methylated CpG islands are intragenic and within gene bodies

(Kobayashi et al., 2013). Studies have identified that whilst many CGIs are methylated in oocytes, most are not related directly to genomic imprinting but, nevertheless, a significant amount of oocyte-derived DNA methylation is present in E3.5 pre-implantation blastocysts (Smallwood 2011, Proudhon 2012). Early studies tackled the timing of gDMR epigenetic marking and elucidated that imprinted gene methylation in oocytes is completed by the metaphase II mature oocyte stage (Lucifero et al., 2004). Characterization of genome-wide DNA methylation dynamics throughout oocyte development has generally been restricted due to the technological limitations of investigating small samples such as scant numbers of available oocytes. Various mechanisms have been identified in the underlying mechanisms involved in *de novo* methylation in oocytes including H3K36me3 deposition at actively transcribed genomic endogenous retroviruses called long terminal repeats (LTRs). These transcribed LTRs, dubbed LTR-initiated transcription units (LITs), have been found to house species-specific CGI promoters that also acquire species specific *de novo* methylation in oogenesis (Brind'Amour et al., 2018). These characterizations of *de novo* methylation through oocyte maturation further suggest that postnatal environmental exposures to oocytes have the potential to disrupt the epigenetic dynamics and landscape. Indeed, localized changes could still be noted at non-CpG sites with significant remodeling during the last stages of oocyte maturation in human oocytes, confirming this potential window of susceptibility (Yu et al., 2017).

1.4.2 Histone Modifications

While the exact mechanisms behind DNA methylation-directed gene silencing are still being fully elucidated, it is understood that other epigenetic factors also control gene expression in conjunction with and independently from DNA methylation. DNA is organized into its tightly regulated structure, by wrapping around octameric protein complexes (Oudet et al., 1975). Each

octameric protein complex contains two of each core histone (H2A, H2B, H3, and H4; Luger et al., 1997, Bentley et al., 1984, Kornberg and Thomas 1974) with 145–147 base pairs of DNA wound around it, with a histone called H1 bound to the outside of this core, forming a full nucleosome, and establishing higher-order chromatin structures (Oudet et al., 1975, Allan et al., 1980). Generally, nucleosomes disrupt expression of DNA, by reducing accessibility to the DNA from transcription factors both by directly limiting access and indirectly through changes in DNA conformation (Luger et al., 1997). An actively investigated function of nucleosomes however, lies in their many post-translational modifications. The list of modifications includes acetylation, methylation, phosphorylation, and ubiquitinylation, amongst others, and continues to grow to this day (Lawrence et al., 2016, Kebede et al., 2015, Kouzarides, 2007).

Post-translational modifications of nucleosomes occur on the N-terminal of histone proteins, which project from the nucleosome and render them accessible to enzymatic activity (Luger et al., 1997). Modifications have been shown to both reduce and increase chromatin compaction, leading to increased and reduced transcription respectively (Shogren-Knaak et al., 2006, Akhtar and Becker, 2000, Lu et al., 2008). Histone modifications also influence gene expression via recruitment and blockage of transcription factors and enzymes (Wysocka et al., 2006, 19 Margueron et al., 2005, Vettese-Dadey et al., 1996). As post-trancriptional histone modifications continue to be discovered and studied, their emerging role in germ cell development is becoming evident and investigating how the germ cell epigenome can be susceptible to external influences is crucial to understanding the heritability of environmental exposures (Lawrence et al., 2016).

1.4.2.1 Histone Modifications in Prenatal Male Germ Cell

Chromatin immunoprecipitation (ChIP) approaches have recently been pivotal in the mapping of histone modifications through germ cell development. ChIP assays in PGCs have identified domains bivalently marked with H3K4me3 and H3K27me3, previously proposed to keep developmental genes in a silenced state poised for activation upon differentiation (Ng et al., 2013, Sachs et al., 2013). Active H3K4me3 marks mapped to promoters and CGI, while H3K27ac and H2BK20ac together mapped to enhancers of germ cell-specific genes (Ng et al., 2013). Importantly, between E11.5-13.5, when DNA methylation is decreasing, repressive marks H3K27me2 occurred more frequently at regions enriched for repeats including various short interspersed nuclear elements (SINEs), long interspersed nuclear elements (LINEs) and Long terminal repeats (LTRs), providing a potential mechanism to stabilize retrotransposons or endogenous retroelements, and protect genomic integrity (Ng et al., 2013). Indeed, a conditional knockout of the H3K9 methyltransferase SET domain bifurcated 1 (*Setdb1*) in E13.5 male germ cells led to a decrease in both repressive H3K27me3 and H3K9me3 marks, a concomitant de-repression of endogenous retroelements, and a decrease in PGC numbers (Liu et al. 2014).

Crosstalk between histone modifications and DNA methylation has begun to unveil how specific genomic sequences may be targeted for or protected from methylation throughout developmental stages. For instance, actively transcribed genes typically have hypomethylated CpG-rich promoters and are instead enriched with H3K4me3 (Plunti et al., 2016). The aforementioned ADD domain of DNMTs classically binds to the H3K4 and its trimethylation blocks this interaction (Law and Jacobsen, 2010, Zhang et al., 2010, Ooi et al., 2007). Lack of H3K4 binding leads to auto-inhibition of DNMT3 activity and spares the region from the DNMTs methylase activity (Guo et al., 2015). Accordingly, genomic regions in

prospermatogonia that are H3K4 trimethylated are marked for protection against DNA methylation via interaction with the ADD domain of DNMTs and H3K4me3 levels at E13.5 correlated with predictably low methylation at later time points up to P2.5 (Singh et al., 2013).

Another highly conserved domain in DNMTs, the PWWP domain, is known to bind H3K36 methylation. Recruitment of DNMT3b with H3K36me3 via the PWWP domain has been shown in male germ cells, suggesting that this histone mark may designate specific regions for DNA methylation (Morselli et al., 2015). Genes with high H3K36me3 levels at E13.5 showed elevated gene body methylation at E16.5. Examples of crosstalk between DNA methylation and histone modifications therefore exist in germ cells, although much remains to be revealed regarding the precise timing of post-translational histone modification acquisition in prenatal germ cells and their sensitivity to external influences.

1.4.2.2 Postnatal Male Germ Cell Chromatin Remodeling During Spermiogenesis

Through spermiogenesis, round spermatids elongate and shed much of their cytoplasm. A remarkable condensation of the nucleus is achieved by replacing histones first with the transition proteins and then with protamines (Prm) (Lesch et al., 2014, Marushige and Marushige, 1975, Allen et al., 1993, Haaf and Ward, 1995). Whether protamine domains or transition proteins serve a role outside genome compaction such as in influencing underlying epigenetic modifications is largely unresolved. Interestingly, mouse protamines, Prm1 and Prm2, were recently reported to carry multiple post-translational modifications, leading the authors to propose a ‘protamine code’ that, similar to the ‘histone code’, provides epigenetic signatures important for embryo development post-fertilization as well as potential mechanisms underlying intergenerational inheritance (Brunner et al., 2014).

Some histones are known to escape remodeling in spermiogenesis and are retained in mature spermatozoa. Akin to DNA methylation, these have been shown to pass environmentally altered epigenetic information across generations (Ben Maamar et al., 2018). Retained H3K4me3 marks have been shown to be enriched at paternally-expressed and unmethylated genes in spermatozoa, while paternally-methylated and maternally-expressed genes lacked H3K4me3 (Hammoud et al., 2009). An enrichment of modified nucleosomes bearing the bivalent marks H3K27me3 and H3K4me3 in spermatids and spermatozoa is present at genes implicated in embryo development (Brykczynska et al., 2010, Lesch et al., 2014, Erkek et al., 2013). Most poised bivalently marked genes identified in PGCs were conserved all through spermatogenesis at genes strongly enriched for developmental pathway regulators. Histones were also determined to be retained at imprinted gene clusters important for embryonic development. Abnormal distribution of genome-wide histone retention has been reported in infertile men (Hammoud et al., 2011). Aberrant DNA methylation at CpGs located at the inappropriately retained histones were found when compared to fertile counterparts. These alterations occurred in regions associated with genes involved in embryonic development and correlated with altered gene expression in the blastocysts stressing the importance of appropriate histone placement and modifications for proper cellular function (Denomme et al., 2017). Altogether, there is emerging evidence that histone modifications can transmit information across generations and alterations in the proper histone code in sperm can have consequences on fertility and offspring health.

1.4.2.3 Histone Modifications in Female Germ Cells and in Sex-Specification of PGCs

To date, investigations regarding the role of histone modifications in development have largely been limited to sperm and embryonic stem cells due to tissue accessibility. Recently, a single cell mapping technique was used to reported increased accessibility to genes significantly

enriched for pathways responsible for histone modifications and chromatin remodelling in early stage growing oocytes (Gu et al., 2019). Deposition of H3K36me3 by the methytransferases SETD1 and SETD2 has been shown to be required for proper oocyte gene expression and *de novo* DNAme of transcribed gene bodies in mouse oocytes (Xu et al., 2019, Brici et al., 2017). Coupled with ChIP, another study used fluorescent activated cell sorting to profile and compare histone modifications in E18.5 female PGCs (primary non-growing oocytes) and P10 meiotically-arrested growing oocytes (Stewart et al., 2015). An anti-correlation between CGI methylation and H3K4 trimethylation was reported in both E18.5 PGCs and PND10 oocytes which was relatively static between the two developmental time points. Instead, H3K4me3 accumulation was found to occur in growing oocyte stages of oogenesis (Hanna et al., 2018, Dahl et al., 2016, Zhang et al., 2016). Importantly, reduction of H3K4me3 at maternally methylated imprinted genes was found to be essential to DNA methylation acquisition in these sequences, as deficiencies in the H3K4 demethylase disrupted DNA methylation of imprinted sequences (Stewart et al., 2015, Ciccone et al., 2009). Other histone modifying enzymes have been implicated in oogenesis and oocyte function; for instance histone methyltransferase G9a deficiency caused developmental arrest associated with decreased H3K9me2 (Yeung et al., 2019). Although the timing of the deposition of histone marks has yet to be fully illustrated, their importance in germ cell development is irrefutable.

Histone modification differences have been reported between male and female germ cells as early as at E13.5, with activating histone modifications localized to their respective regions correlating to gene expression (Kono et al., 2019). These sex-specific marks were identified to aggregate at transcription start sites. Furthermore, sex-specific bivalent domains already appeared in E13.5 PGCs. The sex-specific nature of chromatin marks in male and female PGCs

emphasizes their role in guiding gene expression and cellular function, since sex-specific gene expression is already distinguishable in post-erasure E13.5 PGCs when DNA methylation is globally low.

1.5 Intergenerational Consequences of Perinatal Exposures

1.5.1 Evidence for Intergenerational Effects and Inheritance of Epigenetic Alterations Resulting from Environmental Exposures in Animal Models

Rodent models have been extensively used to examine the effects of environmental exposure in the periconceptional period. Prenatal caloric and protein deficiencies in pregnant dams resulted in litters with decreased birth weights, altered end-organ development, and increased adult-onset sequelae and diseases, such as hypertension (Vickers 2000, Kwong 2000). In these examples, the gestating embryo (F1) is directly exposed to the environmental insult via its pregnant mother (F0) and the outcomes are therefore considered as 'intergenerational' effects (Perez and Lehner, 2019). Truly transgenerational phenomena require effects resulting from non-direct exposure of the affected organism to the stimulus, typically requiring offspring effects in the F2 (in the case of postnatal exposures) or F3 (in pre-/perinatal exposures) generation (Figure 1.1.; Heard and Martienssen, 2014).

Evidence for the susceptibility of germ cells to epigenetic alterations as a result of environmental effects during development has best been studied and characterized for the agouti viable yellow locus (Dickies 1962). Early embryo transfer studies determined that the coat colour-shifting effect seen in the F2 of F0 dams given supplementation of methyl donors during gestation was an epigenetic process and not merely a result of intrauterine environmental effects (Morgan et al., 1999). The shift in coat colour has since been determined to be a result of increased methylation of the intracisternal a particle IAP element found at the locus (Blewitt et

al., 2006; Cropley et al., 2006). Many multigenerational diet effects have hence been elucidated, such as grand-maternal undernutrition in a mouse model causing decreased birth weight, adult-onset obesity, and insulin insensitivity in F2 progeny (Jimenez-Chillaron 2009).

The implication of epigenetic marks, particularly DNA methylation, in transgenerational health is long standing. Impaired folate metabolism via key enzyme mutations (such as in methionine synthase reductase or MTRR) leads to transmission of epigenetic instability with neural tube and placental defects across several generations (Padmananhan et al., 2013). However, mutations in key folate pathway genes do not recapitulate the range of abnormalities produced by maternal folate deficiency in humans (Chen et al., 2001, Deng et al., 2008, Gelineau-van Waes et al., 2008, Pickell et al., 2009, Piedrahita et al., 1999, Swanson et al., 2001). This suggests that underlying epigenetic mechanisms through which folate metabolism acts during development may be more subtle and involve epigenetic marks other than just DNA methylation.

1.5.2 Evidence for Intergenerational Effects of Environmental Exposures in Humans

Studies of the intergenerational effects of environmental exposures date back several decades. Investigations of the historical cohort of the Dutch famine of 1944-1945 demonstrated that direct exposure to severe nutritional deprivation during prenatal periods affected mean placental weight, birth weight, body length, and head circumference of offspring (Stein and Susser, 1975). Consequences of these gestational exposures and their effects on adult health are stressed by the increased rates of obesity, cardiovascular and neurovascular disease, diabetes mellitus and restrictive pulmonary disease (Ravelli et al., 1976, Bygren et al., 2000, Roseboom et al., 2006). These examples illustrate a maternal (F0) exposure and its effect on the directly exposed F1 offspring, an observation of an intergenerational effect. From an epigenetic

standpoint, the effects of environmental exposures during gestation two generations away is intriguing, as these grandchildren (F2) were never submitted to the exposures directly, only as in utero developing F1 germ cells.

Although opportunities to study the intergenerational effects of exposures to nutritional deficits in human populations are rare, natural catastrophes or historical events have allowed for some observations. A study of a famine in China demonstrated intergenerational effects of decreased height in children of parents born during the famine (Li and An, 2015). Similarly, studies of rural Gambian populations also suggest that nutrient restrictions during fetal life can be have intergenerational effects with subsequent generations displaying fetal and postnatal growth effects (Eriksen et al., 2017). Other population studies have linked the impact of nutritional availability during windows of development to the health of future generations. For instance, low parental birth weight has been associated with an increased incidence of offspring with metabolic syndrome, while shifts from low to high food availability in the prenatal to postnatal transition have been linked with increased diabetes mellitus related cause of death in grandchildren (Veena et al., 2007, Bygren et al., 2000, Kaati et al., 2002). Many confounding factors and biases may exist in these population studies and render the study of transgenerational effects of environmental exposures difficult in humans. However strong evidence from animal models has emerged delineating the impact of early life environmental exposures and their extension intergenerationally.

1.6 Folate Pathway and One-Carbon Metabolism

Folate is a B vitamin originally discovered as a cure for megaloblastic anemia (Wills 1931). It has since been identified to be important for various biochemical reactions. As it cannot be internally synthesized by humans, it must be acquired through dietary intake and has been

added to grain products in North America since the early 1990s in efforts to reduce the incidence of neural tube defects (Castillo-Lancellotti et al., 2013). A synthetic form of folate has been produced called folic acid. The term folate has been adopted to synonymously include folic acid, vitamin B9, and all oxidation states of folates.

Folate metabolism contributes to one-carbon metabolism and the generation of nucleotides, amino acids, and notably S-adenosyl methionine (SAM). SAM is the key methyl-donor for methylation reactions, such as DNA and histone lysine methylation (Tibbetts and Apling 2010, Bottiglieri 1996). The initial step for folic acid in the folate cycle following absorption of dietary folate is its conversion to tetrahydrofolate (THF) by dihydrofolate reductase (Figure 1.2). An addition of one-carbon units modifies THF into 10-formyTHF. Notably, following several other steps, the enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR) irreversibly converts 5,10-methyleneTHF into 5-methyltetrahydrofolic acid (5-methylTHF), the primary circulatory form of folate (Frosst et al, 1995). 5-MethylTHF is required for the re-methylation of homocysteine to methionine which is then used for the generation of SAM, the substrate required for DNA methylation reactions via methyltransferases. Disruptions of folate metabolism and the generation of SAM can occur through both dietary and genetic disturbances.

1.6.1 Dietary Disturbances in Folate

Early randomized double blinded trials established a significant protective effect of folic acid supplementation for neural tube defects (0.28 relative risk; MRC Vitamin Study Group). Folic acid is recommended in 0.4 mg daily doses for women of reproductive age with low risk of neural tube defects (NTD). Pregnancies at high risk for NTD are indications for a 10-fold higher dose folic acid supplementation of up to 5 mg/day. High folic acid doses of 5 mg have not been

reported to have maternal risks, but long-term high-dose 5 mg folic acid use has not been well studied in a prenatal population (Mulinare et al., 1988, Czeizel and Dudas, 1992, Robbins et al., 2006).

Fluctuations in SAM availability may impact the DNA methylation patterns established and carry the potential for the development of diseases. This was demonstrated in a mouse model of low paternal dietary folic acid where aberrant DNA methylation in sperm was seen, alongside an increase in offspring birth defect rates (Lambrot et al., 2013). Similarly, mouse models of maternal folate deficiencies have resulted in neurological disturbances and altered brain development in the offspring (Jadavji et al., 2015).

High dose folic acid supplementation in mice has been shown to induce a "pseudo-MTHFR deficiency" associated with abnormal SAM levels and SAM/S-adenosyl homocysteine (SAH) ratios in the liver, causing concern for high doses resulting in similar effects to those seen in folate deficiency (Christensen et al., 2015). In fact, it was reported that very high folic acid supplementation in a mouse model caused global hypomethylation in sperm associated with decreased MTHFR expression (Aarabi et al., 2018). A peri-gestational high folate diet in a mouse model was also associated with adverse reproductive outcomes, specifically with increased incidence of heart defects in F1 progeny (Mikael et al., 2013). Concordantly, a study in humans demonstrated that 10-fold maternal folic acid supplement use induces changes in DNA methylation of the F1 offspring that persists through adulthood (Richmond et al., 2018).

Various studies have sought to examine the effects of excessive folates in humans. Folate exposures in humans have been linked to adverse health outomes, including increased associations to respiratory distress and rates of asthma from gestational exposures, (Bekkers et al., 2012, Haberg et al., 2009). While there is human observational and clinical evidence to

indicate that while inadequate folate is associated with colorectal cancer, data also exists purporting high folate exposures increase risk for different cancers, although the evidence is weak (Mason, J., 2011, Mason et al., 2007, Stolzenberg-Solomon et al., 2006). However, recent cohort and populations studies have linked high RBC folate and maternal folate supplementation with endocrine disorders and increased mortality risk, further underpinning the concern over excess folate supplementation (Chen et al., 2021, Kyte et al., 2015). These studies suggest that while folate deficiency can be deleterious, high folate intake can have wide health implications with the potential of being detrimental to reproductive health.

1.6.2 Genetic Disturbances in Folate

Mutations to key genes of the one-carbon metabolism pathway that lower enzyme activity can lead to a decrease in availability of SAM. A common *MTHFR* polymorphism exists in the North American population, 677 C>T mutation. MTHFR as discussed above is responsible for the irreversible reaction generating the main circulatory form of folate – 5-methylTHF. In the homozygous form, the mutation causes the enzyme to be thermolabile and have a ~50% reduction in activity (Frosst, 1995; Christensen et al., 2009). This variant of MTHFR in the human population has been linked with male factor infertility (Wei et al., 2012). Various studies have linked altered DNA methylation in sperm to infertility and sperm defects in men (Kobayashi et al., 2007; Pacheco et al., 2011). Indeed, high levels of MTHFR expression have been observed in the testes relative to other major organs and perturbations in sperm DNA methylation in 677TT men have also been highlighted (Chan et al., 2019, Garner et al., 2013, Chen et al., 2001). Interestingly, high-dose folic acid supplementation exacerbated the DNA methylation disrupting effects of MTHFR 677TT (Aarabi et al., 2015). Rarer severe mutations in

MTHFR with further reduced activity are associated with microcephaly, developmental delays and cognitive impairment as well as other neurologic symptoms (Whitehead 2006).

While single nucleotide polymorphisms of MTHFR genes of the folate pathway and one-carbon metabolism pathway are a known risk predictor for NTD, inborn errors of other enzymes in the pathway such as MTR, MTRR, MS and TCN2 have been shown to alter levels of substrates in the one-carbon metabolism pathway including SAM, B12, and homocysteine, and have been associated with late-onset disease including neurological, psychiatric, and hematological disorders (Arora et al., 2019, Green et al. 2017, Fischer et al. 2014, Huemer et al., 2015, 2017). Strikingly, MTRR variants were recently linked to increased pregnancy loss in human populations, whilst a mouse model of an *Mtrr* mutation demonstrated transgenerationally inherited epigenetic defects (Blake et al., 2021, Zhang et al., 2020). Altogether, these observations strongly implicate the epigenetic landscape to be important for reproductive health and consequently that the folate cycle likely plays a key role in epigenetic reprogramming.

1.6.3 Placental Response to Methyl-Donor Availability

As the gatekeeper between the embryo and mother, the placenta plays a key role in the fetus' adaptability to the maternal environment, regulating resource allocation, and thereby influencing fetal development (Padmanabhan and Watson, 2013, Sandovici et al., 2012). The placenta has been reported to be particularly sensitive to methyl-donor availability and dietary folate deficiency and one-carbon metabolism pathway mutations are associated with placental disorders in humans (Friso et al., 2002, Furness et al., 2008, Ray and Laskin, 1999, Wen et al., 2008). Notably, the placenta has been shown to be more susceptible to epigenetic perturbations due to environmental exposures than the embryo (Whidden et al., 2016, Denomme et al., 2011).

As the placenta has increased sensitivity to epigenetic perturbations, it would thus be conceivable that intergenerational environmental exposure effects in germ cell development be associated with aberrant epigenetic programming in both embryonic and extra-embryonic tissues of future progeny.

1.7. Hypothesis and Objectives

The fetal period is the key time of DNA methylation pattern acquisition in developing male germ cells, for which an adequate supply of methyl donors is required. The folate cycle is involved in the production of methyl groups necessary for methylation reactions. Using a mouse model, **we hypothesize** that perigestational folate deficiency and folic acid supplementation starting in utero will result in alterations of S-adenosylmethionine availability, and consequently aberrant epigenetic patterns in male and female germ cells. Some of these aberrant epigenetic modifications will escape epigenetic reprogramming and be transgenerationally transmitted.

My first objective was to assess the effects of paternal lifetime folic acid deficiency and high supplementation on male germ cell fitness by assessing offspring outcome and the potential for intergenerational propagation by examining the DNA methylation profiles within mature sperm. The aim of this objective was to assess whether an exposure that encompassed both DNA methylation erasure and re-establishment would cause deleterious effects on germ cell development, epigenetic reprogramming, and progeny health. The results of this objective are described in Chapter II.

My second objective was to assess the effects of maternal prenatal folic acid deficiency and supplementation on female germ cell fitness by assessing offspring outcome and the potential for intergenerational propagation by examining the DNA methylation profiles within maternal and offspring somatic tissues. The goal of this objective was to determine whether

dietary exposures during the time of DNA methylation erasure in female germ cells but that did not encompass DNA methylation re-establishment could still have deleterious effects on development. The results of this objective are described in Chapter III.

My final objective was to assess the efficacy of epigenetic reprogramming in restoring appropriate DNA methylation patterns and ablating any reproductive effects resulting from paternal lifetime folate deficiency and high folic acid supplementation by assessing genome-wide DNA methylation profiles in spermatogonia and spermatozoa following lifetime exposures, and in spermatozoa of the next two generations, following epigenetic reprogramming.

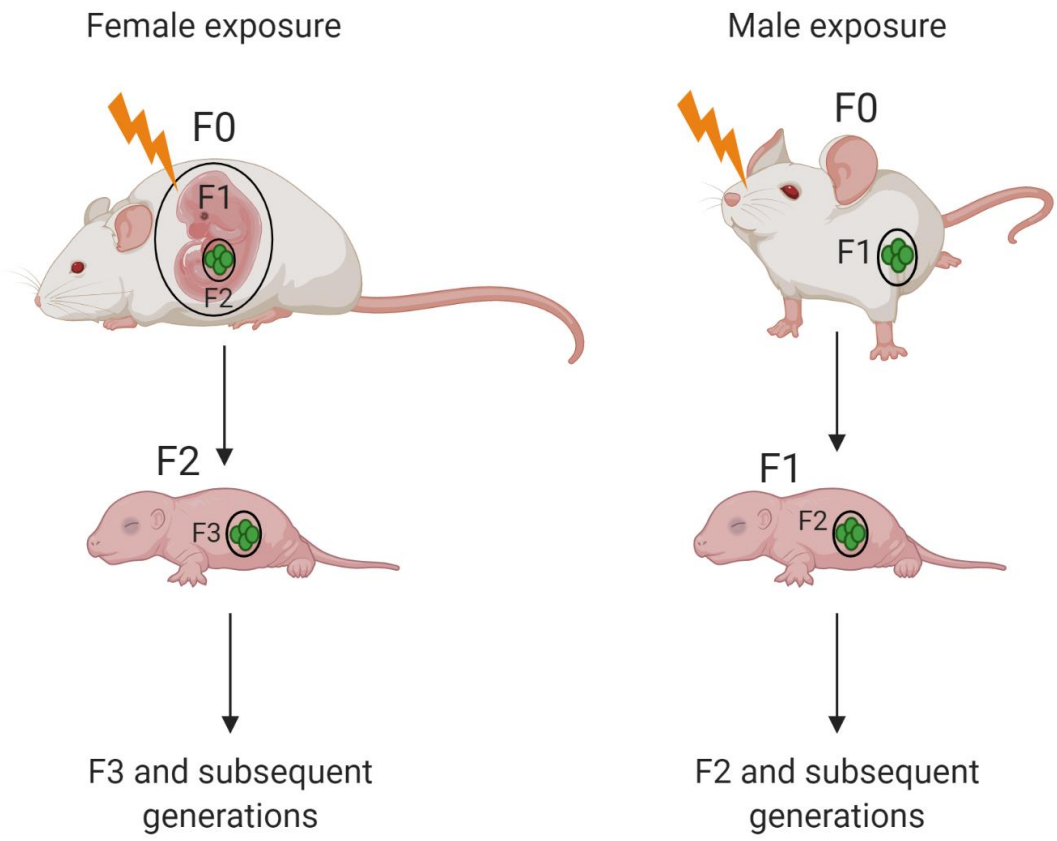

Figure 1.1: Distinguishing intergenerational from transgenerational epigenetic inheritance in females and males.

F0 represents original maternal or paternal exposure to environmental factors. In maternal exposures, the fetus (F1) and its already formed germline (F2) come into contact with the environmental factors. In paternal exposures, the germline (F1) comes into contact with the environmental factors. As a result, a true transgenerational observation can only be attained in the F2 generation in paternal exposures and the F3 generation in maternal studies, as these generations are never exposed to the environmental factors.

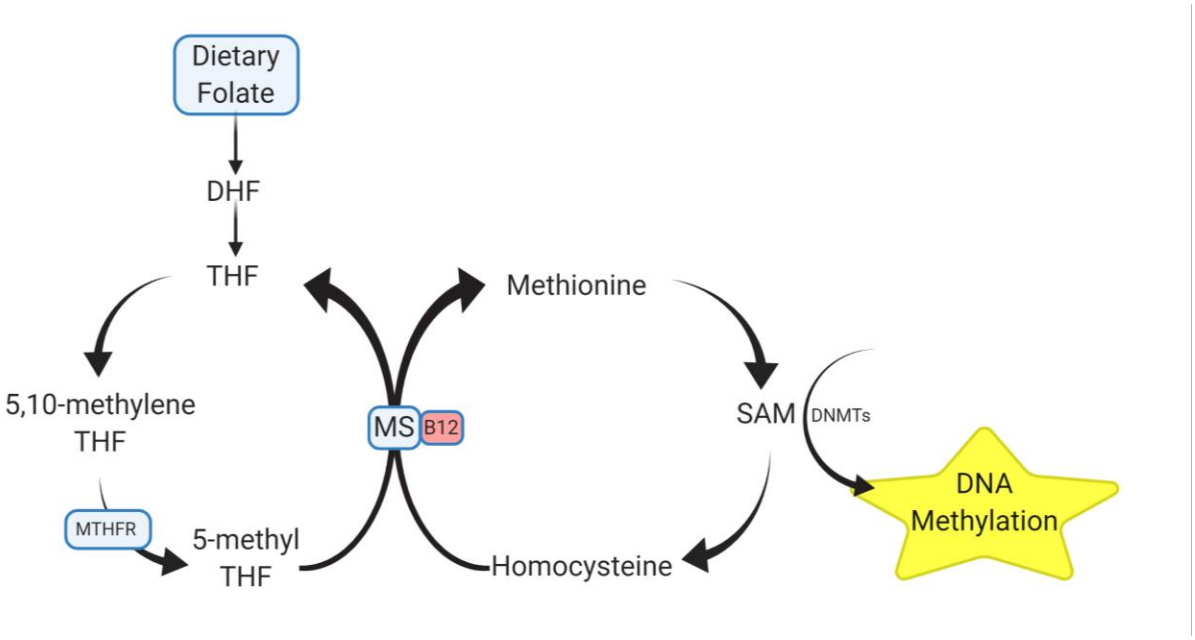

Figure 1.2. Simplified schematic of the folic acid metabolic cycle.

Folic acid is transported into cells, is converted into dihydrofolate (DHF) and subsequently tetrahydrofolate (THF) by dihydrofolate reductase. In the folate metabolic cycle, THF is converted to 5,10-methyleneTHF. Note that multiple steps exist in this transition that are omitted in this representation. 5,10-methyleneTHF reductase (MTHFR). MTHFR will then reduce 5,10-methyleneTHF to 5-methylTHF which can be used to methylate homocysteine to methionine via methionine synthase (MS). Methionine can be used in the methionine cycle to produce S-Adenosyl-methionine (SAM) a universal methylation donor. An alternative pathway to generate methionine from betaine via betaine-homocysteine methyltransferase also exists (not shown).

Chapter II: Intergenerational Impact of Paternal Lifetime Exposures to both Folic Acid Deficiency and Supplementation on Reproductive Outcomes and Imprinted Gene Methylation

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Abstract

Study question: Do paternal exposures to folate deficient (FD), but not folic acid supplemented (FS) diets, throughout germ cell development, including the time of germline epigenetic reprogramming, adversely affect male germ cells and consequently offspring health outcomes and global and imprinted gene DNA methylation?

Summary answer: Males exposed over their lifetimes to both FD and FS diets showed decreased sperm counts and altered imprinted gene methylation with evidence of transmission of adverse effects to the offspring, including variability in imprinted gene methylation.

What is known already: There is increasing evidence that disruptions in male germ cell epigenetic reprogramming are associated with offspring abnormalities and intergenerational disease. The fetal period is the critical time of DNA methylation pattern acquisition for developing male germ cells and an adequate supply of methyl donors is required. In addition, DNA methylation patterns continue to be remodeled during postnatal spermatogenesis. Previous studies have shown that lifetime (prenatal and postnatal) folate deficiency (FD) can alter the sperm epigenome and increase the incidence of fetal morphological abnormalities.

Study design, size, duration: Female BALB/c mice (F0) were placed on one of four amino acid defined diets for 4 weeks before pregnancy and throughout pregnancy and lactation: folic acid control (Ctrl; 2 mg/kg), 7−fold folic acid deficient (7FD; 0.3 mg/kg), 10−fold high folic acid supplemented (10FS, 20 mg/kg) or 20−fold folic acid supplemented (20FS, 40 mg/kg) diets. F1 males were weaned to their respective prenatal diets to allow for diet exposure during all windows of germline epigenetic reprogramming: the erasure, re-establishment and maintenance phases.

Participants/materials, settings, methods: F0 females were mated with chow-fed males to produce F1 litters whose germ cells were exposed to the diets throughout embryonic

development. F1 males were subsequently mated with chow-fed female mice. Two F2 litters, unexposed to the experimental diets, were generated from each F1 male; one litter was collected at E18.5 and one delivered and followed postnatally. DNA methylation at a global level and at the differentially methylated regions of imprinted genes (*H19, Snrpn, Kcnq1ot1, Peg1, and Peg3*) was assessed by luminometric methylation analysis (LUMA) and bisulfite pyrosequencing, respectively in F1 sperm, F2 E18.5 placenta and F2 E18.5 brain cortex.

Main results and the role of chance: F1 males exhibited lower sperm counts following lifetime exposure to both folic acid deficiency and the highest dose of folic acid supplementation (20FS). Post-implantation losses were increased amongst F2 E18.5 day litters from 20FS exposed F1 males. F2 litters derived from both 7FD and 20FS exposed F1 males had significantly higher postnatal-pre-weaning pup death. Sperm from 10FS exposed males had increased variance in methylation across imprinted gene *H19*; increased variance at a few sites within *H19* were also found for the 7FD and 20FS groups. While the 20FS diet resulted in inter-individual alterations in methylation across the imprinted genes *Snrpn and Peg3* in F2 E18.5 placenta, ≥50% of individual sites tested in *Peg1* and/or *Peg3* were affected in the 7FD and 10FS groups. Inter-individual alterations in *Peg1* methylation were found in F2 E18.5 day 10FS group brain cortex.

Large scale data: Not applicable

Limitations, reasons for caution: Further studies are required to understand the mechanisms underlying the adverse effects of folic acid deficiency and supplementation on developing male germ cells. Genome-wide DNA and histone methylome studies as well as gene expression studies are required to better understand the links between folic acid exposures, an altered germ cell epigenome, and offspring outcomes.

Wider implications of the findings: The findings of this study provide further support for paternally transmitted environmental effects. The results indicate that both folic acid deficiency and high dose supplementation can be detrimental to germ cell development and reproductive fitness, in part by altering DNA methylation in sperm.

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Key words: folic acid, paternal effects, DNA methylation, developmental programming, DOHaD, epigenetics, intergenerational effects, sperm, male-mediated, folate

Introduction

Paternal transmission of disease resulting from exposures of male germ cells to environmental factors such as under- and over-nutrition is receiving increased attention due to the emergence of new mechanisms to explain male-mediated effects and the potential for intergenerational consequences (Lane et al., 2014). In particular, environmental exposures can impact the epigenome, including DNA methylation, and result in heritable alterations in gene expression. Folate (vitamin B9) plays a critical role in 1-carbon metabolism involving DNA synthesis and biological methylation reactions such as DNA, histone and protein methylation. Low folate intake and variants in folate metabolism pathway enzymes can alter the availability of cellular methyl groups. Conversely, supplements of folic acid, the synthetic form of folate, are given to pregnant women to prevent neural tube (NTDs) and other birth defects and to subfertile men to improve semen parameters including sperm counts (Ebisch et al., 2007; Wilson et al., 2015). DNA methylation patterning is particularly dynamic in developing male germ cells, which could make them particularly susceptible to low or high folate status during either gestational (in utero) or adult exposures. While there is recent evidence that low paternal folate may alter the sperm epigenome and result in adverse pregnancy outcomes (Lambrot et al., 2013), the potential for male-mediated effects on the offspring mediated by high dietary folate intake has not been examined.

Folate metabolism generates the universal methyl donor S-adenosyl methionine (SAM), the primary source of methyl groups for several cellular reactions including epigenetic modifications such as DNA and histone methylation. It is well established that low peri-conceptional folate status is associated with certain pregnancy complications and NTDs (Czeizel and Dudas, 1992; Czeizel et al., 2011; Gordon N., 1995). As such, supplements containing 0.4 mg per day or more are recommended for women considering pregnancy (Wilson et al., 2015).

Since the late 1990's, many countries have introduced programs to fortify the food supply with folic acid (Miller and Ullrich, 2013) and rates of NTDs have subsequently decreased (De-Regil et al, 2010). Among others, epigenetic mechanisms have been suggested to underlie the effect of folic acid in preventing birth defects.

Higher folic acid doses of 4-5 mg/day (i.e. 10-fold the pregnancy supplement dose) are recommended, for instance, in certain high risk pregnancies (Wilson et al., 2015), and there are ongoing clinical trials assessing the use of 4 mg/day folic acid for the prevention of birth defects other than NTDs, and pre-eclampsia (Bortolus et al., 2014; Wen et al., 2014). Based on the results of small-scale clinical trials showing that high dose folic acid resulted in improved sperm concentrations in semen (Wong et al., 2002; Ebisch et al., 2007), men with idiopathic infertility are often given supplements containing 5mg/day of folic acid. However, use of higher doses of folic acid, in the range of 1-5 mg, is controversial due in part due to questions regarding the effects of circulating un-metabolized folic acid (UFA) (Reynolds, 2016; Smith et al., 2008). High dose folic acid results in the presence of significant levels of UFA in blood which is then reduced by dihydrofolate reductase (DHFR) to a bioactive form. For instance, in elderly men given 5mg/day over 3 weeks, UFA was found in 100% of the group versus 26% at baseline (Obeid et al., 2011). It has been suggested that due to the relatively low activity of DHFR in human liver, increasing the dose of folic acid above a maximum of 1mg/day may not continue to increase the folate pool available for biological methylation reactions (Bailey and Ayling, 2009). Consistent with this, a few recent studies have shown unexpected decreases in DNA methylation associated with the use of folic acid; increasing doses of a methyl donor would have been predicted to result in increases in DNA methylation. For example, a large epigenome-wide study linked higher maternal plasma folate concentrations with lower DNA methylation levels in cord blood (Joubert

et al., 2016). Similarly, DNA hypomethylation was observed in the sperm of infertile men treated with 5 mg/day of folic acid for six months (Aarabi et al., 2015).

Evidence from rodent models also indicates that there can be deleterious developmental effects of high dose folic acid supplements. In rodent studies the baseline diets usually contain the recommended daily intake of folic acid (2 mg folic acid/kg diet), with the supplemented diets ranging from moderate (2.5-fold baseline levels), to high (10-fold) or very high (20-fold), with the 10-fold diet roughly equivalent to the 4-5mg/day human dose. Both 10-fold and 20-fold doses of folic acid given to female mice before and during gestation resulted in embryonic loss and delay, growth retardation and birth defects (Pickell et al., 2011; Mikael et al., 2013). In mouse and rat studies moderate dose (2.0-2.5-fold) gestational ± postnatal folic acid supplementation has been linked to altered DNA methylation patterns in offspring somatic tissues, including the brain (Schaible et al., 2011; Sie et al., 2013; Barua et al., 2016). Here, we chose to study 10-fold and 20-fold folic acid supplements as we were interested in determining the intergenerational effects of clinically relevant doses rather than doses that could be achieved through diet/over the counter supplements.

During their development starting in the prenatal gonad, male germ cells undergo major changes in epigenetic programming involving DNA methylation, histone modifications and non-coding RNAs. For instance, in mice, DNA methylation at most of the ~20 million CpG sites in the genome is erased at mid-gestation in the primordial germ cells and then re-established for the most part in the prospermatogonia of the fetal gonad prior to birth. During postnatal spermatogenesis, DNA methylation is maintained during cell divisions in spermatogonia and most increases and decreases in DNA methylation occur prior to spermiogenesis (Ly et al., 2015). Certain sequences, such as differentially methylated regions (DMRs) of imprinted genes,

have characteristic sperm-specific patterns of DNA methylation which are transmitted to the offspring. Imprinted genes play critical roles in normal fetal growth and development as well as placental function (Tunster et al., 2013). Histone modifications, some of which are transmitted to the offspring, are also remodeled in prenatal and postnatal male germ cells with histone methylation, in particular, a modification potentially sensitive to methyl donor supply (Ly et al., 2015).

The availability of methyl donors, such as folate, is critical for normal DNA methylation patterning in male germ cells. In humans, early studies reported associations between low blood folate concentrations and male subfertility (Bentivoglio et al., 1993; Forges et al., 2007). Also, infertility and aberrant DNA methylation in sperm have been associated with variants in key enzymes involved in folate metabolism such as methylenetetrahydrofolate reductase (MTHFR) (Forges et al., 2007; Gong et al., 2015). In mice, folic acid deficiency resulted in decreased sperm counts (Swayne et al., 2012) and lifetime (in utero and postnatal exposures) dietary folic acid deficiency was associated with evidence of alterations to the sperm epigenome (Lambrot et al, 2013). In the Lambrot (2013) study, embryos fathered by the exposed males showed an increased incidence of malformations. As evidence of its critical role in the testis, MTHFR is expressed at high levels in both prenatal and postnatal male germ cells (Garner et al., 2013) and loss of MTHFR is associated with DNA methylation defects in sperm and infertility (Kelly et al., 2004; Chan et al., 2010). Mutation in the gene for another enzyme necessary for the utilization of methyl groups from the folate cycle, methionine synthase reductase (*Mtrr*), was associated with epigenetic instability and the inheritance of birth defects across a number of generations (Padmanabhan et al., 2017).

While both low and high folate can impact the epigenome of germ cells and cord blood, it is not clear if the epigenetic alterations will translate into effects on the offspring. Here our aim was to determine the effects of lifetime folic acid deficient and supplemented diets in male mice to better understand the physiological impact of folate status on male germ cell development, intergenerational offspring outcomes and global and imprinted gene DNA methylation profiles.

Materials and Methods

Mice and Diets

All procedures were carried out in accordance with the Canadian Council on Animal Care and the study was approved by the McGill University Animal Care Committee. Mice were housed at the Montreal Children's Hospital Research Institute's pathogen-free animal facility under a 12 Light: 12 Dark cycle in a temperature and humidity-controlled environment with access to food and water ad libitum. The breeding and study scheme is outlined in Fig. 1. Eight-week old (F0) female BALB/c mice (n = 15/group) (Charles River, Canada) were fed one of four amino-acid defined diets (Harlan Teklad, USA) for four weeks prior to mating: folic acid control (Ctrl; 2 mg/kg diet) containing the recommended daily intake of folic acid for mice (Reeves 1997), 7-fold folic acid deficient (7FD; 0.3 mg/kg diet), 10-fold folic acid supplemented (10FS; 20 mg/kg diet) or 20-fold folic acid supplemented (20FS; 40 mg/kg diet) diets. All diets contained 1% succinylsulfathiazole in order to prevent de novo synthesis of folate by intestinal bacteria. These diets have been used in several previous mouse studies of the developmental and epigenetic effects of folate deficiency and supplementation (e.g. Pickell et al., 2011; Mikeal et al., 2013; Lambrot et al, 2013; Christensen et al., 2015). Following these four weeks, females were mated with sexually mature, ten-week-old (F0) male BALB/c mice that were fed regular mouse chow diets. Throughout mating, gestation and lactation, the females were fed their diets

and the F1 male offspring were weaned at postnatal day (PND) 20 onto the same diets as their mothers and fed the diets until sacrifice at PND~200.

At 18 weeks of age (approximately three rounds of spermatogenesis), 54 F1 males representative of 10 Ctrl, 11 FD, 10 10FS, and 9 20FS different original F0 litters per diet group were mated with 10 week old female BALB/c mice fed regular mouse chow. Throughout mating, gestation and lactation, the females were fed the regular 18% protein rodent chow diets (Harlan Teklad, USA). F2 male offspring were weaned to and maintained on the regular mouse chow diets.

Breeding studies

In order to assess the outcome in offspring associated with paternal lifetime folic acid exposures, F1 male BALB/c mice (n = 11-15 per diet group representing 8-10 F0 independent litters) were each mated with a second 10-week old female BALB/c mouse. The presence of a vaginal plug on the morning after mating was designated as 0.5 days post coitum (dpc). Isolated pregnant females were fed regular mouse chow until 18.5 dpc, at which point they were sacrificed. Ovaries were collected to determine the number of ovulation sites by counting corpora lutea (CL). Uterine horns were removed and opened to assess implantation by counting viable embryos and resorptions. Preimplantation loss was calculated for each female as the difference between the number of CL and implantation sites. The difference between the numbers of implantation sites and resorptions was used as a measure of post-implantation loss. Placentas, viable embryos and resorptions were removed and weighed. Embryos were sexed by ano-genital distance (and later verified by PCR to ensure accuracy), their crown-rump length measured and evaluation of gross morphological abnormalities performed. Referring to “The Atlas of Mouse Development” (Kaufman, 1992), embryos and late resorptions were examined

for developmental delay and malformations such as cleft palate, closed eyelids, pointy nose, thick neck, curved tail, back and limb malformations. Embryos that deviated by two standard deviations from the means of the mean embryo weights, calculated per litter, across the Ctrl, were considered either growth restricted (two standard deviations below mean of means) or growth enhanced (two standard deviations above mean of means).

Sperm and tissue collection

F1 male mice (n = 17-20 per group, representative of 10 Ctrl, 11 7FD, 10 10FS, and 9 20FS independent F0 litters) were sacrificed upon generation of male F2 pups, at PND ~200 and weighed. Paired testes, epididymides and emptied seminal vesicles were removed and weighed. The right testis was frozen for testicular sperm counts. Mature spermatozoa from paired cauda epididymides were collected as previously described (Chan et al., 2012) and kept frozen at -80^{o}C until use.

Sperm counts

Frozen testes (n = 6 per diet group) of F1 males in each diet group were used for hemocytometric testicular sperm counts as described by Robb et al., with modifications (Kelly et al., 2003). To prepare for counting, a weighed portion of the left testis was homogenized (Polytron, setting 5; Brinkmann Instruments Inc, Westbury, NY) for 2 × 15-second periods, separated by a 30-second interval, in 5 mL of 0.9% NaCl, 0.1% thimerosal, and 0.5% Triton X-100. Elongated spermatid nuclei with a shape characteristic of step 17-19 spermatids and resistant to homogenization were counted.

Plasma and red blood cell total folate

Whole blood (n = 5/group) from F0 females and F1 and F2 males was taken by cardiac puncture, maintained on ice and plasma separated and processed within an hour of sampling.

Samples were shipped frozen to the Health Canada Nutrition Laboratory for analysis. The Lactobacillus casei microbiological assay was used to measure plasma and red blood cell (RBC) folate as previously described (Horne and Patterson, 1988). RBC folate content was normalized to total protein, which was determined using the modified Lowry assay (Bensadoun and Weinstein, 1976).

DNA methylation analyses

Frozen tissues were homogenized using mortar and pestle. DNA was isolated using the DNeasy Blood and Tissue kit (Qiagen, Germany) as per the manufacturer's protocol from less than ~10 mg of homogenized frozen placenta and brain frontal cortex tissues. To control for the heterogeneity of placental and brain frontal cortex samples, whole tissues were homogenized by mortar and pestle to produce a uniform powder representative of the full tissue of each type for DNA isolation. Sperm DNA was extracted using the QIAmp DNA Microkit (Qiagen) with modifications. Sperm samples were incubated overnight at 56^{o} C in sperm lysis buffer containing EDTA, Tris, dithiothreitol and proteinase K. Quantitative measurement of the DNA methylation levels on CpG dinucleotides was accomplished on isolated genomic DNA that was subjected to bisulfite treatment using the EpiTect Bisulfite kit (Qiagen, Germany). Imprinted gDMDs were amplified using primers specific to pyrosequencing applications. Pyrosequencing was performed as previously described by Ronaghi et al., 1998. Amplified sequences were sequenced using the PyroMark Q24 kit (Qiagen) and the PyroMark Q24 Vacuum Workstation (Qiagen) using the manufacturer's protocol. Designed primers for assessment of *H19, Snrpn, Kcnq1ot1, Peg1* and *Peg3* are listed in Supplementary Material, Table S1.

Genome wide DNA methylation was assessed using the Luminometric Methylation Assay (LUMA) as previously described (Lutropp et al., 2015) with a few modifications using the

PyroMark Q24. Briefly, duplicate digestion was carried out for both restriction enzymes HpaII and MspI (Thermo Fisher Scientific, USA). For each digestion, 500 ng of DNA diluted in 10 µl of DNase-free water was digested in 10 µl of enzyme restriction master mix [7 µl DNase-free water, 2 µl 10X Tango Buffer, 0.5 µl EcoRI (10 U/ µl), 0.5 µl restriction enzyme (HpaII or MspI) (10 U/ µl)]. Following the 4h digestion at 37 °C, samples were analyzed on a Pyrosequencer Q24 from Qiagen under the "AQ" mode. To do so, 20 µl of Pyrosequencing Annealing Buffer was added to each digested DNA sample for a total of 40 µl and 30 µl (375 ng of DNA) was loaded onto a Pyrosequencing plate. The nucleotides were not diluted and the Pyrosequencing reagents were added to the cartridge according to the volumes determined from the run preparation using the PyroMark Q24 software. Percentage methylation was calculated using the normalized peak ratios of HpaII over MspI as in the following formula: % Methylation = 100 [1-(HapII / EcoRI / MspI / EcoRI)].

Statistical Analysis

Unless otherwise specified, results are expressed as the mean ± SEM. Data were graphed and analyzed with Prism 5 (GraphPad Software Incorporated, USA). Comparisons were made by Fisher's exact test, ANOVA followed by the Dunnett's multiple comparison test compared to control, or 1-way ANOVA. Increased variance at individual CpGs was calculated by F-test between deficient or supplemented versus control diet groups. Increased variance across an imprinted gene locus was calculated by ANOVA followed by the Dunnett's multiple comparison test on the mean of the all CpG variances across the imprinted locus, supplemented or deficient versus control. A level of significance for all analyses was set at $p<0.05$.

Results

Serum and red blood cell folate concentrations

To determine effects of the different diets on folate status of the males in each of the study generations (Fig. 1), plasma and red blood cell (RBC) total folate concentrations were evaluated in the F0 mothers (as a measure of in utero exposure of male pups), F1 and F2 adult males. Relative to the control diet (Ctrl), the deficient 7FD diet resulted in significantly lower RBC and plasma folate concentrations in the F0 mothers and F1 males (Fig. 2A-D). In contrast, compared to the Ctrl F0 mothers and F1 males, both the 10FS and 20FS diets resulted in significantly higher RBC and plasma folate concentrations (Fig. 2A-D). All F2 males were fed regular chow based diets throughout their lives from in utero to postnatal stages of development (Fig. 1). No differences were found in the RBC or plasma folate concentrations of the F2 males, regardless of the paternal diet group (Fig. 2E-F). It should be noted that the plasma folate concentrations were higher in F2 chow fed mice compared to Ctrl fed F1 male mice, which likely reflects the higher folate content of regular rodent chow compared to the defined diet.

Health and reproductive effects of lifetime exposure to folic acid deficient or supplemented diets

We next assessed whether in utero and postnatal exposures to folic acid deficiency and supplementation affected the general and reproductive health of the F1 males. F1 litter sizes at birth did not differ between folic acid control and supplementation diet groups (Fig. 3A). Adult F1 male body weights at necropsy (18 weeks of age) were similar among the groups (Fig. 3B). In addition, there were no differences in reproductive organ weights (paired epididymides, paired testes and seminal vesicle weights) among the groups (Suppl. Fig. 1). As an indicator that germ cells were affected by the diets, testicular sperm counts were measured and found to be significantly lower in the 7FD and 20FS groups (Fig. 3C) compared to the control. Thus although the F1 males showed no signs of compromised general or reproductive health, the

decreased sperm counts indicated adverse effects of the in utero and postnatal exposure to the 7FD and 20FS folic acid diets on developing germ cells.

Differential effects of paternal lifetime folic acid deficiency and supplementation on offspring (F2) outcomes

To determine whether the decreased sperm counts seen in F1 males resulted in compromised reproductive outcomes, lifetime diet-exposed F1 males were mated to chow-fed females and effects of the paternal diets on pregnancy outcomes determined at embryonic day 18.5 (E18.5) for the F2 generation. Preimplantation loss did not differ between the groups (Fig. 4A). At E18.5, mean F2 litter sizes were similar for the four groups (Fig. 4B). There were no differences among diet groups in embryo and placenta weights (Fig. 4C, D). F2 embryo outcomes for individual litters from at least 6-7 different F1 males per group are shown in Fig. 4E. The incidence of resorptions, as well as the incidence of embryos with any abnormality, including resorption, growth restriction, or growth enhancement, were significantly increased in the litters sired by 20FS exposed F1 males (Fig. 4E). Congenital malformations such as craniofacial and limb abnormalities and cleft palate were not found in any of the groups. The E18.5 results gave a preliminary indication of adverse reproductive outcomes in the 20FS group.

The F2 offspring were followed up in more detail by examining pups after birth in the early postnatal period. Litter sizes did not differ significantly between the groups (Fig. 5A)**.** Corresponding F2 litter sizes were re-assessed at the time of weaning on PND21. On PND21, a significantly lower litter size was observed in F2 litters from 20FS fathers (Fig. 5B); a similar trend ($p=0.07$) was seen for the 7FD group. To further examine the effects of paternal diet on the occurrence of pup mortality in early postnatal life, the proportion of pups that died prior to weaning was compared to those that survived to adulthood for each litter (Fig. 5C). While no

evidence of postnatal mortality was found in litters sired by Ctrl males, significant increases in postnatal mortality were found in the 7FD and 20FS litters. For the F2 7FD litters, postnatal-preweaning death affected both some and all pups in affected litters. Taken together, the results indicate that both paternal (F1) folate deficiency and the 20FS level of supplementation have intergenerational consequences, adversely affecting the F2 offspring.

Effects of folic acid diets on sperm DNA methylation patterns

Based on the role of folic acid as a methyl donor and the proposed roles of epigenetic abnormalities in mediating intergenerational effects, DNA methylation was assessed in the sperm of the F1 males. Both whole genome (global methylation) and sequence-specific approaches were used. Sperm from F1 males from at least five original F0 litters per group were chosen for DNA methylation analysis. Using LUMA, DNA methylation levels were assessed at CCGG sites (about 20% of all CpG sites) across the genome. Sperm DNA methylation levels of 70-75% were similar for all four groups of F1 males (Fig. 6A). Next, imprinted genes were targeted since they possess distinct sperm-specific patterns that escape preimplantation reprogramming, are critical for normal fetal and placental development, and are affected in men with low sperm counts. The germline differentially methylated regions (DMRs) of maternally and paternally methylated imprinted genes are expected to have low (0-10%) and high (90-100%) levels of methylation, respectively, in sperm. Bisulfite pyrosequencing was used to examine the methylation of the DMRs of the three maternally methylated imprinted genes *Snrpn, Kcnq1ot1, and Peg1* (Fig. 6B-D). All three maternally methylated imprinted loci possessed normal low levels of DNA methylation (1-11%) in sperm of the F1 males and were not affected by the diets. The paternally methylated imprinted gene *H19* had high levels of DNA methylation (85-99%) in sperm in all F1 males, regardless of diet group (Fig. 6E). However, whereas sperm *H19* DNA methylation

values for the control Ctrl sperm clustered closely together, more variation in sperm DNA methylation was noted in some of the diet groups. Significant increases in variances at a few individual sites were observed in two groups, at CpGs 1 and 3 for sperm from 7FD F1 males and at CpGs 1 and 4 for sperm from the 20FS F1 males; in contrast, increases in variances were seen at all but CpG5 for sperm from 10FS F1 males. In particular, a significant increase in methylation variance across the *H19* DMR locus was found across the six assayed CpGs following 10FS exposure in F1 males (Fig. 6F). Thus, subtle but potentially important effects of the diets on the paternally methylated imprinted gene H19 were found in the sperm of the F1 males.

Epigenetic effects in the F2 Offspring

To determine if increased variance of DNA methylation in F1 sperm could escape epigenetic reprogramming during preimplantation development and be inherited in the offspring, global and imprinted gene DNA methylation were examined in F2 E18.5 placentas and embryonic tissue. Brain tissue was chosen as it is a key embryonic tissue that depends on normal DNA methylation patterning for its development and has been found to be susceptible to periconception exposures (e.g. Ecker et al., 2004; deWaal et al., 2015). E18.5 F2 litters were chosen to represent offspring from F1 males of five different F0 females per group; DNA methylation levels were assessed for a male and female from each litter. Mean global methylation in placentas was lower than that for sperm, ranging from 55-57% (Fig. 7A). For the F2 placentas, no differences in global mean DNA methylation were observed between the groups (Fig. 7A). However, the 20FS group showed two distinct clusters, one within the expected range and one of lower global methylation; males and females were equally distributed between the two clusters. Interestingly, we observed that two of four embryos in the lower 20FS cluster were

sired by F1 males that also sired litters with postnatal death; in contrast none of those in the upper cluster were sired by F1 males siring litters with postnatal deaths (Fig. 7A).

The possibility of aberrant DNA methylation inheritance from F1 males to F2 offspring was assessed at a gene-specific level in placentas by examining F2 imprinted gene methylation at the paternally methylated imprinted gene *H19* and the maternally methylated imprinted genes *Snrpn, Kcnq1ot1, Peg1, and Peg3*. Methylation levels averaged ~50% for all imprinted genes as would be expected for a somatic or non-germ cell tissue. No differences between groups in overall mean placental DNA methylation were observed at any of the imprinted loci (Fig. 7B, C, D, E, and G). Placental *H19* methylation values were tightly clustered at most CpGs examined for all groups. In contrast, for the maternally methylated imprinted genes, there were significant increases in the variance of DNA methylation levels at individual CpGs across ≥50% of CpGs in a given locus for *Peg1* (10FS group- CpGs 1 and 3), *Snrpn* (20FS group-CpGs 1,2,3,5) and *Peg3* (7FD group- CpGs 3,4,6; 10FS group- CpGs 1,3,4,6 and 20FS group- CpGs 3,4,5,6) in the placentas of F2 embryos (Figure 7C, D, E, G). Additionally, there were significant increases in the variance of DNA methylation levels across the *Snrpn and Peg3* loci in the placentas of F2 embryos from the 20FS group (Fig. 7 F, H).

Global and gene-specific DNA methylation were also assessed in the brains of the embryos corresponding to the placentas examined (Fig. 8). Global DNA methylation levels in the cortex averaged 70-72% and did not differ significantly between the groups or show evidence of variance between embryos (Fig. 8A). No increases were found in the variance of DNA methylation levels at individual CpGs across the *H19, Snrpn, Kcnq1ot1, Peg1, and Peg3* loci in the cortices of F2 embryos from the 7FD, 10FS, and 20FS group (Figure 8B-E). While the *H19, Snrpn, Kcnq1ot1 and Peg3* imprinted genes showed normal ~50% DNA methylation levels and

little evidence of variance across the entire locus between embryos in all groups, a significant increase in DNA methylation variance across the *Peg1* locus was observed for the 10FS group compared to the Ctrl group (Fig. 8F). Taken together, there was evidence of inter-individual DNA methylation variation in the F2 offspring, most notable in the placentas, associated with paternal folic acid deficiency and supplementation.

Paternal lifetime exposure to folic acid deficient or supplemented diets effects on surviving male offspring health

To determine if paternal lifetime folic acid deficiency or supplementation could affect postnatal development of F2 pups that survived post-weaning, we investigated the body weights, reproductive organ weights and sperm counts of adult males. Adult F2 male body weights (Suppl. Fig. 2A) at necropsy (~20 weeks of age) and male reproductive organ weights (paired testes, paired epididymides, and seminal vesicles, Suppl. Fig 2B, C, and D) were similar for all the groups. Furthermore, unlike in the F1 generation, testicular sperm counts of the F2 males did not differ between the groups. Thus, adult F2 males did not show signs of male reproductive abnormalities persisting beyond the F1 generation.

Discussion

Our data indicate that a male's lifetime exposure to both folic acid deficient and highly supplemented diets result in decreased sperm counts, adverse outcomes in his offspring and evidence of epigenetic alterations (see summary and model in Figure 9). While folic acid deficient diets have previously been linked to paternally-mediated intergenerational effects in a different strain of mouse, C57BL/6 (Lambrot et al., 2013), than the BALB/c strain used in the current study, it was unexpected that a folic acid supplemented diet could produce similar outcomes. This is potentially concerning since, although the treatment duration was longer than

that clinically used, the doses of folic acid supplements used in the current study, 10- and 20-fold the daily recommended intake of folic acid for mice, are within the range used clinically in high risk pregnancies and for the treatment of male subfertility. Male germ cells undergo extensive epigenomic reprogramming, including that of DNA methylation, during their development from primordial germ cells in the fetal gonad through postnatal spermatogenesis, including mitotic, meiotic and postmeiotic phases. Folic acid is an important source of methyl groups needed for DNA methylation and thus decreases and increases in folic acid intake have the potential to impact DNA methylation programs. Our study was designed to expose male germ cells to low, high and very high folate levels throughout male germ cell development.

Plasma and RBC folic acid concentrations reflected the folic acid content of the experimental diets consumed by the mice. For example, F0 dams and F1 sires that consumed a folic acid deficient diet had lower plasma and RBC folate concentrations, whereas F0 and F1 mice consuming the supplemented diets had higher plasma and RBC folate concentrations. All F2 males fed the chow diet had a similar folate status regardless of parental or grand-maternal diet. The effects of the defined diets on plasma folate concentrations are in keeping with previous studies utilizing the same diets with the 7FD diet resulting in a near four-fold decrease and the 10FS and 20FS diets leading to approximately two-fold increases in plasma folate concentrations, respectively (Li et al., 2006; Pickell et al., 2011; Mikael et al., 2013). The relationship between folic acid intake and RBC folate concentration is nonlinear suggesting a threshold for folate accumulation in tissues. In relation to our study, the germ cells of the F1 males were exposed to higher or lower folate both in utero (F0) and post-natally during periods in which they were developing from primordial germ cells to mature spermatozoa and reprogramming their DNA methylation levels. Since F2 males were not exposed directly to the

diets, it can be assumed that differences in their placenta and tissue methylation profiles are due to exposure of the F1 germ cells to the different diets.

The lifetime folic acid diets did not appear to affect the general health of the F1 males, as indicated by a lack of difference in body and reproductive organ weights. However, we observed a decrease in sperm counts in the testes of F1 males fed the 7FD and 20FS diets. This is similar to findings by Swayne et al. (2012) in which male BALB/c mice fed a folic acid deficient diet for 15 weeks during the post-weaning period resulted in a ~40% reduction in cauda sperm number. In contrast, Lambrot et al. (2013) did not find a decrease in sperm counts in C57BL/6 male mice exposed over a lifetime to the same 7FD diet we used. The discrepancy may be due to the different mouse strains, BALB/c versus C57BL/6, used in the various studies. For instance, testes of BALB/c mice appear to be more susceptible to perturbations in the folate metabolic pathway (Chan et al., 2010). The folate dependent enzyme MTHFR is found in particularly high levels in the prenatal and postnatal testis (Chen et al., 2001; Garner et al., 2013). MTHFR deficient BALB/c strain mice have very few germ cells in their testes and are infertile in contrast with MTHFR deficient C57BL/6 strain mice that have decreased sperm counts (50% of normal) and are fertile (Kelly et al., 2005; Chan et al., 2010). A previous study characterized differences in folate metabolizing and DNA repair enzymes between the two strains and reported that BALB/c mice are more prone than C57BL/6 mice to accumulate DNA damaged cells (resulting in cell death) when one carbon donors are low (Knock et al., 2011). Folic acid is required for nucleotide synthesis and biological methylation reactions through the folate metabolic pathway. The folic acid deficient diet could affect either of these pathways leading to the decreased sperm counts. These findings are consistent with observations in humans where low serum folate has been correlated with lower sperm counts (De Sanctis et al., 2011)

The decrease in F1 sperm counts associated with the 20FS diet, which was similar to that observed in the 7FD group, was unexpected and its basis may provide clues to the adverse pregnancy outcomes in the offspring of both groups of males. A few lines of evidence indicate that excess folic acid results in functional folate deficiency. Down regulation of folate metabolic pathway enzymes may be related to the presence of circulating UFA in individuals taking high dose folic acid supplements (Kelly et al., 1997; Bailey et al., 2010). UFA in turn results in the accumulation of cellular dihydrofolate, an inhibitor of folate metabolism pathway enzymes such as MTHFR (Matthews and Daubner, 1982). In in vitro studies, excess folic acid inhibits MTHFR activity in brain extracts (Hollinger et al., 1982). In addition, in mouse studies excess dietary folic acid has recently been shown to result in the both decreased MTHFR protein levels as well as production of a less active form of MTHFR in liver (Christensen et al,. 2015). Together, our data suggest that both folate deficiency and high dose folic acid supplements may impact male germ cells in a similar manner either by directly (7FD) or indirectly (e.g. MTHFR inhibition) reducing the provision of methyl groups from folic acid.

In addition to the reduction in testes sperm number, we also have evidence of adverse outcomes in the offspring (F2) of the F1 males exposed to the folic acid deficient and supplemented diets. One of the most striking findings of this study was an increase in postnatal-preweaning mortality in F2 litters from F1 males fed the 7FD and 20FS. To our knowledge, this is the first study to examine the intergenerational implications of paternal lifetime exposure to both folate deficiency and folic acid supplementation in the postnatal period of F2 offspring. The increased mortality in litters (F2 generation) from FD exposed fathers could be a result of aberrant development, similar to findings by Lambrot et al. (2013) in which embryos demonstrated developmental delay and abnormalities. Unfortunately, since the pups died at

different times and were in most cases not recovered before the mothers cannibalized them, we were unable to perform autopsies to determine the cause of death. Interestingly, another study on male-mediated effects (sperm based) of drugs used to treat testicular cancer also reported postnatal mortality of unknown cause in the offspring of the exposed males (Bieber et al., 2006). The increased mortality in the postnatal window in the current study suggests that paternal lifetime low or high folate exposures affected male germ cells and that the effects were transmitted to offspring that survived beyond the embryonic period.

As DNA methylation patterns are erased and reset in the developing male germ cells, we postulated that altered methyl donor supply due to the folate diets would impact epigenetic programming in sperm. Global DNA methylation levels measured using the LUMA assay provided an initial assessment of DNA methylation levels at ~20 million sites across the mouse genome. In previous human studies, we have shown decreases in overall sperm DNA methylation associated with folic acid supplement consumption and MTHFR deficiency (Aarabi et al., 2015). In the current study, sperm global DNA methylation levels were unaffected by the diets, giving a preliminary indication that there were no major global alterations in male germ cell DNA methylation reprogramming. However, as the bulk of DNA methylation takes place in repeat sequences, more subtle effects at developmentally important sequences would be missed with a global assay. We were particularly interested in imprinted genes since germ cell processing of these sequences is unique. In both male and female primordial germ cells methylation is erased on all imprinted sequences. In the male germline, DNA methylation is re-established on the DMRs of paternally methylated genes such as *H19* while maternally methylated genes such as *Snrpn* remain unmethylated. Lower or higher intakes of folate could alter the availability of methyl co-factors and thus potentially interfere with the erasure or re-

establishment of methylation on imprinted genes. In a mouse study of the effects of gestational folate supplements on fetal (F1) brain, alteration in imprinted gene methylation was reported, suggesting that germ cells might also be susceptible (Barua et al., 2014).

Overall, mean imprinted gene methylation levels were unaffected in sperm. Other studies have similarly reported imprinted gene mean methylation in spermatozoa to be unaffected by lifetime folate deficiency in mice or postnatal folic acid supplementation in human studies (Lambrot et al., 2013; Aarabi et al., 2015). However, by examining only overall mean imprinted gene methylation on millions of sperm, inter-individual differences may be missed. An initial indication of perturbed epigenetic marks associated with the diets was seen in the F1 sperm at the paternally methylated imprinted gene *H19*. We observed a significant increased variance of DNA methylation levels at 5 out of 6 assayed individual CpGs within the *H19* locus, as well as across the locus, between individual animals in the 10FS group; individual H19 CpGs were also affected in the 7FD and 20FS groups. Interestingly, the most marked sperm H19 variability was found in the 10FS group when it might have been expected in the 7FD and 20FS groups. It is possible that, as reflected by the decrease in sperm counts, the sperm epigenome in the 7FD and 20FS groups was more severely affected than that in the 10FS group. In a previous study on cultured enriched populations of mouse spermatogonial stem cells, a similar variability in male germ cell DNA methylation, in response to *Mthfr* haploinsufficiency and varying concentrations of methyl groups, was found (Garner et al., 2013). It is possible that such inter-individual variability in germ cell DNA methylation is a common response to folate metabolic pathway and methyl donor perturbations or that it is secondary to effects on other epigenetic modulators such as histone methylation which undergoes significant remodeling during male germ cell development (Figure 9). The results in the current study suggest the possibility that DNA

methylation alterations may contribute to the observed folate diet related intergenerational effects.

Another way to examine the significance of epigenetic abnormalities residing in sperm is to examine the resulting offspring for epigenetic defects. We sampled both the extra-embryonic and embryonic tissues. The placenta is particularly useful for assessing epigenetic effects of periconceptional exposures including germ cell exposures, as it typically shows higher levels of epigenetic perturbation than the corresponding embryo; this is thought to be due to the retention or tolerance of epigenetically abnormal cells in the placenta but not in the embryo (Rivera et al., 2008; Fortier et al., 2008). Thus the placenta is thought to provide a more sensitive indicator of epigenetic perturbations than the embryo. Increases in the variance of both individual CpGs as well as pan-locus imprinted gene methylation were noted for maternally methylated imprinted genes but not for the paternally methylated gene *H19* in the E18.5 day placentas of all the F2 diet groups. Alterations in variance but not in mean DNA methylation of imprinted genes suggests that a subset of F1 aberrant gametes, not detected in whole sperm studies (Figure 6), from FD and FS exposed males, develop into functional spermatozoa and can result in viable embryos that survive at least until the end of gestation. The observed increased placental DNA methylation variability for some but not all imprinted genes examined could also reflect underlying epigenetic instability induced by the diets and perhaps based on another epigenetic modification other than DNA methylation; this possibility is supported by the fact that DNA methylation of maternally methylated imprinted genes was not affected in the F1 sperm.

The paternal diets resulted in less evidence of epigenetic effects on global DNA and imprinted gene methylation in the brain cortex as compared to the placenta. While the placenta is thought to provide a more sensitive indicator of epigenetic perturbations, somatic tissues remain

equally important to investigate as they may result in abnormal tissue structure or function in the offspring. Due to its complexity, the brain has been postulated to be particularly sensitive to germ cell or early embryo perturbations in DNA methylation patterns. Previous studies have shown that gestational folic acid supplementation can result in altered gene expression and DNA methylation in the cerebellum of the offspring (Barua et al., 2014; Barua et al., 2016); these studies employed genome-wide approaches to DNA methylation profiling. In the current study, there was little evidence of altered global or imprinted gene methylation in the cortex with the exception of effects on *Peg1* in F2 brain cortex from the 10FS group. It is unclear why the 10FS group was the only one affected, although as discussed above for the sperm *H19* methylation results, it is possible that affected sperm are more viable in the 10FS groups as compared to the two other diet groups. It is clear that much more detailed profiling will be required in our model to determine the extent and type of epigenetic effects in brain and other somatic tissues of the F2 offspring.

Lambrot (2013) also identified aberrant DNA methylation in precursors of spermatozoa associated with the administration of folate deficient diets. However, these differentially methylated loci were no longer present is mature spermatozoa, possibly through mechanisms of correction or elimination of affected gametes by apoptosis. Instead, the authors noted abnormal patterns of chromatin marks persisting in mature spermatozoa. Together, results from the Lambrot and the current study suggest that lifetime folic acid deficiency and folic acid supplementation may not only transiently affect DNA methylation of sperm, but additionally affect other epigenetic marks such as chromatin modifications. Subsequently, the inheritance of an altered epigenome or perhaps epigenetic instability may result in abnormal development in

the offspring leading to increased morphological abnormalities and/or death (see model in Figure 9).

Our findings do not support a simple model of diet-induced altered DNA methylation as the sole basis for the prenatal and postnatal F2 progeny death. We thus propose a more complex model (Figure 9) on which to base further studies. The decreased F1 sperm counts demonstrate that reproductive fitness has been affected and is possibly impaired. The observed postnatal death in the F2 generation suggests that exposed F1 male germ cells are functional but abnormal. Finally, increased imprinted gene DNA methylation variance in F1 sperm and in F2 somatic tissues hints that there is underlying epigenetic dysregulation or instability as a result of the paternal diets. The mild variability in imprinted gene methylation is unlikely to be the basis of the F2 offspring death. DNA methylation alterations may still play a role if genome-wide effects, not examined here, are present at susceptible regions such as regulatory elements of developmental genes. It is equally likely that other epigenetic modifications, such as histone methylation are affected by the diets. We suggest that the folate diets may affect more than one epigenetic mechanism in exposed developing male germs and result in epigenetic instability. In the Padmanabhan (2013) study, a mutation in the folate metabolism gene *Mtrr* in the first generation caused congenital defects in the mutant progeny as well as in wild-type descendants for up to four generations; it is postulated that the *Mtrr* mutation resulted in epigenetic instability, however, the underlying germline inherited epimutations have not been identified.

It is clear that further studies will be required to understand the basis and more fully explore the mechanisms underlying the adverse effects of folate deficiency and folic acid supplementation on developing male germ cells and the offspring sired by the exposed males. Our results demonstrate that the epigenetic phenotypes can be highly variable and underline the

fact that numerous offspring will need to be examined to better understand the spectrum of epigenetic defects induced in developing male germ cells by folate diets. Genome-wide DNA methylome studies, histone modification profiles, as well as gene expression studies to get at functional outcomes will be required. It will also be important to separate out effects of prenatal (gestational) and postnatal exposures to male germ cells, as is being done for somatic exposures (e.g. Sie et al, 2013). It is possible that gestational exposures, when the major phase of epigenetic reprogramming of male germ cells takes place, may represent a particularly susceptible window for the induction of DNA methylation defects in male germ cells.

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Figures and Tables

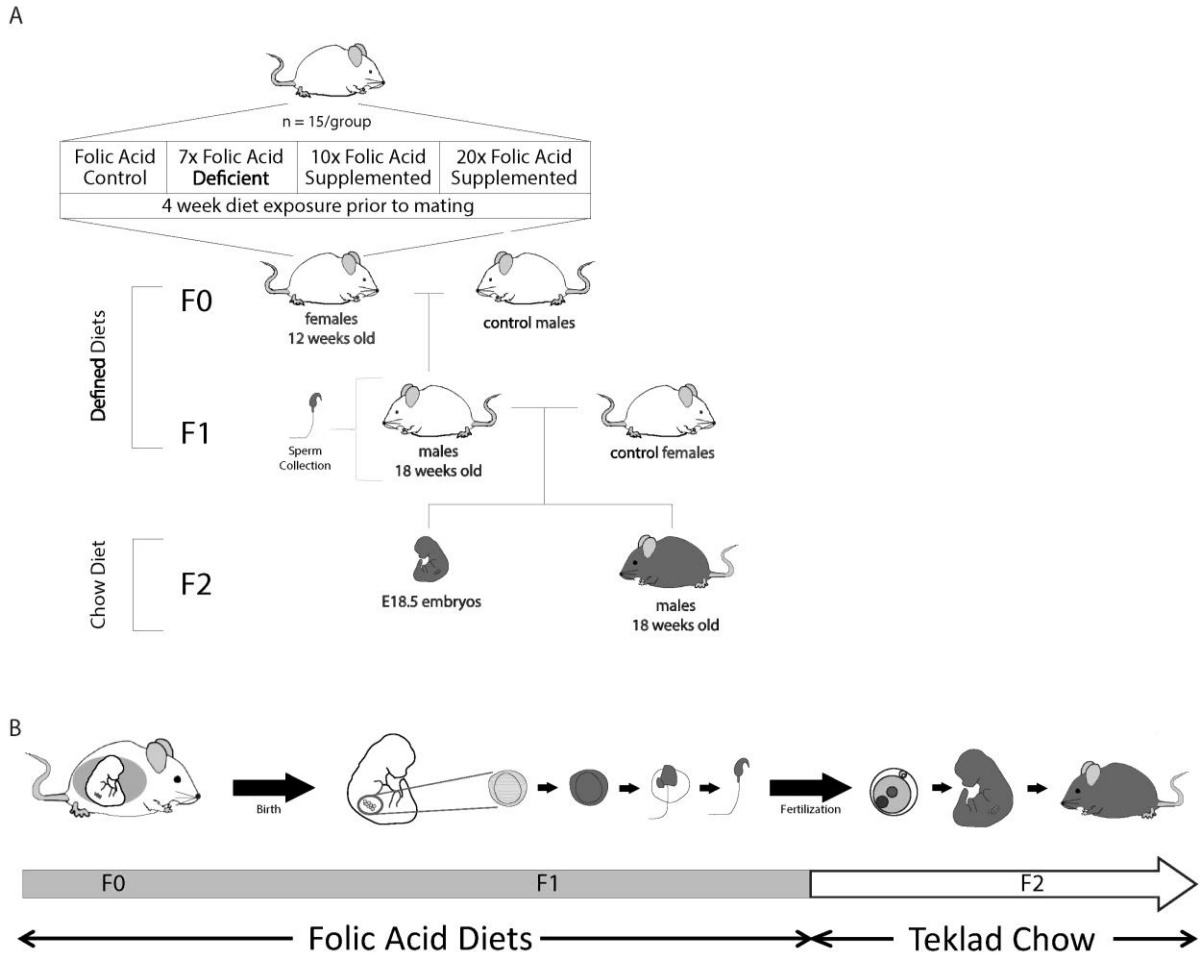


Figure 2.1. Intergenerational reproductive effects of lifetime folate deficiency and supplementation.

Eight week old BALB/c F0 females were fed either a Ctrl, 7FD, 10FS or 20FS diet, (n = 15 for each) for 4 weeks prior to breeding with BALB/c males fed with regular rodent chow. Females were sacrificed, F1 male pups received the same experimental diet as their mother. At 18 weeks of age, one F1 male from each litter was mated with a female fed with rodent chow. Females were maintained on the rodent chow through pregnancy and lactation. From weaning until

sacrifice, F2 male pups received rodent chow. (Ctrl = Folic Acid Control Diet, 7FD = 7x Folic Acid Deficient, 10FS = 10x Folic Acid Supplemented, 20FS = 20x Folic Acid Supplemented).

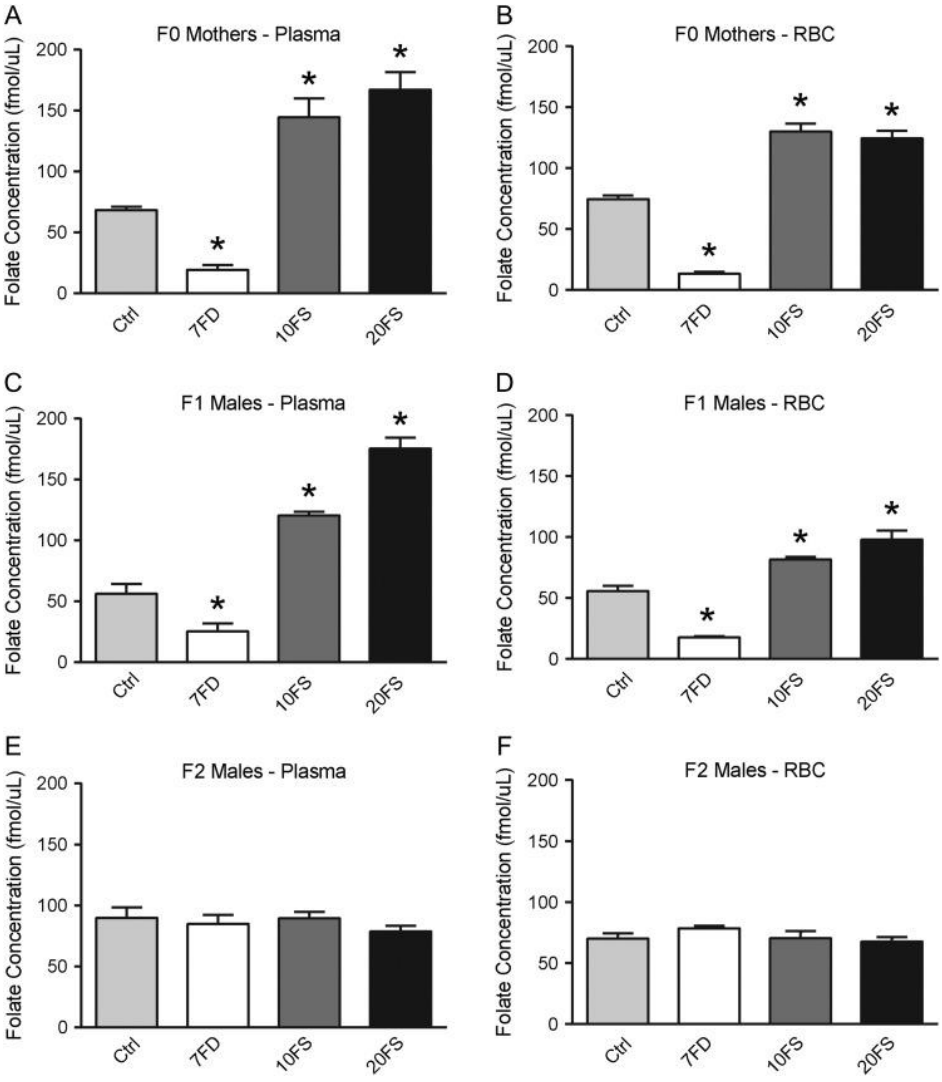


Figure 2.2. Plasma and red blood cell (RBC) folate concentrations.

Plasma and red blood cell (RBC) folate concentrations in F0 dams (A-B), F1 male progeny (C-D) and F2 male progeny (E-F) at sacrifice (n = 5). (Ctrl = Folic Acid Control Diet, 7FD = 7x Folic Acid Deficient, 10FS = 10x Folic Acid Supplemented, 20FS = 20x Folic Acid Supplemented). * = $p<0.05$ by one-way ANOVA with Dunnett's multiple comparisons test versus Ctrl.

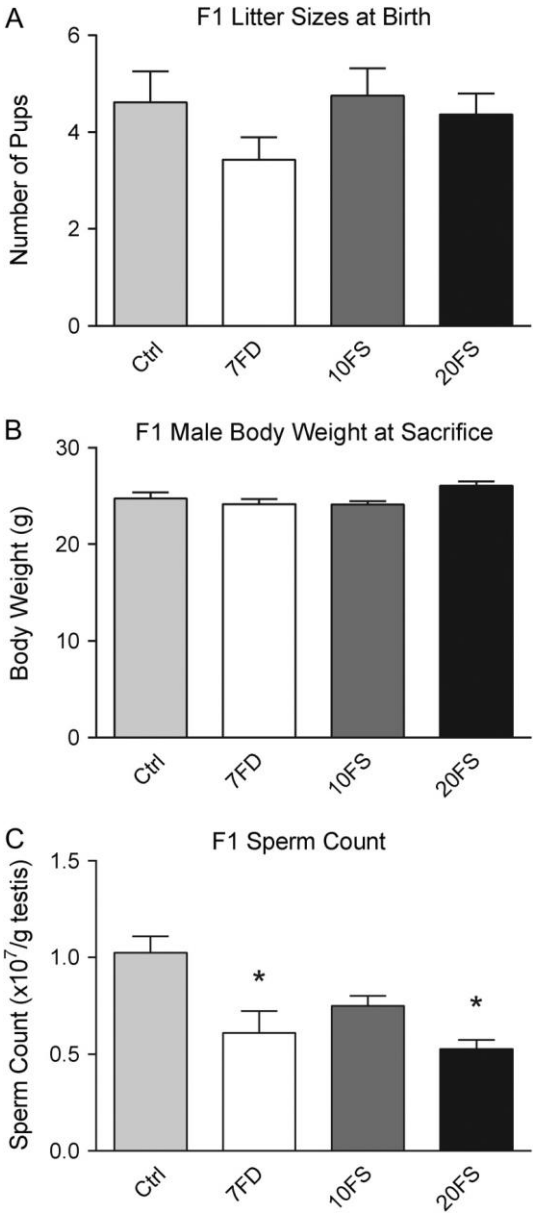


Figure 2.3. Lifetime folate deficiency and 20FS supplementation decrease sperm count.

Effect of lifetime folate deficiency and supplementation on F1 litter sizes at birth (A; n = 11-15 litters/group), F1 adult male body weight (B; n = 17-20/group), and F1 sperm count (C; n = 6/group). (Ctrl = Folic Acid Control Diet, 7FD = 7x Folic Acid Deficient, 10FS = 10x Folic Acid Supplemented, 20FS = 20x Folic Acid Supplemented). * = $p<0.05$ by one-way ANOVA with Dunnett's multiple comparisons test.

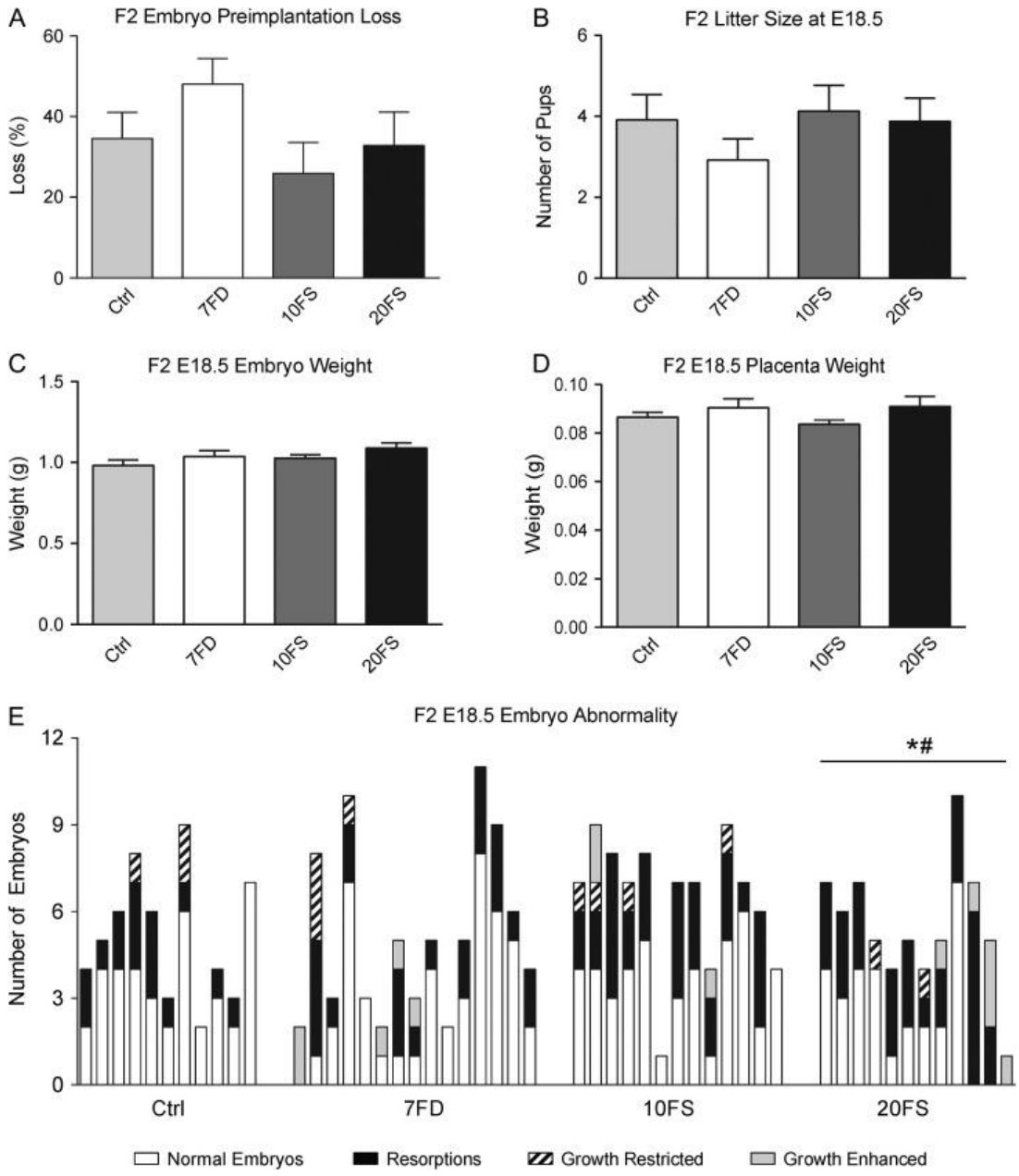


Figure 2.4. Effects of lifetime folate deficiency and supplementation on pregnancy outcomes at E18.5.

Preimplantation loss of F2 at E18.5 (A; n = 11-15 F2 litters, representing n = 7-9 original F1 litters), F2 litter sizes at E 18.5 (B), F2 embryo weights at day 18.5 (C; n = 25-38 embryos, representing n = 7-9 original F1 litters), and F2 placental weights at embryonic day 18.5 (D). Incidence of fetal abnormalities at embryonic day 18.5 per litter (E; n = 11-15 litters); growth

restriction and enhancement are defined as a 2-fold standard deviation difference of embryo weight to the group mean of litter mean weights.(Ctrl = Folic Acid Control Diet, 7FD = 7x Folic Acid Deficient, 10FS = 10x Folic Acid Supplemented, 20FS = 20x Folic Acid Supplemented). * = $p<0.05$ by one-way ANOVA with Dunnett's multiple comparisons test.

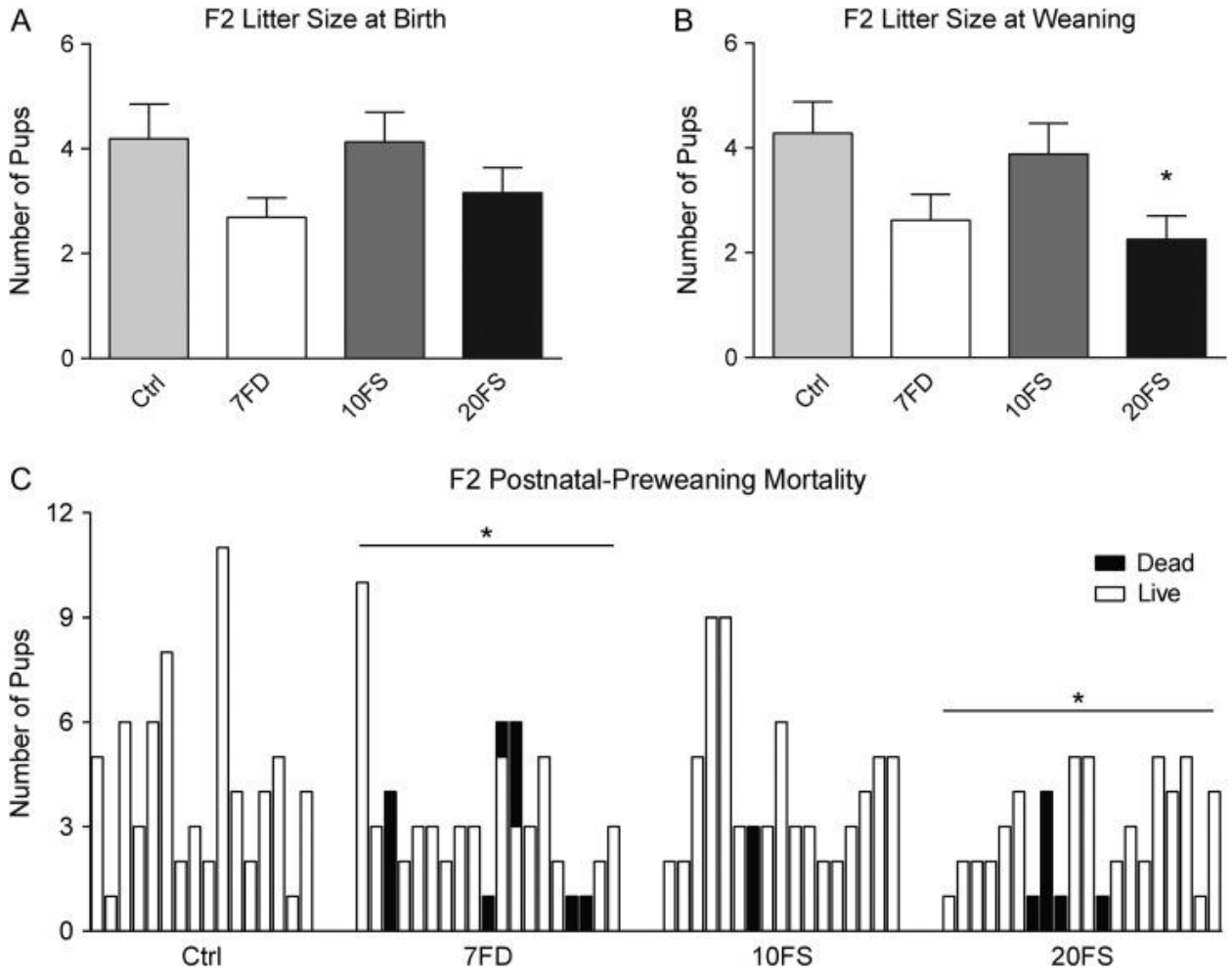


Figure 2.5. Lifetime folate deficiency and 20FS supplementation result in postnatal-preweaning mortality.

Effect of lifetime folate deficiency and supplementation on F2 litter sizes at birth (A; n = 16-20 F2 litters, representing n = 9-11 original F1 litters), F2 litter sizes at weaning (PND21) (B), and incidence of pup postnatal mortality per litter (C). (Ctrl = Folic Acid Control Diet, 7FD = 7x Folic Acid Deficient, 10FS = 10x Folic Acid Supplemented, 20FS = 20x Folic Acid Supplemented). * = $p<0.05$ by one-way ANOVA with Dunnett's multiple comparisons test or by Fisher's exact test.

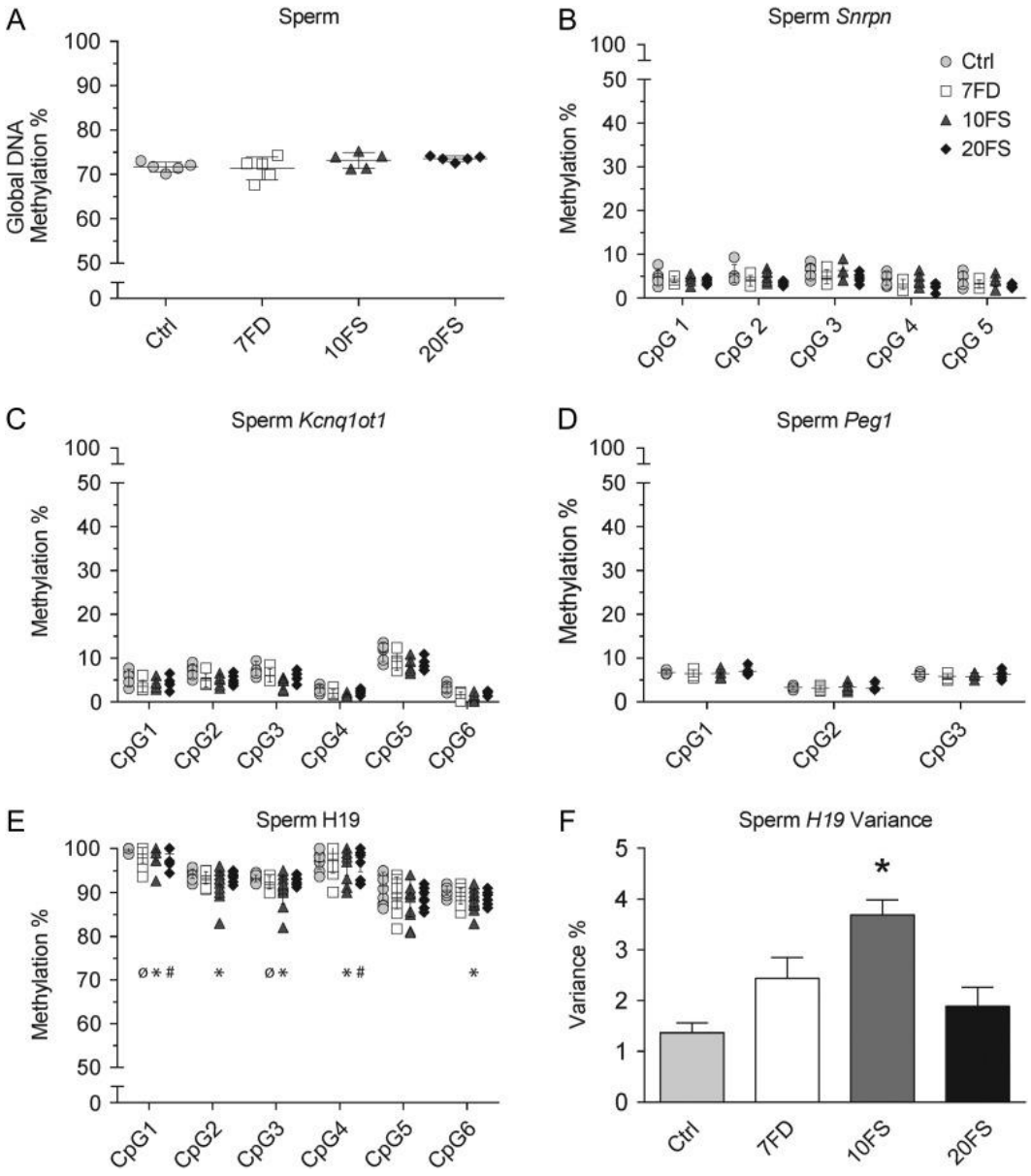


Figure 2.6. F1 sperm global DNA methylation and DMR methylation at imprinted genes uncovers variance of H19 methylation.

Global DNA methylation was measured using LUMA (A). Loci of maternally methylated genes *Snrpn* (B; n = 5/group), *Kcnq1ot1* (C; n = 5/group), and Peg1 (D; n = 5/group) and paternally methylated gene *H19* (E; n = 10-13/group) methylation levels were quantified by bisulfite pyrosequencing. *H19* variance (F) was measured as a mean of variances of all six CpGs (Ctrl = Folic Acid Control Diet, 7FD = 7x Folic Acid Deficient, 10FS = 10x Folic Acid Supplemented,

20FS = 20x Folic Acid Supplemented). For individual CpG variance (panel E), ø (7FD), * (10FS), # (20FS) = $p<0.05$ by F-test between deficient or supplemented versus control diet groups. For across-locus variance analysis (panel F), * = $p<0.05$ by one-way ANOVA with Dunnett's multiple comparisons test.

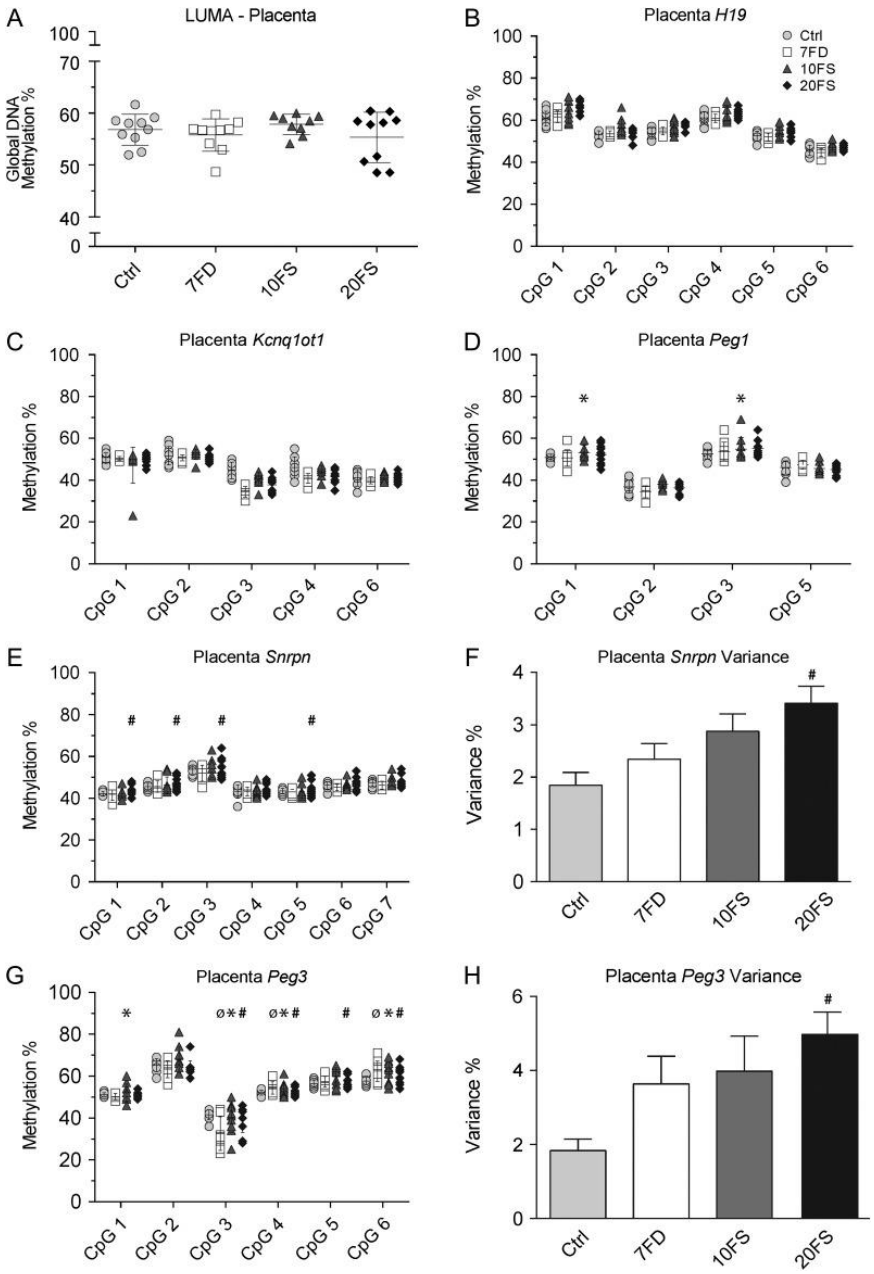


Figure 2.7. F2 E18.5 placenta global DNA methylation and DMR methylation at imprinted genes uncovers variance of Snrpn and Peg3 methylation in F2 pups sired by 20FS exposed males.

Global DNA methylation was measured using LUMA (A; n = 10/group). Loci of paternally methylated gene H19 (B) and maternally methylated genes *Kcnq1ot1* (C), *Peg1* (D), *Snrpn* (E), and *Peg3* (G) methylation levels were quantified by bisulfite pyrosequencing (n = 5/group).

Snrpn and *Peg3* variances were measured as a mean of variances of all seven and six CpGs, respectively (Ctrl = Folic Acid Control Diet, 7FD = 7x Folic Acid Deficient, 10FS = 10x Folic Acid Supplemented, 20FS = 20x Folic Acid Supplemented). For individual CpG variance (panels D, E, G), ø (7FD), * (10FS), # (20FS) = $p<0.05$ by F-test between deficient or supplemented versus control diet groups. For across-locus variance analysis (panels F, H) * = $p<0.05$ by one-way ANOVA with Dunnett's multiple comparisons test.

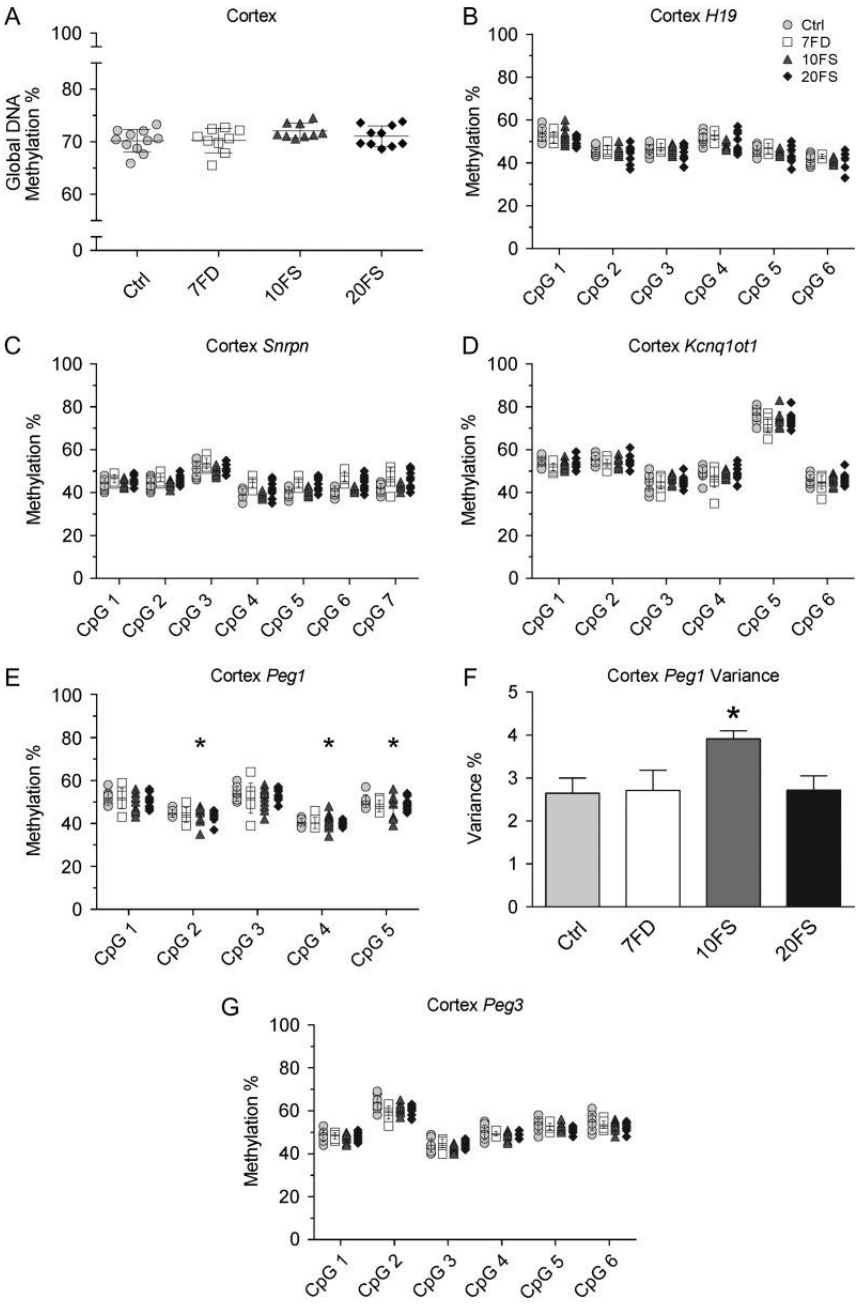


Figure 2.8. F2 E18.5 cortex global DNA methylation and DMR methylation at imprinted genes uncovers variance of Peg1 methylation in F2 pups sired by 10FS exposed males.

Global DNA methylation was measured using LUMA (A, n = 10/group). Loci of paternally methylated gene *H19* (B) and maternally methylated genes *Snrpn* (C), *Kcnq1ot1* (D), *Peg1* (E), and *Peg3* (G) methylation levels were quantified by pyrosequencing (n = 5/group). *Peg1*

variance was measured as a mean of variances of all five CpGs (Ctrl = Folic Acid Control Diet, 7FD = 7x Folic Acid Deficient, 10FS = 10x Folic Acid Supplemented, 20FS = 20x Folic Acid Supplemented). For individual CpG variance (panel E), * (10FS) = $p<0.05$ by F-test between deficient or supplemented versus control diet groups. For across-locus variance analysis (panels F) * = $p<0.05$ by one-way ANOVA with Dunnett's multiple comparisons test.

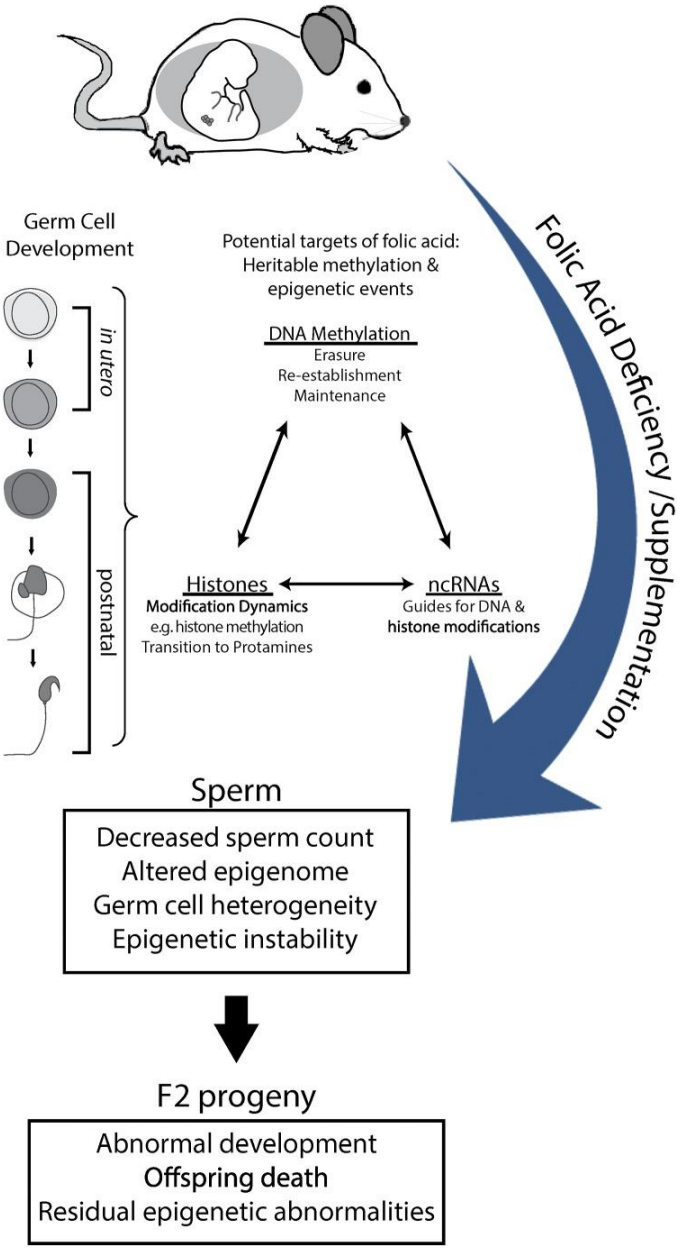
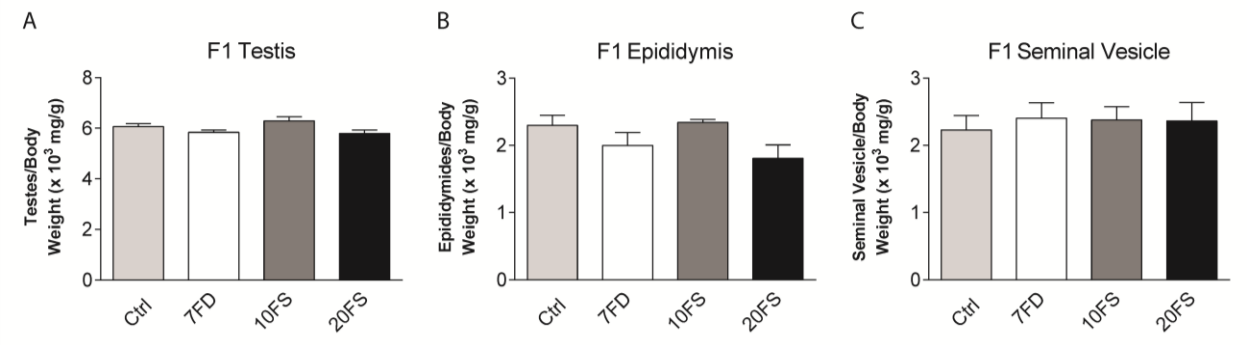


Figure 2.9. Model of postulated epigenetic mechanisms and interactions which underlie the adverse outcomes in offspring of males exposed to folic acid deficient (FD) and highly supplemented (FS) diets starting from fertilization.

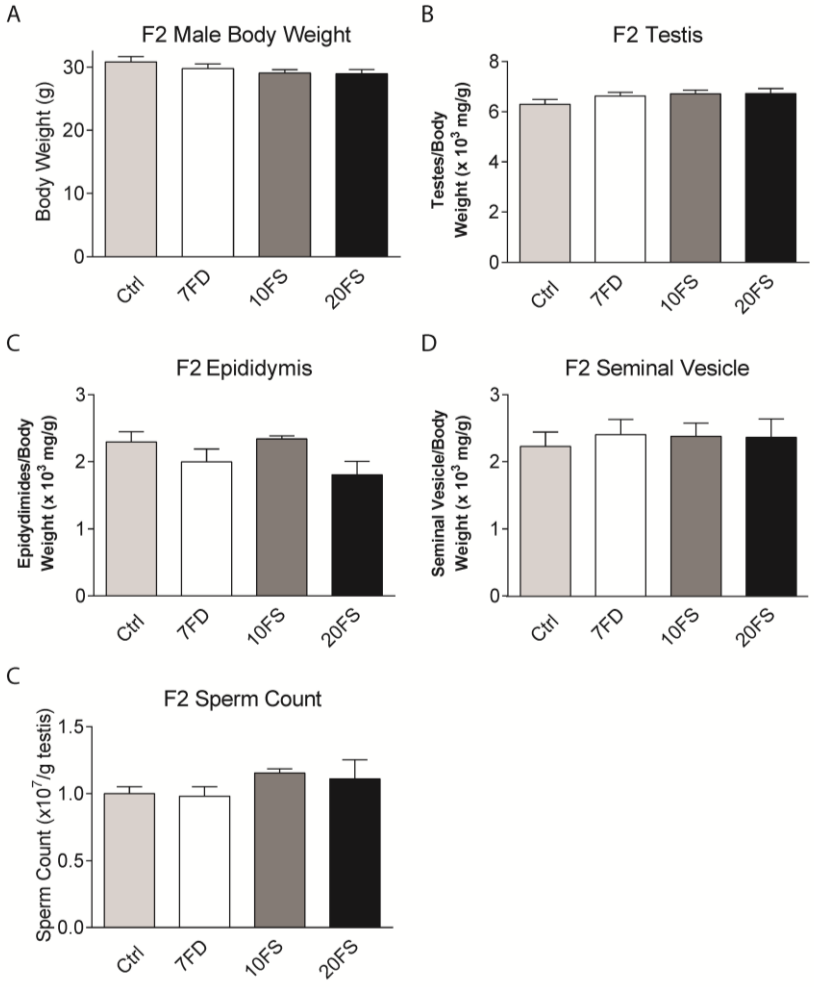
Lifetime exposure to both FD and FS spans two vulnerable windows of epigenetic reprogramming during germ cell development: in utero and postnatal. Herein each window of development, both window-specific (e.g. DNA methylation erasure and histone-protamine

transition) and development-long (e.g. DNA methylation re-establishment/maintenance and histone modification dynamics) events are at risk of being influenced by external exposures of folic acid. Interactions between DNA methylation, histone modifications, and ncRNA expression all have the potential to contribute to the decreased reproductive fitness of sperm observed as decreased sperm count, altered epigenome (DNA methylation variance), germ cell heterogeneity, and epigenetic instability. Ultimately, the cumulative and interacting epigenetic effects of lifetime exposures on the sperm manifests in the F2 progeny as abnormal development, increased offspring death, and residual epigenetic abnormalities.



Supplementary Figure 2.1. Lifetime folate deficiency and high dose supplementation do not affect reproductive organ weights.

F1 adult male paired testes, seminal vesicles, and epididymides weights relative to body weight (A-C; n = 17-20 F2 males, representing n = 9-11 original F1 litters). (FCD = Folic Acid Control Diet, 7FD = 7x Folic Acid Deficient, 10FS = 10x Folic Acid Supplemented, 20FS = 20x Folic Acid Supplemented).



Supplemental Figure 2.2. Paternal (F1) lifetime folate deficiency and supplementation does not affect adult male progeny (F2) sperm counts or reproductive organ weights.

F2 sperm count (A; n = 7 males, representing n = 5 original F1 litters), F2 adult male paired testes, seminal vesicles, and epididymides weights relative to body weight (B-D; n = 16-31 males, representing n = 8-11 original F1 litters). FCD = Folic Acid Control Diet, 7FD = 7x Folic Acid Deficient, 10FS = 10x Folic Acid Supplemented, 20FS = 20x Folic Acid Supplemented).

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Connecting Text – Chapter II to Chapter III

The work in Chapter II was driven by two primary objectives. First, to determine whether either lifetime folate deficiency or high dose folic acid supplementation in males resulted in reproductive effects seen in future sired litters, and second, whether the diets resulted in detectable alterations in DNA methylation of the directly exposed sperm or of the non-directly exposed offspring tissue. The exposure was designed to include the entirety of germ cell development, which encompassed both waves of epigenetic reprogramming. Therefore, the effects of the folate exposures on methyl donor availability would coincide with DNA methylation erasure and *de novo* methylation in both the early embryo, and early germ cell, as well as maintenance methylation during adult spermatogenesis.

The results in Chapter II demonstrated not only that lifetime exposure in males to FD or FS both surprisingly decreased reproductive fitness, but also suggested the heritability of environmental exposure effects in sperm to future offspring. The evidence of this vertical transmission of effects was observed in the form of increased variability of DNA methylation in imprinted genes of both directly exposed sperm and non-directly exposed offspring. These results suggested that an epigenetic mechanism may be underlying the reproductive effects of lifetime male FD and FS. We postulated that the window of development comprising the re-establishment of DNA methylation requiring the availability methyl donors may be most susceptible to environmental insults affecting the methyl donor pool. As such, in Chapter III we designed an experimental mouse model of FD and FS strictly isolated to the prenatal window of female germ cell development.

De novo methylation in female germ cell epigenetic reprogramming occurs during oocyte growth, in the postnatal stages of germ cell maturation. By limiting our exposures to a window

that excluded *de novo* methylation in female germ cell reprogramming, we hypothesized that reproductive and epigenetic disturbances would be limited. We employed a base resolution genome wide technique, reduced representation bisulfite sequencing, to probe for subtle genome-wide DNA methylome effects in the offspring.

Chapter III: Impact of Mothers' Early Life Exposure to Low or High Folate on Progeny Outcome and DNA Methylation Patterns

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Abstract

The dynamic patterning of DNA and histone methylation during oocyte development presents a potentially susceptible time for epigenetic disruption due to early life environmental exposure of future mothers. We investigated whether maternal exposure to folic acid deficient (FD) and supplemented (FS) diets starting *in utero* could affect oocytes and cause adverse developmental and epigenetic effects in next generation progeny. Female BALB/c mice (F0) were placed on one of four amino acid defined diets for 4 weeks before pregnancy and throughout gestation and lactation: folic acid control (rodent recommended daily intake or RDI; Ctrl), 7−fold folic acid deficient (7FD), 10−fold folic acid supplemented (10FS) or 20−fold folic acid supplemented (20FS) diets. F1 female pups were weaned onto Ctrl diets, mated to produce the F2 generation, and the F2 offspring were examined at E18.5 for developmental and epigenetic abnormalities. Resorption rates were increased and litter sizes decreased amongst F2 E18.5 day litters in the 20FS group. Increases in abnormal embryo outcomes were observed in all three FD and FS groups. Subtle genome-wide DNA methylation alterations were found in the placentas and brains of F2 offspring in the 7D, 10FS and 20FS groups; in contrast, global and imprinted gene methylation were not affected. The findings show that early life female environmental exposures to both low and high folate prior to oocyte maturation can compromise oocyte quality, adversely affecting offspring of the next generation, in part by altering DNA methylation patterns.

Key words: folic acid, maternal effects, DNA methylation, histone methylation, developmental programming, DOHaD, epigenetics, intergenerational effects, oocyte

Introduction

Folic acid supplementation for the prevention of neural tube defects (NTDs) has been well established through randomized trials, with a reported 93% reduction in NTDs [1, 2]. Consequently, in many countries, the food supply is currently fortified with folic acid and periconceptional folic acid supplementation ($\geq$0.4 mg/day) is recommended for women of reproductive age [3, 4]. While high doses of folic acid of 4-5 mg/day are often used for women at elevated risk for NTDs, concerns have been raised regarding the safety of high dose folic acid supplements in terms of offspring health [5]. The great success of folic acid fortification measures across North America has led to women exceeding the recommended daily intakes. For instance, in a recent Canadian study, 90.4% of surveyed pregnant women exceeded the upper limit of 1 mg/day, adding to the concern [6]. However, at the same time, it should be noted that a subpopulation (6.7%) of this Canadian cohort had folate intakes below the Estimated Average Requirements. Similarly, in a recent study from the United States, 21.2% of children and adults studied were reported to have inadequate folate levels with increased risks associated with low economic status [7]. Thus, despite folic acid supplementation programs, conditions of both low and high folate occur within the North American population.

The vitamin folate or its synthetic form used in supplements, folic acid, is important for one-carbon metabolism and biological methylation reactions including those involved in the key epigenetic modifications of DNA and histone methylation. Thus, exposures of females to low or high folate have the potential to impact epigenetic patterning in their offspring during development. A number of studies have found evidence of epigenetic perturbations in somatic tissues of offspring exposed to low or high folate during gestation. For example, mouse and rat studies have examined the effects of moderate to high dose supplementation (2.0-20-fold rodent

daily recommended intake) and reported altered DNA methylation patterns in offspring somatic tissues, including the brain [8-12]. In a large human study, increased maternal plasma folate status was linked to decreased DNA methylation levels in cord blood [13]. In contrast, tissues that have received little attention regarding low or high folate exposures during gestation are the germ cells of the embryo and fetus. The epigenome, including both DNA and histone methylation, is reprogrammed in the embryonic gonad and thus is likely to represent one of the most susceptible tissues to the induction of epigenetic defects [14, 15]. Furthermore, altered epigenetic profiles in germ cells have the potential to be transmitted by mature oocytes or sperm and result in intergenerational effects.

A few studies have examined the epigenetic impact of low and or high folate on developing male germ cells. Paternal lifetime folate deficiency, starting in utero and continuing postnatally, in a mouse model, resulted in altered histone and DNA methylation in sperm and led to increased birth defects in the next generation offspring [16]. In a previous study, we showed that a similar lifetime exposure to both folic acid deficient and supplemented diets in males was associated with altered DNA methylation in sperm, and adverse effects in the next generation offspring including increased death in the early postnatal period and altered epigenetic patterning in somatic tissues [12]. Together, these studies indicate that gestational exposures to low or high folate can affect epigenetic patterning in germ cells with intergenerational consequences.

Like sperm, oocytes transmit key epigenetic information from parent to offspring. As in male germ cells, DNA methylation patterns are, for the most part, erased in female primordial germ cells at mid gestation [15]. However, while in males DNA methylation reacquisition begins in the prenatal gonad, in the female, DNA methylation patterns in germ cells are only acquired after birth when oocytes enter the growth phase preceding ovulation [17, 18]. Histone

methylation dynamics during female germ cell development are complex with certain marks, such as histone 3 lysine 36 trimethylation (H3K36me3), preceding and necessary for DNA methylation and evidence of interactions between different types of histone methylation marks [18-23]. Thus, it is likely that the timing during germ cell development for female and male germ cells to be susceptible to the effects of low and high folate will be different.

A few studies suggest that early female germ cells will be susceptible to the induction of epigenetic alterations by differing gestational environments. For the well-studied agouti viable yellow locus, when females of the F0 generation were given methyl donors on days 8.5 to 15.5 of gestation, a shift of coat colour, associated with increased methylation of the intracisternal a particle (IAP) element found at the locus, was seen in F2 offspring of the F1 dams [24, 25] The results indicate that DNA methylation, at least at a single locus, can be influenced by methyl donor supply in early female germ cells. In an additional example, involving impaired folate metabolism (methionine synthase reductase or *Mtrr* mutation) rather than diet, there was evidence of maternal grandparental transmission of epigenetic instability and birth defects across several generations [26].

The rationale for the current study was based on accumulating evidence that women are exposed to too little and too much folic acid during pregnancy and that both types of exposures can impact the epigenome and potentially be passed on to the next generation. Here, our goal was to use a well characterized mouse model to examine the intergenerational impact of both low and high folate on early female germ cells.

Materials and Methods

Mice and diets

All procedures respected the Canadian Council on Animal Care and the study was approved by the McGill University Animal Care Committee. Animals were housed at the

Research Institute of the McGill University Health Centre pathogen-free animal facility under a 12 Light: 12 Dark cycle in a temperature and humidity-controlled environment with ad libitum food and water.

Figure 1 outlines the diet exposures and breeding schemes. Eight-week old (F0) female BALB/c mice (n = 15/group) (Charles River, Canada) were fed one of four amino-acid defined diets (Harlan Teklad, USA) for four weeks prior to mating: folic acid control (Ctrl; 2 mg/kg diet) containing the RDI of folic acid for mice [27], 7-fold folic acid deficient (7FD; 0.3 mg/kg diet), 10-fold folic acid supplemented (10FS; 20 mg/kg diet) or 20-fold folic acid supplemented (20FS; 40 mg/kg diet) diets. *De novo* synthesis of folate by intestinal bacteria was prevented by supplementing diets with 1% succinyl-sulfathiazole. These same diets have been used in other studies of developmental effects of folate deficiency and supplementation [12, 16, 28-30]. After four weeks on the diets, females were mated with sexually mature, ten-week old F0 male BALB/c mice maintained on regular mouse chow diets (Product #2018, Harlan Teklad, USA). Throughout mating, gestation and lactation, the F0 females remained on their respective diets until the F1 female offspring were weaned at postnatal day (PND) 20 onto Ctrl diets.

At 8-10 weeks of age, 54 F1 females, representative of 10 Ctrl, 11 FD, 10 10FS, and 9 20FS different original F0 litters per diet group, were mated with 10-week-old male BALB/c mice fed regular mouse chow. Throughout mating and gestation, the F1 females were fed the folic acid control diet (Ctrl; 2mg/kg diet) (Harlan Teklad, USA). The presence of a vaginal plug on the morning after mating was designated as 0.5 days post coitum (dpc). At E18.5, ovaries from the F1 females were collected to determine the number of ovulation sites by counting corpora lutea (CL). Uterine horns were removed and opened to assess implantation by counting viable embryos and resorption sites. Preimplantation loss was calculated for each female as the

difference between the number of CLs and implantation sites. The difference between the numbers of implantation and resorption sites was used as a measure of post-implantation loss. Placentas, viable embryos and resorption sites were removed and weighed. Embryos were sexed by anogenital distance (and later verified by sexing PCR to ensure accuracy as described in [31], their crown-rump length measured, and evaluation of gross morphological abnormalities performed. Referring to "The Atlas of Mouse Development" [32], embryos and late resorptions were examined for developmental delay and malformations such as cleft palate, closed eyelids, pointy nose, thick neck, curved tail, back and limb malformations. Embryos that deviated by two standard deviations from the means of the mean embryo weights, calculated per litter, across the Ctrl, were considered either growth restricted (two standard deviations below mean of means) or growth enhanced (two standard deviations above mean of means).

Tissue collection

Tissue collection was done as previously described [12]. Briefly, following sacrifice of F1 female mice at 18.5 dpc, placenta and brain frontal cortices (anterior 1mm of whole brains) from embryos were isolated, flash frozen, and kept at -80^{o}C until use.

DNA methylation analyses

Analysis of imprinted gene DNA methylation was performed as previously described [12]. Frozen tissues were homogenized using mortar and pestle. DNA was isolated from ~10mg of samples using the DNeasy Blood and Tissue kit (Qiagen, Germany). Bisulfite treatment of genomic DNA was performed using the EpiTect Bisulfite kit (Qiagen, Germany). Imprinted germline differentially methylated domains (gDMDs) were amplified using primers specific to

pyrosequencing applications and sequenced using the PyroMark Gold Q24 Reagents (Qiagen) and the PyroMark Q24 Vacuum Workstation (Qiagen). Previously established primers used for assessment of *H19 [31], Snrpn [31], Kcnq1ot1* [33], *Peg1* [34], and *Peg3* [33] are listed in Supplementary Material, Supplementary Table S1.

Global DNA methylation was assessed using the Luminometric Methylation Assay (LUMA) as previously described [12]. Briefly, duplicate digestion was carried out for both restriction enzymes HpaII and MspI (Thermo Fisher Scientific, USA). Samples were analyzed on a Pyrosequencer Q24. Percentage methylation was calculated using the normalized peak ratios of HpaII over MspI as in the following formula: % Methylation = 100 [1-(HapII / EcoRI / MspI / EcoRI)].

Reduced representation bisulfite sequencing (RRBS) libraries were generated using previously used and published protocols and the gel-free technique [35-37]. RRBS was carried out on n=5-6 samples/diet/tissue (placenta and brain cortex). DNA samples of 500 ng were digested using the methylation-sensitive restriction enzyme, MspI, followed by end repair and A-tailing. Fragment size filtration was performed with AMPure XP beads (Beckman Coulter, Brea, CA, USA). After ligation to methylated adapters (Illumina), DNA samples were bisulfite converted twice followed by a subsequent AMPure bead clean-up. RRBS libraries were prepared by large scale PCR. Placenta and brain cortex DNA libraries were then multiplexed for paired-end sequencing with twelve samples per lane of a HiSeq 2000 sequencer (Illumina) followed by initial data processing and alignment of reads by the software pipeline bsmap version 2.6 [38]. MethylKit software (version 0.5.3) was used for identification of differentially methylated tiles (DMTs) after folate deficiency or folic acid supplementation. This software implements the Benjamini-Hochberg false discovery (FDR)-based method for P-value correction and only

DMTs passing the q-value threshold (q = 0.01) were considered [39]. Analysis was based on 100-bp step-wise tiling windows, containing ≥2 CpGs per tile and ≥10 × CpG coverage of each tile per sample. The methylation level of a 100-bp tile was the average of all CpGs within the tile. If significant changes of DNA methylation after folate deficiency or folic acid supplementation exceeded 10%, the tile was designated as a DMT; further annotation of DMTs was performed by the HOMER software version 3.51. Summary statistics for RRBS are shown in Supplementary Table S2. DAVID Bioinformatic Functional Annotation Tool (v6.7) was used for gene ontology analysis.

As done previously, validation of RRBS results was carried by pyrosequencing on a subset of DMTs in placental samples [31, 37]. Details of the individual pyrosequencing assays are shown in Supplementary Table S3.

We also determined whether any DMTs were found in regions that would normally inherit DNA methylation marks from the maternal allele but not the paternal allele. To do this, we used publicly available methylation data (GSE34864) from mouse sperm [36], germinal vesicle oocytes (GVO- DRA00570) [40] and from the maternal allele from the inner cell mass (ICM) (GSE56697) [41]. Methylation data were obtained from each germ cell type/developmental stage and processed to obtain DNA methylation across 100-bp regions, similar to RRBS. In order to target regions where DNA methylation was specifically inherited from the maternal allele, we included only regions where sperm methylation was low (≤10%) and GVO/ICM methylation was greater than or equal 25%; these regions were then intersected with our DMTs using the intersectBed function of bedtools (version 2.26.0).

Statistical analysis

Results are expressed as the mean ± SEM, unless stated otherwise. Data were graphed and analyzed with Prism 5 (GraphPad Software Incorporated, USA). Comparisons and statistical calculations were made by Fisher's exact test, ANOVA followed by the Dunnett's multiple comparison test compared to control, or 1-way ANOVA. A level of significance for the analyses was set at $p<0.05$.

Results

F1 Female Body Weight was not Affected by Gestational and Early Postnatal Folic Acid Deficient and Supplemented Diet Exposures

First, we evaluated the impact of the diets on outcomes in the F1 progeny and on the F1 female body weight as a general measure of health. The impact of the diets on F1 litter sizes has been reported previously; there were no significant differences observed in litter sizes or sex ratios between the diet and control groups [12]. Adult body weight did not differ between females exposed to control diets as compared to females exposed to the different folic acid deficient and supplemented diets administered prenatally from conception to birth until weaning (Supplementary Fig. S1; mean 28.7 g, 28.4 g, 28.0 g, and 26.5 g in Ctrl, 7FD, 10FS, and 20FS respectively). Thus, as previously shown for F1 males [12], there was no apparent major effect of the diets on the health of the F1 females.

Pre-weaning Folate Deficiency or Folic Acid Supplementation During F1 Mothers' Early Life Resulted in Decreased Offspring (F2) Litter Sizes and Increased Abnormal Outcomes

Next, we assessed whether the oocytes of the F1 mothers had been impacted by the folate diets by examining effects on offspring of the F2 generation. F2 litters produced from F1 mothers from 20FS exposed groups displayed decreased litter sizes at E18.5 (Fig. 2A). F2 preimplantation loss, and embryo and placenta weights were not affected by the mothers' diet

(Fig. 2B, C, D). However, increased resorptions amongst F2 litters at E18.5 from 20FS exposed F1 females were observed (Fig. 2E). Resorptions, growth enhancement, or growth restriction were designated as abnormal outcomes. A trend was noted for a higher proportion of embryonic abnormalities among litters coming from F1 mothers exposed to the folic acid deficient and supplemented diets as compared to mothers on the control diet (Fig. 2F). Whereas 4 of 10 control group litters had more than one abnormal outcome per litter, there were 24 out of 34 litters amongst the 7FD, 10FS, and 20FS groups with more than one abnormal outcome per litter ($p=0.08$, Fisher's exact test). Together the results suggest that the diets, in particular the 20FS, adversely affected F2 pregnancy outcomes. However, placenta weights were unaffected and those offspring that survived had normal weights at E18.5.

Global and Imprinted Gene DNA Methylation in Offspring Was Not Affected by F1 Mothers' Early Life Exposure to Folate Deficiency or Folic Acid Supplementation

To determine if the decreased litter sizes and increased abnormal outcomes in F2 litters at E18.5 could be associated with epigenetic disturbances in the F1 oocytes that were inherited by the offspring, global and imprinted gene DNA methylation were determined in F2 E18.5 placentas (Fig. 3) and embryonic brain cortex (Fig. 4). E18.5 F2 embryos were chosen to represent offspring from F1 mothers of 10 different original F0 females per group. No evidence of sex differences was found in global methylation by LUMA ($n = 5–6$/group/sex) or in imprinted gene methylation ($n = 9–11$/sex/group) in either placental or cortex tissues (Supplementary Figs. S3 and S4, respectively). Therefore, both males and females were combined for downstream analysis.

Mean global methylation in placenta and brain cortex ranged from ~55-73%, with no significant differences observed between the groups (Fig. 3A, Fig. 4A). An assessment was performed at a gene-specific level in placenta and brain cortex for the well characterized gDMDs of the paternally methylated imprinted gene *H19* and the maternally methylated imprinted genes *Snrpn*, *Kcnq1ot1*, *Peg1* and *Peg3*. Methylation levels averaging ~50% for all imprinted genes were observed, as expected for a somatic or non-germ cell tissue (Fig. 3B-F & Fig. 4B-F). No evidence of sex differences was found. Overall, no differences were observed for global and mean imprinted gene DNA methylation of placenta or cortex tissues.

F1 Mothers' Early Life Folic Acid Deficient and Supplemented Diets are Associated with Subtle Genome-wide DNA Methylation Alterations in the Placenta and Brain Cortex of F2 offspring

To assess whether more subtle changes in genome-wide DNA methylation may have occurred in the F2 offspring, RRBS was performed on the placenta and brain cortex tissues at E18.5. RRBS was carried out on matched placenta and cortex sample of males (n = 5–6/tissue/group) as no evidence of sex differences had been found for the methylation of imprinted genes. Analysis of ~1.5 million CpGs was performed in 100-bp tiles to identify regions with differential methylation between the low or high folate and control groups (i.e.100-bp tiles exhibiting gain or loss of 10% methylation from the FD and FS exposures: DMTs). Heatmaps of the RRBS results are shown in Supplementary Fig. S5. For all samples, as well the individual groups, the cortex and placenta samples clearly clustered within the individual tissue type. For the data as a whole, within placenta or cortex, there was no evidence of clustering between groups suggesting that overall genome-wide methylation differences between the groups were small, requiring a more detailed analysis of individual tiles using MethylKit.

Using MethylKit, the F1 mothers' early life exposures to the 7FD, 10FS and 20FS diets resulted in 907, 1163 and 1116 DMTs, respectively, in placentas of F2 offspring (Fig. 5). Most alterations were of low magnitude of 10–15% (Fig. 5A). However, some DMTs displayed higher magnitude (15–20%) changes in methylation (184/907, 191/1163 and 202/1116 in 7FD, 10FS and 20FS exposure groups, respectively), while considerably less DMTs displayed a greater than 20% change (56/907, 56/1163 and 96/1116 in 7FD, 10FS and 20FS exposure groups, respectively). Regardless of the diet group, the majority of the DMTs showed a gain of methylation, with a range of 76–79% hypermethylated DMTs (Fig. 5A and Supplementary Table S4). We observed a significant enrichment of intergenic regions in DMTs following both FD and FS perinatal maternal exposures compared to all tiles sequenced (average of 47% with range of 37–56% for diet-associated intergenic DMTs vs 25%, all tiles sequenced that were intergenic; Fig. 5B and C–I and Supplementary Table S5).

RRBS was also performed on placenta-matched frontal cortex of E18.5 F2 embryos and DNA methylation of FD and FS groups was compared to Ctrl (Fig. 6). Perinatal maternal exposures to 7FD, 10FS and 20FS diets resulted in 421, 481, and 926 DMTs, respectively, in F2 offspring frontal cortices, with the magnitude of change mostly in the 10-15% range (Fig 6A). Fewer DMTs displayed a higher magnitude (15-20%) change in methylation (75/421, 107/481, and 218/926 in 7FD, 10FS and 20FS exposure groups, respectively), while even less DMTs displayed changes of 20% or greater (35/421, 51/481, and 64/926 in 7FD, 10FS and 20FS exposure groups respectively). Similar to the placenta, both FD and 10FS exposures resulted in a higher prevalence for gain of methylation with 60-77% of DMTs hypermethylated for the cortex (Fig. 6A and Supplementary Table S4). In contrast, 20FS exposures in the F1 mothers' early life resulted in a greater number of hypomethylated versus hypermethylated DMTs (68% vs 32%,

respectively). Again, for all groups, DMTs were significantly enriched in intergenic regions (average of 44% for diet- associated intergenic DMTs vs 27% all tiles sequenced that were intergenic; Fig. 6B-I and Supplementary Table S6)

DNA methylation for a subset of affected loci (hypomethylated and hypermethylated sites) identified by RRBS for the placenta was examined using a second assay, bisulfite pyrosequencing, in all cases validating the RRBS results (Supplementary Table S7).

Genes and Pathways Associated with DNA Methylation Alterations in F2 Offspring

DNA methylation alterations associated with nearby genes (i.e. genic DMTs) were assessed to determine potential functional impacts on the placenta and cortex. In F2 placentas, all three diets resulted in genic region associated DMTs with statistically significant enrichment of various pathways. Notably pathways implicated in transcription, multicellular organism development and nervous system development were commonly affected in all three exposures (Table 1). Two pathways consistently had the greatest number of differentially methylated genes with 26 genes implicated in multicellular organism development and 25 genes involved in transcription and common to all three diet exposures (Supplementary Table S8).

In the cortex, when only genic DMTs were considered, the top five pathways are listed in Table 2. Only one significantly enriched pathway was identified after correction (substrate adhesion-dependent cell spreading -8 genes) for the 7FD group.

We intersected the DMT lists from the placenta and the cortex tissues to identify regions that had DNA methylation simultaneously altered in both tissue types. Of the DMTs found within genic regions 46, 55 and 71 were conserved across both placenta and cortex tissue types within the 7FD, 10FS and 20FS groups, respectively (Supplementary Table S9). Of the tissue-

conserved DMTs in the 7FD group, 45 of 46 showed the same directionality of methylation change in both tissues; similar directionality was seen for 49 of 55 loci for the 10FS exposure and 51 of 71 loci for the 20FS group. When considering tissue-conserved DMTs with the following two criteria: same directionality of effect and at least one of the two tissues having greater than a 20% change in methylation, there were 11, 9 and 15 DMTs meeting these criteria in the 7D, 10FS and 20FS groups, respectively. A number of genes were affected in more than one diet group: *Map1lc3b* (hypomethylated), *Arhgef26* (hypermethylated) and *Ckb* (hypermethylated) in the three groups, *Olfr136* (hypermethylated) and *Mir153* (hypermethylated) in the 7FD and 20FS groups and *Rab11fip3* in the 10FS and 20FS groups.

DMTs coincide with loci retaining methylation on the maternal allele during post-fertilization development

Next we wanted to assess whether the folate diets had affected oocyte loci of potential significance for the health of the offspring of the next generation. A number of loci, including the maternally methylated differentially methylated regions (DMRs) of imprinted genes show high levels of methylation in oocytes and this methylation is maintained during the demethylation wave that occurs during pre-implantation development. For such maternally methylated loci, that also have low methylation on the paternal allele, offspring thus inherit methylation from their mothers and it may have functional significance in the progeny. For instance, in the case of imprinted genes, failure to inherit the normal maternal methylation patterns impacts growth and development of the offspring. Here we determined whether any DMTs were found at loci that normally inherit methylation from the maternal allele. Previously published datasets that characterized genome-wide methylation states in GVO and the ICM were analysed in order to

identify DMTs in loci for which methylation patterns have been shown to be inherited from the oocyte, resisting demethylation in pre-implantation embryos [40, 42, 43]. For the ICM we restricted our analysis to the maternal allele (ICMm). We cross-referenced our DMTs to regions associated with low methylation in sperm (≤10%) but moderate to high methylation in GVO and ICMm (≥25%) for placenta and cortex (Table 3 and 4, respectively). For placenta, there were 50, 67 and 55 DMTs, and for cortex there were 19, 22 and 37 DMTs, representing the 7FD, 10FS and 20FS groups, respectively, localized to regions methylated in GVO and ICMm (Supplementary Table S10). For each tissue the specific DMTs identified are listed along with the associated genes, the degree of alteration in DNA methylation and the level of methylation of the loci taken from the published GVO and ICMm data (Supplementary Table S11). Notably, the majority of DMTs in each group and tissue were characterized as having levels of methylation ≥75% in GVO.

Among the placenta DMTs identified, the majority were hypermethylated (80%, 94% and 80%) and were predominantly in genic regions (77–100%; Supplementary Tables S10A and S11). For the cortex, while most identified DMTs in the 7FD and 10FS groups were hypermethylated (84% and 77%, respectively), the majority of DMTs in the 20FS diet group were hypomethylated (65%). Cortex DMTs were also predominantly in genic regions (66.7–87.55%; Supplementary Table S10B). Most of the groups for each tissue had one DMT localized to an imprinted (*Wars* within the *Dlk1-Gtl2* region, *Snurf*, *Peg13*, *Plagl*) or imprinted-like region (*L3mbt11*) [35]. The DMTs identified for both tissues were enriched in pathways implicated in transcription, multicellular organism development and nervous system development. However, no statistical significance of pathway enrichment remained upon correction (Supplementary Table S12).

Discussion

The effects of maternal folate deficient and folic acid supplemented diets during gestation and their direct consequences on offspring health have been well studied [44, 45]. Less is known about the impact of gestational exposures to low and high folate on the epigenome of developing germ cells, in particular those of females, and whether subsequent generations are affected. Using a mouse model, we demonstrated that an exposure to either folate deficiency or folic acid supplementation in females, including their developing germ cells, during early life can have adverse effects on their future reproductive health and progeny. Both reproductive loss and alterations in DNA methylation were found in offspring as a result of these exposures. Both the timing and doses were relevant to clinical folic acid use in high risk pregnancies. By weaning the F1 females onto control diets, our study design restricted the diet exposures of the F1 mothers to the *in utero* stages of female germ cell development along with the pre-weaning phase of perinatal life. Effects observed in the F2 offspring would thus be due to disruptions in the F1 oocytes resulting from exposures to low or high folate prior to the primordial follicle stage.

The effects of diet exposures on red blood cell (RBC) and plasma folate concentrations, as well as the reproductive outcomes of F0 females in this study, have been previously reported [12]. Briefly, when compared to the control diets, F0 dams that consumed a FD diet had ~4× lower plasma and RBC folate concentrations, whereas mice consuming the supplemented diets had ~2× higher plasma and RBC folate concentrations. In the early lifetime diet exposed F1 females, no differences in mean adult body weights were observed. Although the F1 females were exposed to their mothers' low and high folate levels prenatally and up until weaning, as adults they were of normal weight and apparent health when they initiated their own pregnancies to produce the F2 generation.

In our previous study, we explored effects of male germ cell exposure to the same diets used here on offspring reproductive and epigenetic outcomes [12]. Due to its role in one-carbon metabolism we expected the low and high folate diets to perturb methyl donors needed for the dramatic reprogramming of DNA methylation that takes place in the germline. Before being mated to produce the F2 generation, F1 males were exposed to the diets during prenatal development, when DNA methylation in germ cells is erased across much of the genome, followed by reacquisition at most sites just before birth, as well as postnatally when DNA methylation patterns continue to be remodeled during spermatogenesis. In the male study, the F2 generation exhibited decreased litter sizes, fetal abnormalities and increased postnatal death that were highest in the 20FS group as compared to the 7FD and 10FS groups; all three groups showed evidence of epigenetic perturbations in imprinted genes. As similar reproductive and epigenetic effects were seen in the three diet groups, we proposed that the 10FS and 20FS diets resulted in the equivalent of a folate deficient state due to down regulation of folate metabolism pathway enzymes. In support of this, we and others have shown that high doses of folic acid in the 10FS to 20FS range can downregulate enzymes such as MTHFR in the one-carbon metabolism pathway, paradoxically leading to a decrease in the availability of SAM for cellular methylation reactions [30, 46].

In this study, female germ cells in the F1 mothers were only exposed to the folate diets during the DNA methylation erasure but not the remethylation phase in female germ cells. We thus postulated that effects on reproductive and epigenetic outcomes in the F2 might be less severe than those seen in the male study. Contrary to our expectations, an increase in resorptions and decrease in litter size were found in the 20FS group. Post-implantation loss was higher than control in the 7FD group but did not reach significance. As a caveat, we cannot rule out the

possibility that uterine defects in the F1 females may have contributed to the resorptions; however, we consider this interpretation unlikely due to the apparent normal general health of the F1 females. A trend to increased abnormal outcomes (growth restriction, growth enhancement and resorptions) was observed across all three diet exposure groups. These results gave us the first indication that the folate diets had adversely affected the oocytes of the F1 mothers.

To better understand the mechanisms underlying the reproductive effects of the folate diets, as a measure of potentially heritable epigenetic effects of the exposures, we next examined DNA methylation patterns in the offspring. Two tissues in the F2 offspring were chosen for study. The placenta was chosen as it often shows higher levels of DNA methylation defects associated with perturbation of peri-conceptional epigenetic reprogramming events than do somatic tissues in the fetus [47, 48]. The brain cortex was chosen as a representative embryonic somatic tissue to examine. Assessment of global DNA methylation by LUMA showed no difference in the mean levels of methylation in placenta or cortex among the diet groups. The LUMA results indicated that large-scale changes in DNA methylation were not present in viable fetuses as a result of maternal folate exposures, a similar finding to that of the male study with the same diets.

The imprint control regions (ICRs) or gDMDs of imprinted genes are reprogrammed in primordial germ cells in order to take on maternal- or paternal-specific methylation patterns that dictate allele-specific expression in offspring. Thus, we examined the methylation of the well characterized gDMDs of several maternally and paternally methylated imprinted genes and did not reveal any perturbations in the placenta or cortex of male or female offspring. These results contrast with those of the male folate diet experiments where F2 offspring showed variation in

methylation of the same gDMDs of imprinted genes examined in this study, suggesting the induction of epigenetic instability at these loci in developing male germ cells. We suggest that imprinted genes may have been more affected in the male experiment as germ cells were exposed to the low and high folate diets during both DNA demethylation and remethylation. In contrast, in the female experiment described in this paper, germ cells were only exposed during the DNA demethylation phase of germ cell development.

Uncovering subtle effects at developmentally important sequences that might help explain the abnormal pregnancy outcomes requires higher resolution genome-wide approaches. Therefore, we performed RRBS. Large numbers of DMRs were found in all three diet groups for both tissues, with roughly twice as many regions affected in placenta as in brain cortex. When compared to Ctrl diets, with the exception of the 20FS cortex group, F2 placenta and cortex for all the other diet groups had more hypermethylated DMTs (60–79%) than hypomethylated DMTs (21–40%). The presence of predominantly hypermethylated DMTs in the offspring, despite de novo DNA methylation not occurring in the oocyte during the exposure window is intriguing. The fact that the folate deficient and supplemented diets showed remarkably similar effects, resulting in both hyper- and hypomethylation, supports the suggestion of a common underlying biochemical/molecular explanation.

The question thus arises as to how the folate diets resulted in DNA methylation defects in the F2 offspring when the diet exposures preceded the DNA remethylation phase in F1 oocytes. Recent high-resolution studies have indicated that histone modifications and transcriptional activity control the unique DNA methylation landscape of the oocyte genome (reviewed in [49]). DNA methylation in oocytes occurs for the most part in actively transcribed regions including gene bodies; in contrast, intergenic, transcriptionally inactive regions have low levels of DNA

methylation. Dynamic changes in histone modifications precede DNA methylation in oocytes [22]. H3K36me3 marks regions of DNA methylation whereas sites of H3K4me3 are found in regions where DNA methylation levels are absent or low [23, 43, 50]. Thus, it is possible that altered methyl donor availability from the folate diet exposures could disrupt normal H3K4me3 and H3K36me3 dynamics and subsequently DNA methylation patterns. For instance, in the oocyte-specific SETD2 knockout, H3K36me3 depletion in oocytes results in a decrease in gene-body and imprinted gene ICR DNA methylation and the appearance of DNA methylation in regions of the genome that are normally unmethylated [23].

Interestingly, in contrast to all other comparisons, the F2 20FS brain cortex samples showed predominantly hypomethylated DMTs rather than hypermethylated DMTs. For the cortex, the 20FS group also had the highest number of DMTs compared to the 7FD and 10FS groups. Reproductive outcomes were also most marked in the 20FS group. Notably, the 20FS cortex DMTs were found in genic regions, in introns and exons. As transcription is important in setting DNA methylation patterns in female germ cells, it is possible that altered transcription in oocytes exposed to the 20FS dose could contribute to the hypomethylated genic DMTs. However, if altered transcription was occurring, it is unclear why the 20FS placenta samples did not show predominantly hypomethylated DMTs as well. Compared to other tissues, brain has particularly high levels of 5-hydroxymethylcytosine (5-hmC) [51]. As RRBS cannot distinguish between 5-methylcytosine (5-mC) and 5-hmC, an alternate possibility is that altered 5-hmC may also help explain the predominance of hypomethylated DMTs in the 20FS group cortex.

A full understanding of the molecular mechanisms underlying the effects of the folate diets on developing oocytes will require high-resolution studies of histone modifications and DNA methylation in isolated oocytes from the fetal and neonatal ovaries of the F1 females. In

the meantime, more detailed examination of the DNA methylation perturbations in the placenta and cortex can shed light on the types of sequences affected and the DNA methylation defects that persist in association with folate diet effects on developing female germ cells. With our gene ontology analysis of the DMT lists, several interesting pathways and genes emerged. In the placenta, an enrichment in genes related to both multicellular organism development and transcription was found, regardless of exposure group. Disturbances in either pathway, but especially the multicellular organism development pathway, could explain or contribute to the increased resorptions and other developmental abnormalities. For instance, *Alx3* expression has been reported to prevent malformations and deficiencies are associated with NTDs and craniofacial abnormalities [52, 53]. Concurrently, another consistently affected gene *Angpt2*, has been linked to pre-eclampsia, general placental function and fetal health [54, 55]. Finally, transcription factors *Tbx1* and *Hoxa10* have both been shown to have wide reproductive and developmental roles, whilst having epigenetic functions and being under epigenetic regulation, respectively [56, 57].

Interestingly a minority of DMTs (7–11%) in the cortex corresponded to DMTs found in the placenta, suggesting that there are tissue-specific responses and susceptibilities to environmental exposures in the pre-conceptus oocyte. Additionally, no consistent biological pathways were enriched in the cortex DMTs. Genic DMTs conserved in both placenta and cortex frequently affected 1st introns, which have been shown to be highly conserved and play roles in expression regulation [58]. Although the effects of these epigenetic changes in tissues of the offspring and their developmental outcome are difficult to infer, transcriptional dysregulation remains a viable hypothesis. The majority of DMTs occurred in intergenic regions, areas known

to house regulatory elements. Indeed, placental DMTs were found to have a ~3.3- to 3.6-fold enrichment of placenta-specific regulatory elements in the 10FS and 20FS exposures.

Examination of loci known to inherit their DNA methylation patterns from the oocyte, but not the sperm allowed us to further probe the potential functional impact of the folate diets on DNA methylation in specific genomic regions. Parent-of-origin inherited methylation has important roles in embryonic growth and development; such marks have classically been shown to be critical in the case of imprinted genes. In our analysis, many interesting genes were identified and, although no pathways were significantly affected following correction, similar pathways to those emerging from the overall RRBS data analysis were identified. The finding that genic regions were predominantly affected by the diets suggests that regions where DNA methylation normally takes place in oocytes, targeted by H3K36me3 marks, may be particularly susceptible to the low and high folate exposure.

Together, our results indicate that low and high dietary folate exposure of oocytes in the time preceding reacquisition of DNA methylation can impact oocyte health, leading to altered reproductive and epigenetic outcomes. The mechanisms underlying the adverse reproductive outcomes will require further study by examining offspring at earlier stages of development and performing DNA methylation along with gene expression studies. The identification of DNA methylation defects in neurodevelopmental pathways suggests a potential target for study of offspring during postnatal development. This study tested the effects of low and high folate diets on early oocytes, an exposure relevant to the clinical use of folic acid supplements during pregnancy. In future studies, as women take folic acid supplements prior to becoming pregnant, it will be interesting to study the offspring and epigenetic impacts of exposure to the same folate diets during the window of DNA methylation acquisition in growing oocytes.

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Data availability: All RRBS sequencing data have been submitted to Gene Expression Omnibus (GEO) Series and are available under the accession number GSE150629.

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Author Contributions: LL and JT conceived the study. LL and JT designed the study. LL, ML, CA and JM performed the experiments and acquired the data. LL, DC, ML, CA, JM analysed the data. LL, DC and JT wrote the manuscript. All authors edited and approved the manuscript.

Conflict of interest statement: None declared.

Figures and Tables

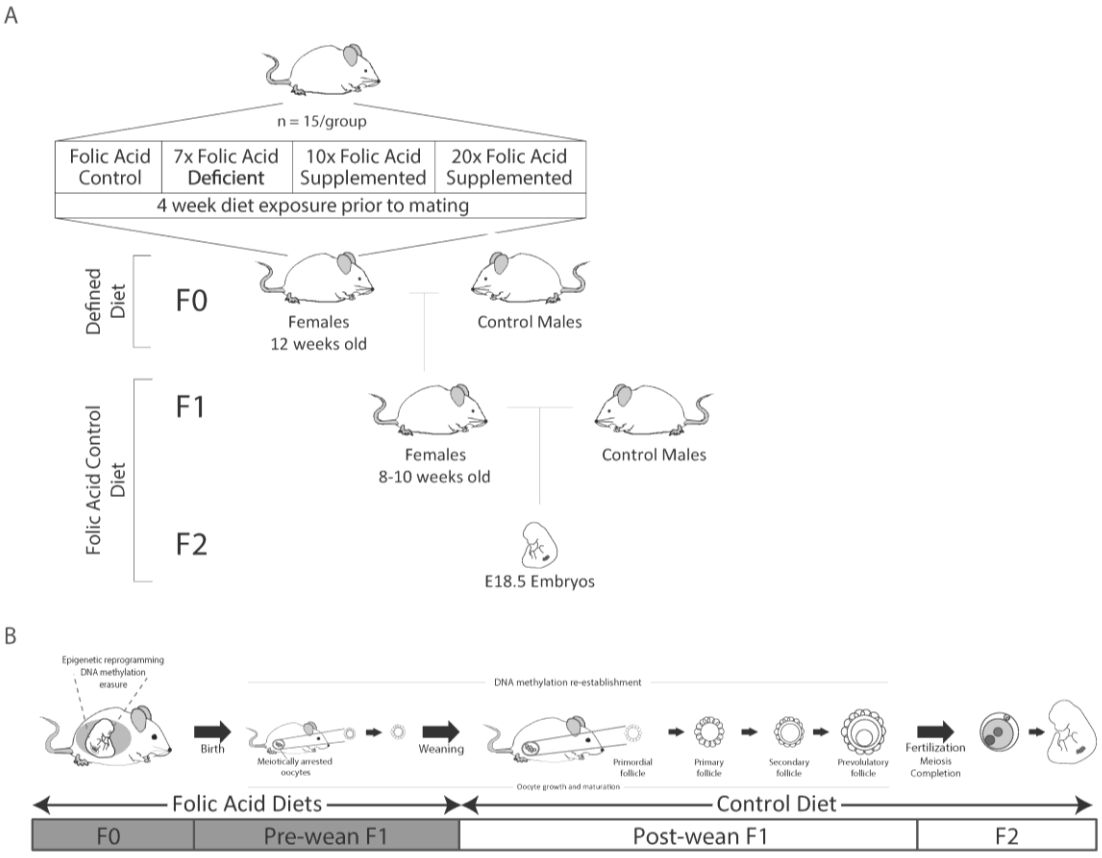


Figure 3.1. Study design for effects of prenatal female exposure to folate deficiency and folic acid supplementation

A) Scheme outlining times of exposure to folic acid diets at each generation. BALB/c F0 ♀ (n = 15) were exposed to one of four defined diets: folic acid control diet (Ctrl; 2 mg/kg folic acid), 7X folic acid deficient diet (7FD; 0.3 mg/kg), 10X folic acid supplemented diet (10FS; 20 mg/kg), or 20X folic acid supplemented diet (20FS; 40 mg/kg). F1 females were weaned onto their Ctrl diets. B) Outline of folic acid exposures and coinciding epigenetic events in female germ cell development.

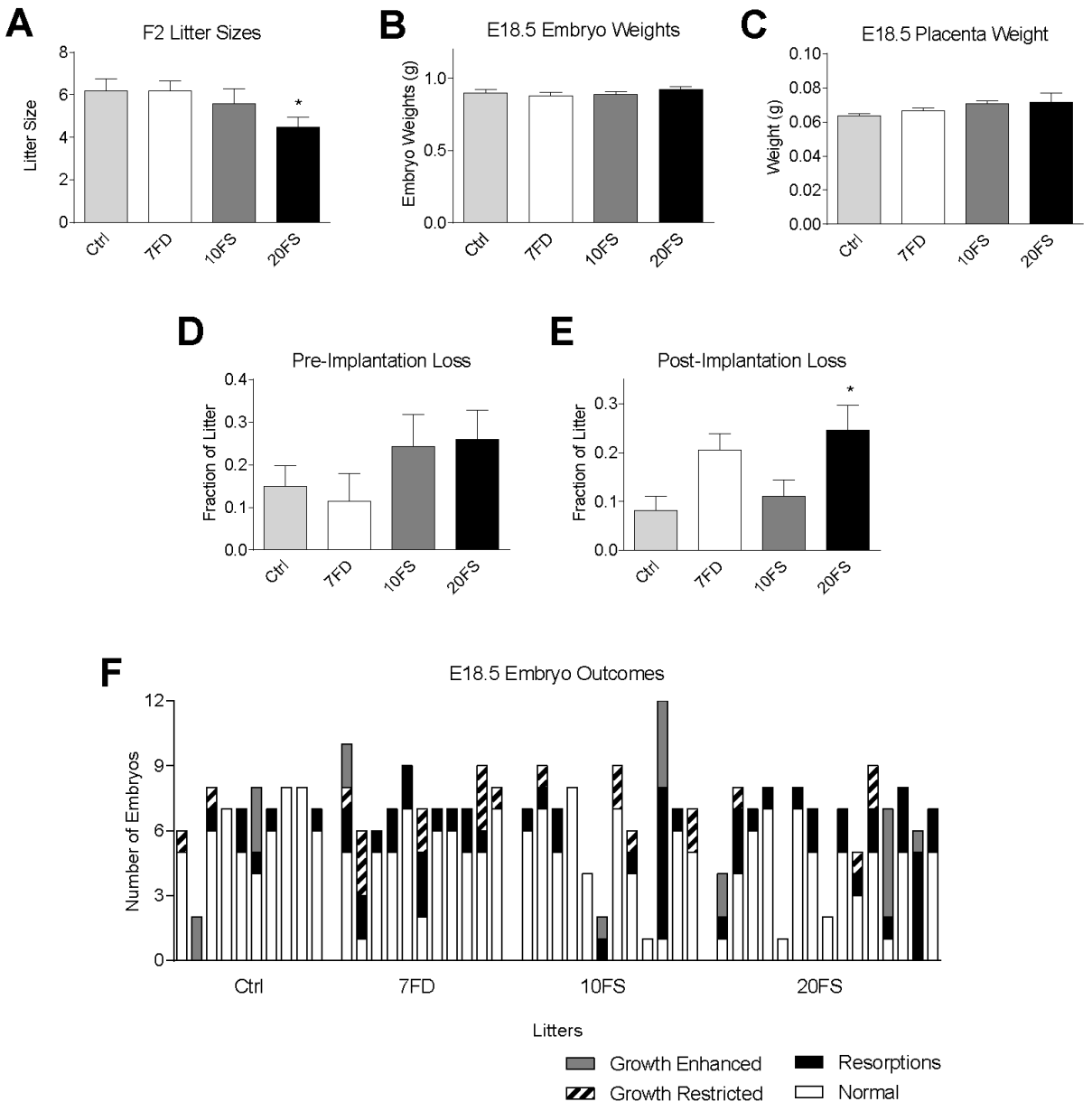


Figure 3.2. Effects of mothers' preweaning exposure to folate deficiency and folic acid supplementation on future reproductive outcomes in F2 at E18.5.

A) F2 litter sizes at E18.5 (n = 10-15 F2 litters). B) F2 embryo weights at E18.5 and C) F2 placental weights at embryonic day 18.5 (n = 49-59 embryos). D) Pre-implantation loss of F2 at E18.5 (n = 10-15 F2 litters), E) Post-implantation loss of F2 at E18.5. F) Incidence of fetal abnormalities at embryonic day 18.5 per litter (n = 10-15 litters); growth restriction and enhancement are defined as a 2-fold standard deviation difference of embryo weight to the group

mean of litter mean weights.(Ctrl = Folic Acid Control Diet, 7FD = 7x Folic Acid Deficient, 10FS = 10x Folic Acid Supplemented, 20FS = 20x Folic Acid Supplemented). * = $p<0.05$ by one-way ANOVA with Dunnett's multiple comparisons test.

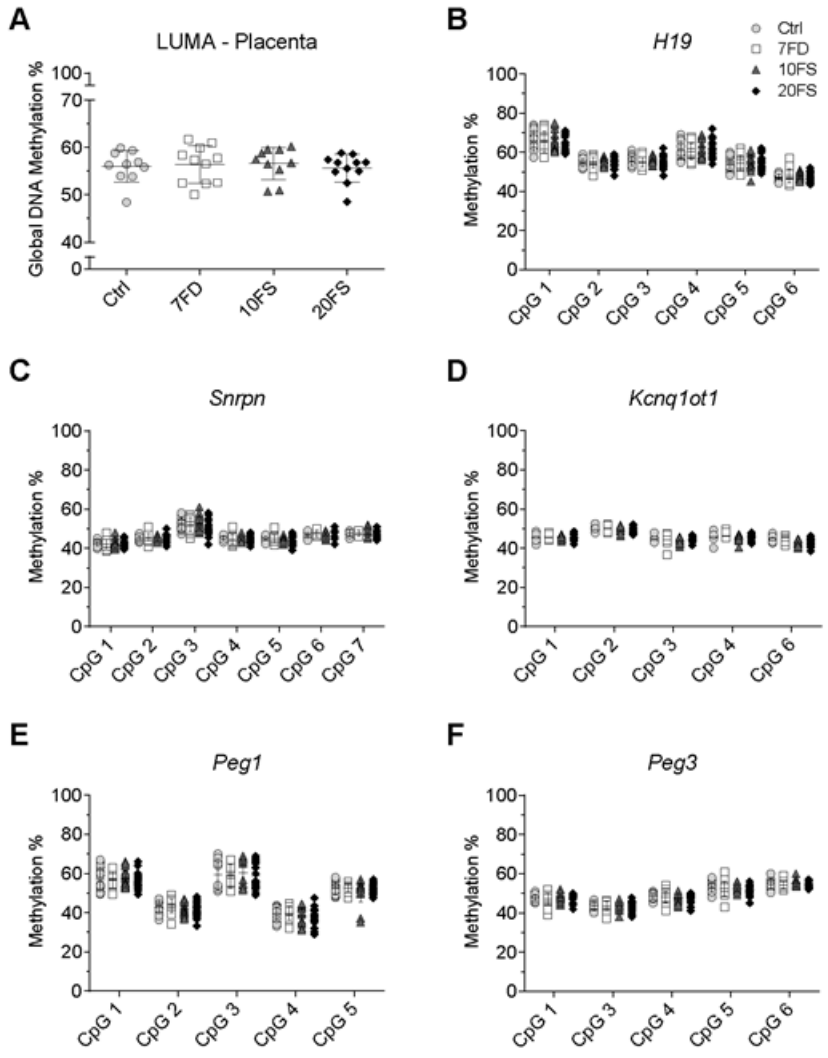


Figure 3.3. F2 E18.5 placenta global DNA methylation and DMR methylation at imprinted genes.

(A) Global DNA methylation was measured using LUMA (n = 5–6/group/sex). Loci of paternally methylated gene (B) *H19* (n = 9–11/group/sex) and maternally methylated genes (C) *Snrpn*, (D) *Kcnq1ot1*, (E) *Peg1* and (F) *Peg3* (n = 9–11/group/sex) methylation levels were quantified by bisulphite pyrosequencing (Ctrl, folic acid control diet; 7FD, 7-fold folic acid deficient; 10FS, 10-fold folic acid supplemented; 20FS, 20-fold folic acid supplemented).

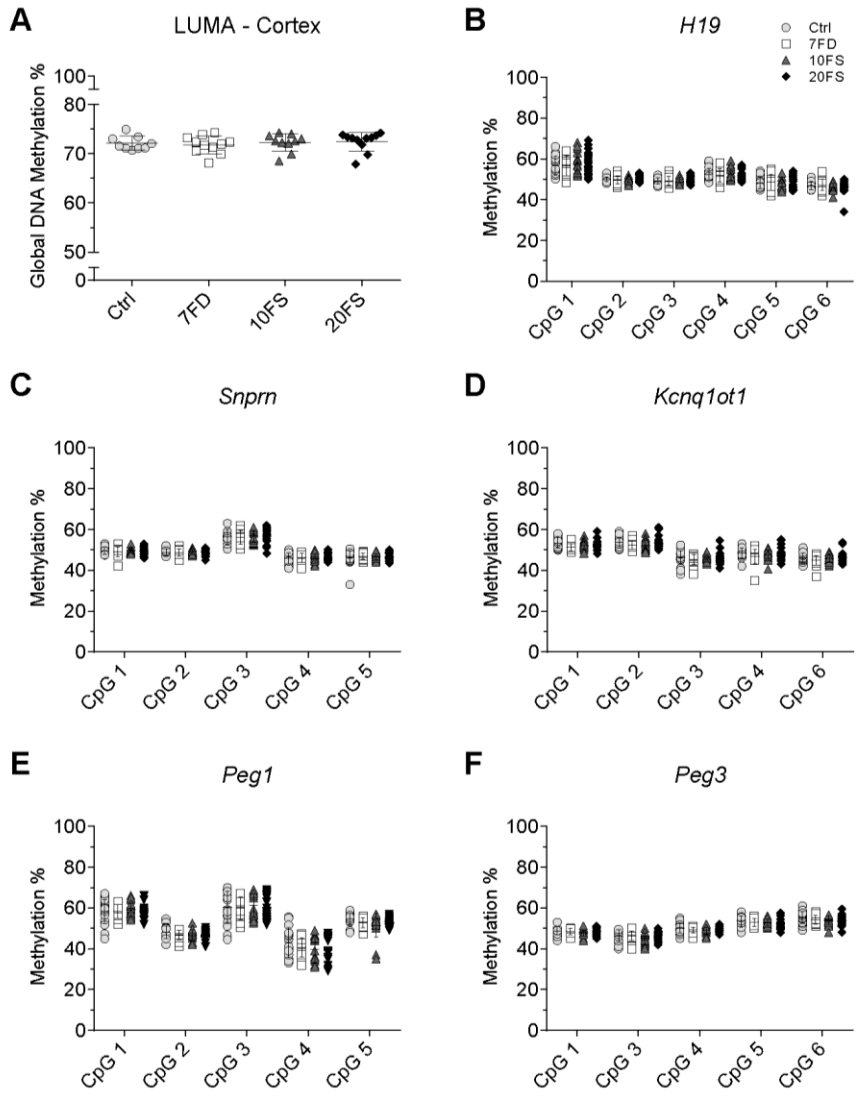


Figure 3.4. F2 E18.5 cortex global DNA methylation and DMR methylation at imprinted genes.

A) Global DNA methylation was measured using LUMA (n=10 males/group). Loci of paternally methylated gene B) *H19* (n = 9-11/group/sex) and maternally methylated genes C) *Snrpn*, D) *Kcnq1ot1*, E) *Peg1* and F) Peg3 (n = 9-11/group/sex) methylation levels were quantified by bisulfite pyrosequencing. (Ctrl = Folic Acid Control Diet, 7FD = 7x Folic Acid Deficient, 10FS = 10x Folic Acid Supplemented, 20FS = 20x Folic Acid Supplemented).

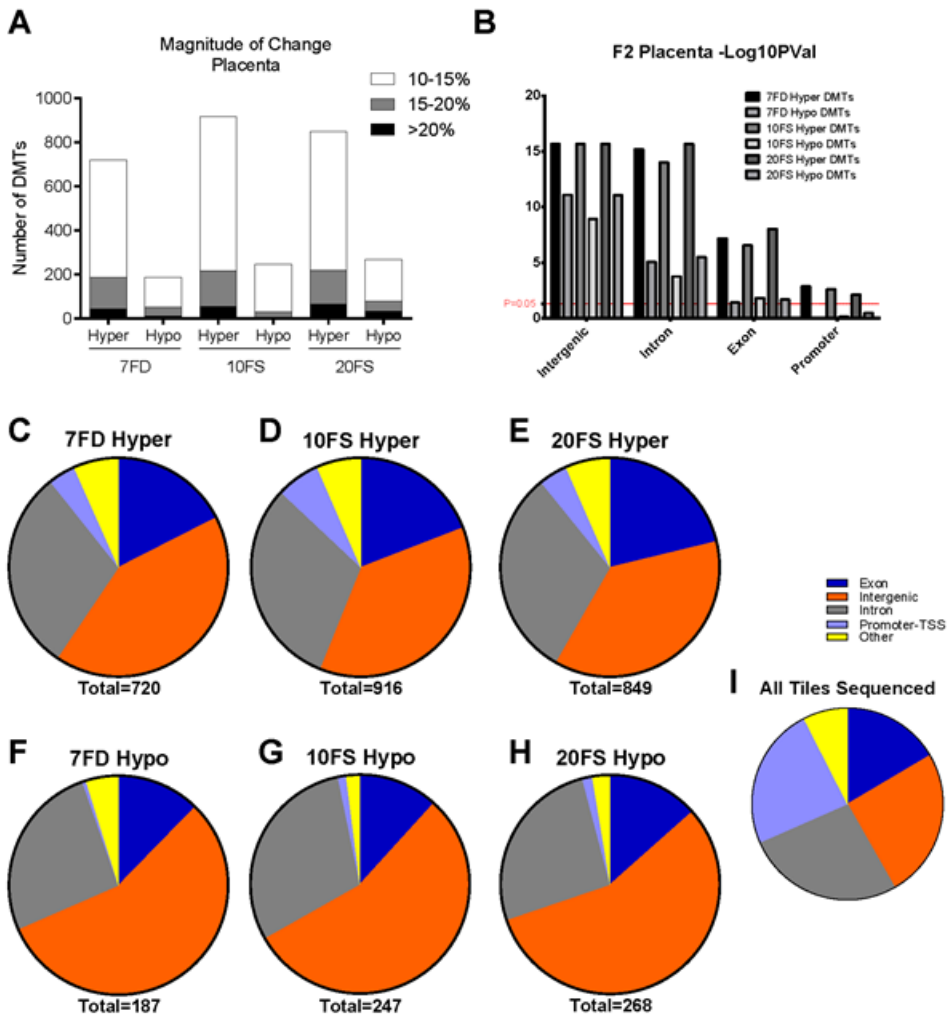


Figure 3.5. Distribution of DMTs in placenta by genomic regions following perinatal maternal FD and FAS exposure.

A) Magnitude of methylation change in DMTs in post treatment groups. B) Statistical significance (-log(PVal)) of difference in methylation within genomic region DMTs compared to Ctrl. C-H) Prevalence of genomic regions affected within DMTs. I) Genomic region distribution among all tiles covered and sequenced by RRBS. (n = 6 males/group; 7FD = 7x Folic Acid Deficient, 10FS = 10x Folic Acid Supplemented, 20FS = 20x Folic Acid Supplemented).

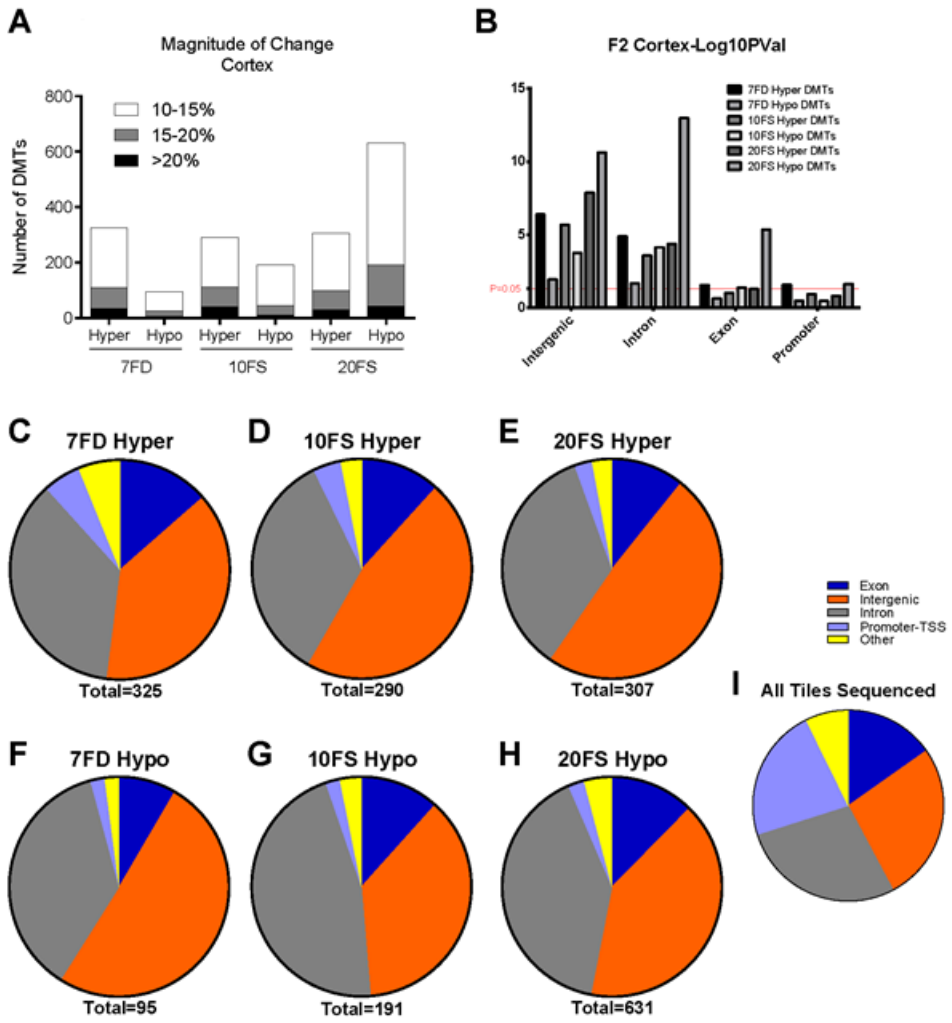


Figure 3.6. Distribution of DMTs in cortex by genomic regions following perinatal maternal FD and FAS exposure.

A) Magnitude of methylation change in DMTs in post treatment groups. B) Statistical significance (-log(PVal)) of difference in methylation within genomic region DMTs compared to Ctrl. C-H) Prevalence of genomic regions affected within DMTs. I) Genomic region distribution among all tiles covered and sequenced by RRBS. (n = 6 males/group; 7FD = 7x Folic Acid Deficient, 10FS = 10x Folic Acid Supplemented, 20FS = 20x Folic Acid Supplemented).

Table 3.1: DAVID bioinformatics analysis showing all significantly enriched biological pathways for all genic DMTs in the F2 placenta, for each exposure group.

7FD DMTs			
Tissue	**Pathway**	**No. of Genes**	**Benjamini**
Placenta	Multicellular organism development	53	6.97E-05
	Transcription, DNA templated	71	4.91E-04
	Regulation of transcription from RNAPII promoter	44	1.26E-03
	Angiogenesis	17	7.08E-03
	Nervous system development	21	1.63E-02

10FS DMTs			
Tissue	**Pathway**	**No. of Genes**	**Benjamini**
Placenta	Multicellular organism development	67	2.76E-09
	Transcription, DNA-templated	87	2.04E-05
	Regulation of transcription from RNA polymerase II promoter	52	4.52E-04
	Epithelial cell differentiation	11	2.64E-03
	Nervous system development	25	1.05E-02
	Establishment of planar polarity	6	1.99E-02
	Anterior/posterior pattern specification	12	2.77E-02
	Regulation of ion transmembrane transport	13	2.92E-02
	Cochlea morphogenesis	6	4.06E-02
	Planar cell polarity pathway involved in neural tube closure	5	3.92E-02
	Axon guidance	13	5.69E-02

20FS DMTs			
Tissue	**Pathway**	**No. of Genes**	**Benjamini**
Placenta	Multicellular organism development	71	3.73E-11
	Regulation of transcription from RNAPII promoter	54	1.93E-04
	Transcription, DNA templated	84	1.52E-04
	Nervous system development	25	1.39E-02
	Transmembrane receptor protein tyrosine kinase signaling pathway	11	3.29E-02

Table 3.2*:* DAVID bioinformatics analysis showing all significantly enriched biological pathways for all genic DMTs in the F2 cortex, for each exposure group.

7FD DMTs				
Tissue	**Pathway**	**No. of Genes**	**PValue**	**Benjamini**
Cortex	Substrate adhesion-dependent cell spreading	8	2.08E-05	4.19E-02
	Protein phosphorylation	28	1.43E-04	2.55E-01
	Positive regulation of gene expression	22	1.66E-04	2.90E-01
	Cellular response to retinoic acid	8	4.88E-04	6.34E-01
	Regulation of neurotransmitter secretion	5	7.85E-04	8.02E-01

10FS DMTs				
Tissue	**Pathway**	**No. of Genes**	**PValue**	**Benjamini**
Cortex	Cytoskeleton organization	7	2.81E-03	9.76E-01
	Transcription, DNA-templated	40	4.21E-03	9.40E-01
	Ovarian follicle development	5	7.20E-03	9.60E-01
	Multicellular organism development	24	1.17E-02	9.80E-01
	Regulation of alternative mRNA splicing, via spliceosome	4	1.53E-02	9.84E-01

20FS DMTs				
Tissue	**Pathway**	**No. of Genes**	**PValue**	**Benjamini**
Cortex	Protein phosphorylation	33	3.09E-05	6.26E-02
	Phosphorylation	32	2.17E-04	2.03E-01
	Protein autophosphorylation	14	8.25E-04	4.37E-01
	Synapse organization	6	2.30E-03	7.00E-01
	Wnt signaling pathway	14	3.20E-03	7.38E-01

Table 3.3*:* DMTs in placenta which intersect with methylation data from GVO and ICM studies by Shirane et al., 2013 and Wang et al., 2014.

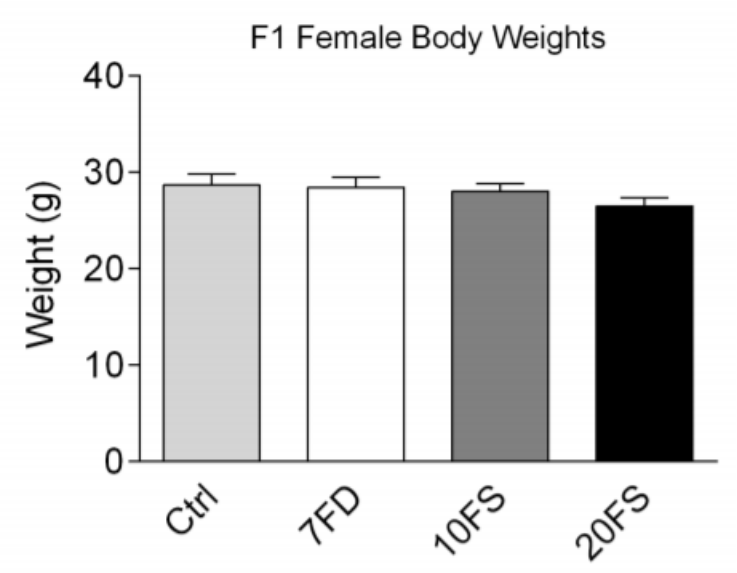
Distribution of DMTs is done among regions that undergo de novo DNA methylation (gain) or DNA methylation erasure (loss) on the maternal allele during the transition from germinal vesicle stage oocyte to the inner cell mass. (Ctrl = Folic Acid Control Diet, 7FD = 7x Folic Acid Deficient, 10FS = 10x Folic Acid Supplemented, 20FS = 20x Folic Acid Supplemented, Hyper = hypermethylated DMTs, Hypo = hypomethylated DMT, Gain and loss of methylation was defined as an increment or decline in methylation of ≥10% from GVO to ICM).

Methylation Change from GVO→ICM	Number of Placenta DMTs								
	7FD			10FS			20FS		
	Hyper	*Hypo*	*All DMTs*	*Hyper*	*Hypo*	*All DMTs*	*Hyper*	*Hypo*	*All DM*
Gain	7	4	11	6	1	7	5	4	9
Stable	196	37	233	269	46	315	222	61	283
Loss	133	33	166	167	38	205	170	41	211
Total	336	74	410	442	85	527	397	106	503

Table 3.4: DMTs in cortex which intersect with methylation data from GVO and ICM studies by Shirane et al., 2013 and Wang et al., 2014.

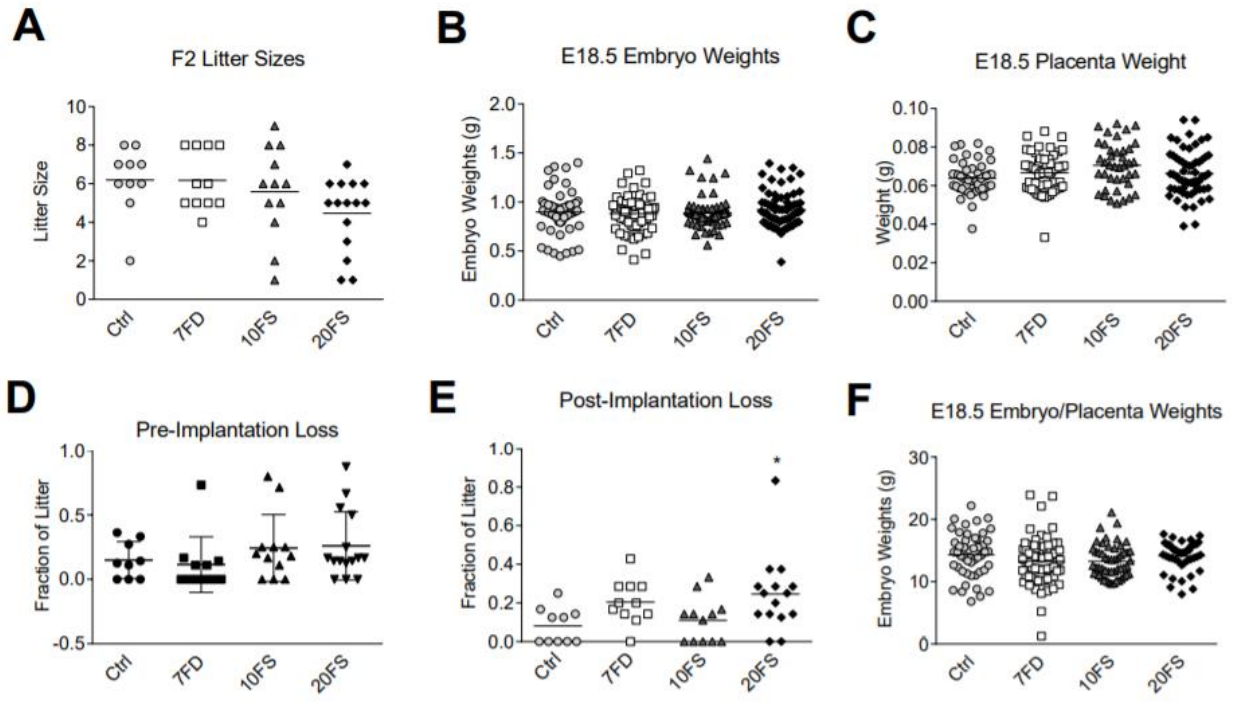
Distribution of DMTs is done among regions that undergo de novo DNA methylation (gain) or DNA methylation erasure (loss) on the maternal allele during the transition from germinal vesicle stage oocyte to the inner cell mass. (Ctrl = Folic Acid Control Diet, 7FD = 7x Folic Acid Deficient, 10FS = 10x Folic Acid Supplemented, 20FS = 20x Folic Acid Supplemented, Hyper = hypermethylated DMTs, Hypo = hypomethylated DMT, Gain and loss of methylation was defined as an increment or decline in methylation of ≥10% from GVO to ICM).

Methylation Change from GVO→ICM	Number of Cortex DMTs								
	7FD			10FS			20FS		
	Hyper	*Hypo*	*All DMTs*	*Hyper*	*Hypo*	*All DMTs*	*Hyper*	*Hypo*	*All DM*
Gain	4	1	5	3	0	3	3	5	8
Stable	81	26	107	82	30	112	82	135	217
Loss	63	9	72	56	39	95	53	128	181
Total	144	35	179	138	69	207	135	263	398



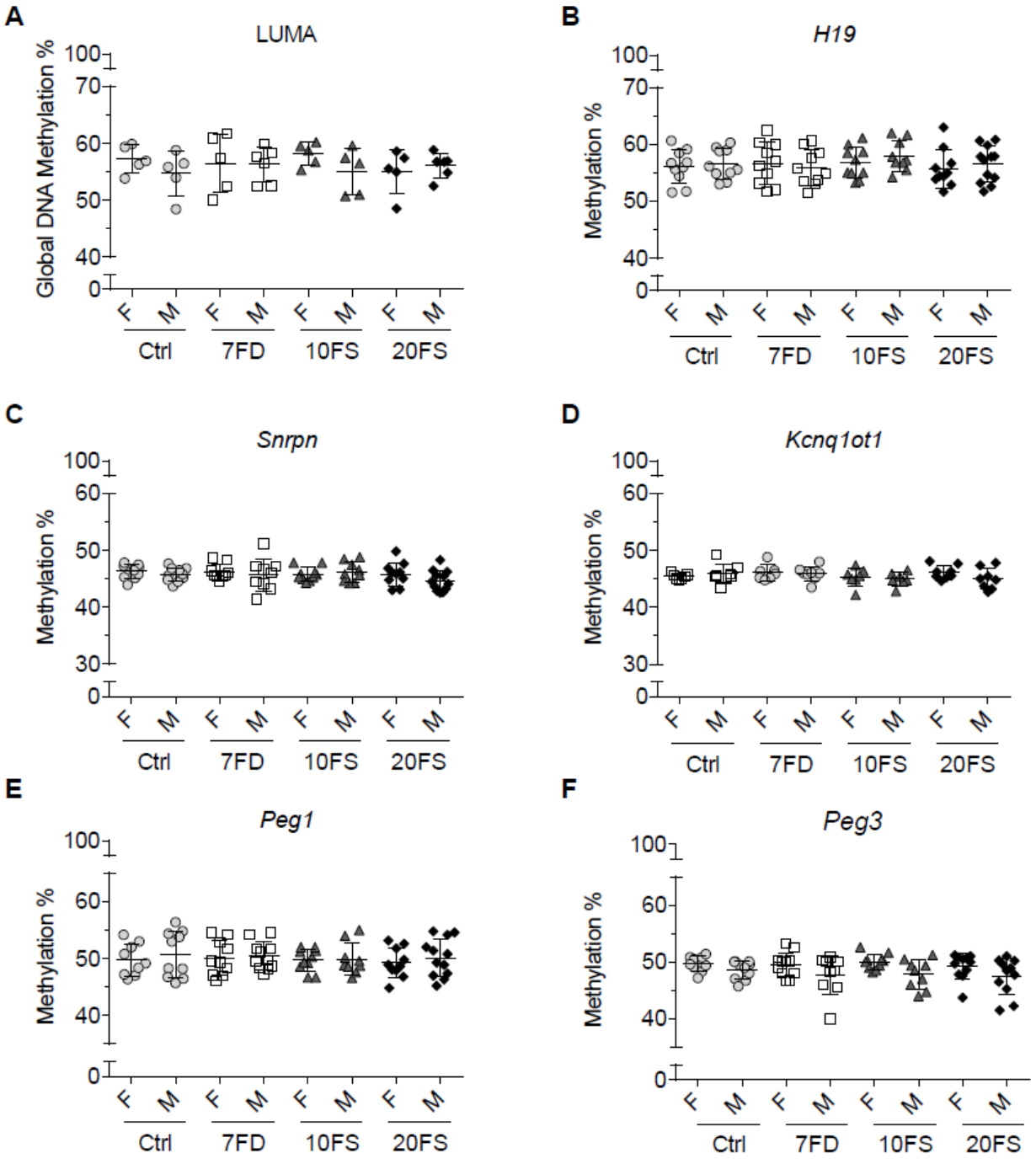
Supplementary Figure 3.1. Effect of prenatal folate deficiency and supplementation on F1 adult female body weight

(n = 17-20/group) (Ctrl = Folic Acid Control Diet, 7FD = 7x Folic Acid Deficient, 10FS = 10x Folic Acid Supplemented, 20FS = 20x Folic Acid Supplemented). * = $p<0.05$ by one-way ANOVA with Dunnett's multiple comparisons test



Supplementary Figure 3.2. Effects of mothers' preweaning exposure to folate deficiency and folic acid supplementation on future reproductive outcomes in F2 at E18.5.

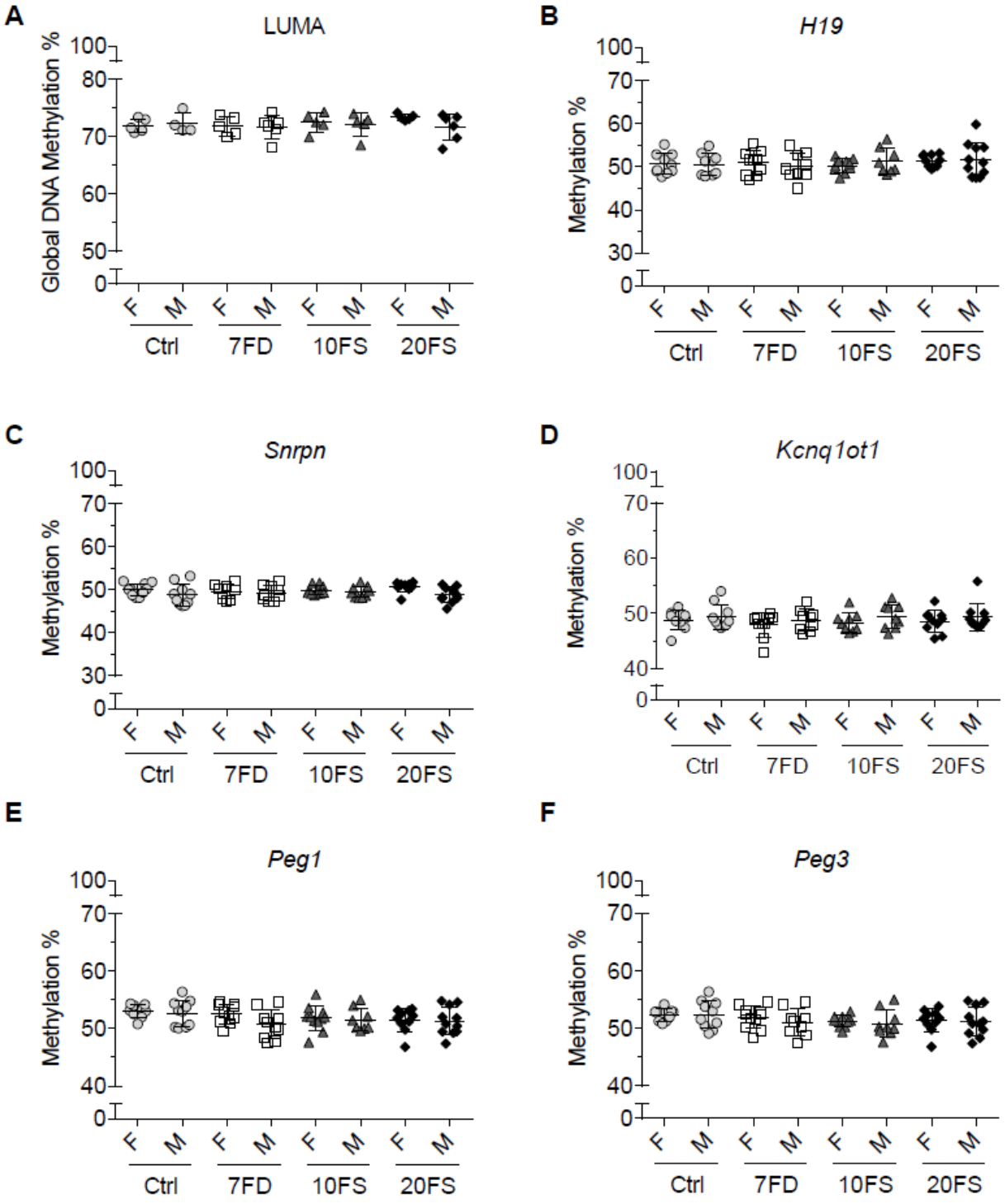
Individual data points were plotted for (A) F2 litter sizes at E18.5 (n = 10-15 F2 litters), (B) F2 embryo weights at E18.5, (C) F2 placental weights at embryonic day 18.5 (n = 49-59 embryos), D) pre-implantation loss of F2 at E18.5 (n = 10-15 F2 litters), (E) Post-implantation loss of F2 at E18.5 and (F) the ratio of embryo to placenta weight (n = 49-59). (Ctrl = Folic Acid Control Diet, 7FD = 7x Folic Acid Deficient, 10FS = 10x Folic Acid Supplemented, 20FS = 20x Folic Acid Supplemented). * = $p<0.05$ by one-way ANOVA with Dunnett's multiple comparisons test.



Supplementary Figure 3.3. Individual data points for F2 E18.5 placenta global DNA methylation and DMR methylation at imprinted genes.

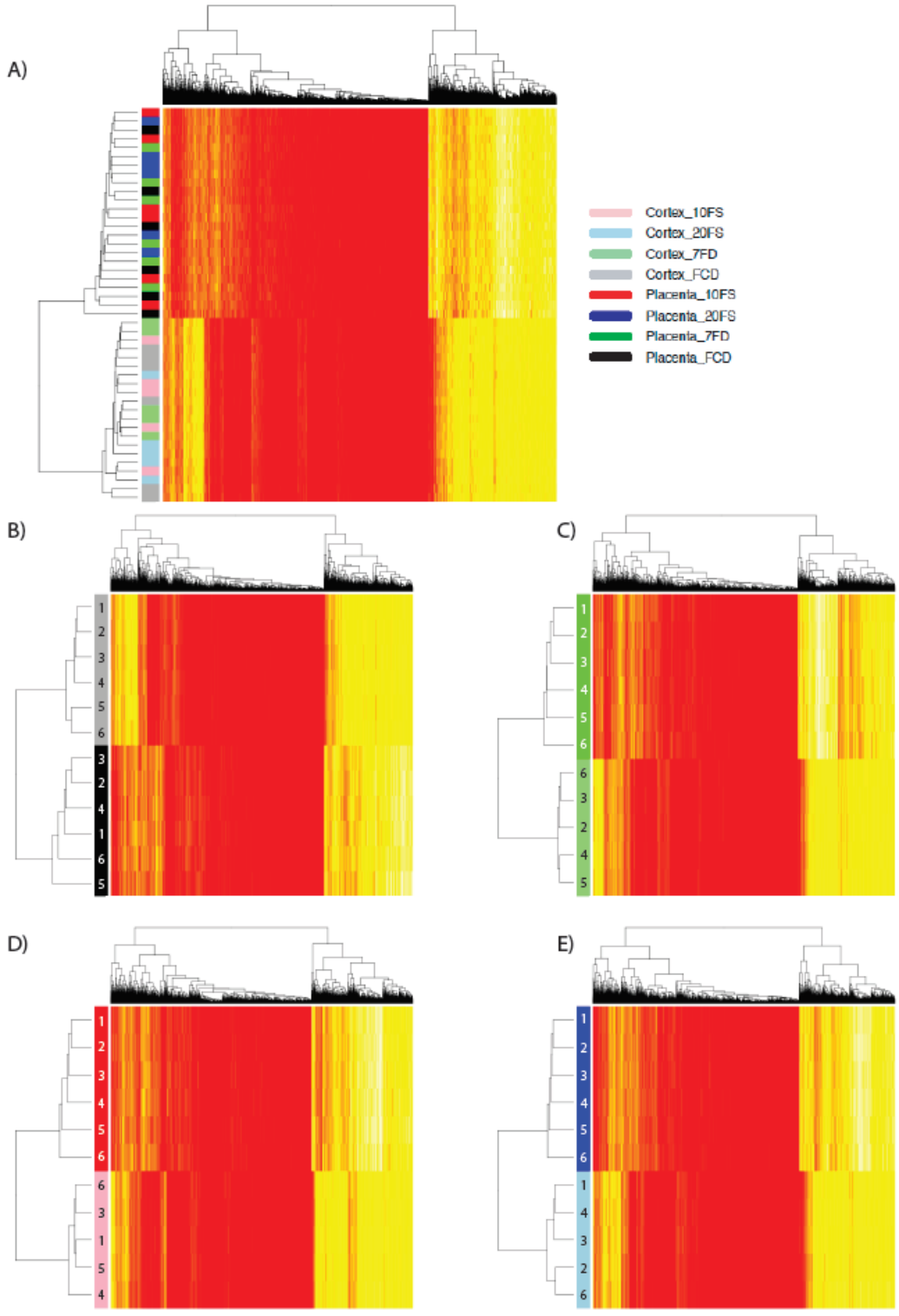
A) Global DNA methylation was measured using LUMA (n =5-6/ group/sex). Loci of paternally methylated gene B) H19 (n =9-11/group/sex) and maternally methylated genes C) Snrpn, D)

Kcnq1ot1, E) Peg1 and F) Peg3 (n = 9-11/group/sex) methylation levels were quantified by bisulfite pyrosequencing. (Ctrl = Folic Acid Control Diet, 7FD = 7x Folic Acid Deficient, 10FS = 10x Folic Acid Supplemented, 20FS = 20x Folic Acid Supplemented).



Supplementary Figure 3.4. Individual data points for F2 E18.5 cortex global DNA methylation and DMR methylation at imprinted genes.

Global DNA methylation was measured using LUMA (n =5-6/group/sex). Loci of paternally methylated gene A) H19 (n = 9-11group/sex) and maternally methylated genes B) Snrpn, C) Kcnq1ot1, D) Peg1 and E) Peg3 (n = 9-11/group/sex) methylation levels were quantified by bisulfite pyrosequencing. (Ctrl = Folic Acid Control Diet, 7FD = 7x Folic Acid Deficient, 10FS = 10x Folic Acid Supplemented, 20FS = 20x Folic Acid Supplemented).



Supplementary Figure 3.5. Heatmap and hierarchal clustering.

Heatmap and hierarchal clustering of commonly sequenced CpG sites at 20x coverage from A) all samples and from cortex and placental samples of B) Ctrl, C) 7FD, D) 10FS and E) 20FS diet groups. Numbers indicate matching cortex and placenta tissues from the same animal. (n = 5-6 males/group; Ctrl = Folic Acid Control Diet, 7FD = 7x Folic Acid Deficient, 10FS = 10x Folic Acid Supplemented, 20FS = 20x Folic Acid Supplemented).

Supplementary Table 3.1: Pyrosequencing primers for germline differentially methylated regions (DMRs).

Germline DMR (No. of CpGs)	Methylated Allele	Original Sequences of Pyro Primers (Bisufite Converted Sequences)	Reference
H19 (6)	Paternal	Forward: 5'-GGGGGGTAGGATATATGTATTTTT Reverse: 5'-biot-ACCTCATAAAACCCATAACTATAAAATCAT Sequencing: 5'-GTGTGTAAAGATTAGGG	Susiarjo et al, 2013
SNRPN (7)	Paternal	Forward: 5'-TTGGTAGTTGTTTTTTGGTAGGAT Reverse: 5'-biot-TCCACAAACCCAACTAACCTTC Sequencing: 5'- GTGTAGTTATTGTTTGGGA	Susiarjo et al, 2013
Peg1 (5)	Maternal	Forward: 5'-GGTTGGGTTTGGATATTGTAAAG Reverse: 5'-biot-TTCCCTAAAATTCTAACAACCTAAACA Sequencing: 5' ATTGTAAAGTTAAAGTTGTAGTAAA	de Waal et al, 2014
Peg3 (6)	Maternal	Forward: 5'-GGTTTTTAAGGGTAATTGATAAGG Reverse: 5'-biot-CCCTATCACCTAAATAACATCCC Sequencing: 5'-AATTGATAAGGTTGTAGATT	de Waal et al, 2015
Kcnq1ot1 (5)	Maternal	Forward: 5'-AGGTTTTGGTAGGTGGTTT Reverse: 5'-biot-CTAACTAAACCAAAATACACCATCATA Sequencing: 5'-GTTAGGAGGAATAGTTGTTTTA	de Waal et al, 2014

H19 Imprinted Maternally Expressed Transcript (Non-Protein Coding)

Snrpn Small Nuclear Ribonucleoprotein Polypeptide N

*Kcnq1ot1*Opposite Strand/Antisense Transcript 1 (Non-Protein Coding)

Peg 1 and 3 Paternally-Expressed Gene 1 and 3

Supplementary Table 3.2: Summary RRBS performance for individual samples

	Mean	± SEM	Min.	Max.
Total Reads	18, 262, 640	374,466	12,777,040	18,971,750
1× Coverage CpG Count	1, 629, 169	15, 307	1, 219, 880	1, 971, 469
CpGs with >=10× Coverage	64.58%	0.36%	56.76%	69.29%

SEM: Standard Error of Mean; Min.: minimum; Max.: Maximum

Supplementary Table 3.3: Primers used for the validation of RRBS results.

Pyrosequencing Assay	Target Region	Primers
1	Chr 5:112467185-210	For 5′-biot-GGATTGAGATTGTTGGTTATAAAGG-3′ Rev 5′-CCAAAACACTACATTAAAACAAACCCA-3′ Seq 5′-AAACCACAAATCATATATAATATC-3′
2	Chr 5: 112467230-250	For 5′- ATGGATTGAGATTGTTGGTTATAAAG -3′ Rev 5′-biot-TCCAAAACACTACATTAAAACAAACCC -3′ Seq 5′- AGGTGATATTATATATGA -3′
3	Chr x: 95461105-145	For 5′-biot-TGTTGGGGGTATATATGAGGAG -3′ Rev 5′- AACTTACCAATACTACTATTCCTCAACTAA -3′ Seq 5′- ATCCACAAAATCCTTAAAT -3′
4	Chr 7: 80390945-985	For 5′-biot-GGGGTAGTTTTTTGTAGGAGAT -3′ Rev 5′- TCAACAACAACCTCCTACACTA -3′ Seq 5′- ACCTCTCACCTACCC -3′

For: Forward PCR primer; **Rev**: Reverse PCR primer; **Seq**: Pyrosequencing primer.

Supplementary Table 3.4: RRBS comparison of 7FD, 10FS, and 20FS versus Ctrl placenta and cortex.

Comparison	Tissue	Number of DMTs		
		Total	Hypermethylation (%)	Hypomethylation (%)
7FD vs Ctrl	Placenta	907	720 (79.4%)	187 (20.6%)
	Cortex	420	325 (77.3%)	95 (22.7%)
10FS vs Ctrl	Placenta	1163	916 (78.7%)	247 (21.3%)
	Cortex	481	290 (60.2%)	191 (39.8%)
20FS vs Ctrl	Placenta	1117	849 (76.0%)	268 (34.0%)
	Cortex	938	307 (32.7%)	631(67.3%)

Supplementary Table 3.5: Summary Placenta RRBS Data

Values represent average number of tiles of all samples within tissue across all exposures.

		Placenta			
		All sequenced tiles	All DMTs	Hypermethylated	Hypomethylated
Genic Regions	*Intergenic*	35244 (27%)	225 (47%)	319 (39%)	131 (56%)
	Promoter-TSS	29523 (23%)	22 (3%)	41 (5%)	3 (1%)
	Exon	19871 (15%)	95 (16%)	160 (19%)	29 (12%)
	Intron	36415 (28%)	159 (29%)	253 (39%)	64 (27%)
	Other	9525 (7%)	32 (5%)	45 (6%)	10 (3%)

Supplementary Table 3.6: Summary Cortex RRBS Data

Values represent average number of tiles of all samples within tissue across all exposures.

		Cortex			
		All sequenced tiles	All DMTs	Hypermethylated	Hypomethylated
Genic Regions	*Intergenic*	35346 (27%)	131 (44%)	137 (45%)	125 (43%)
	Promoter-TSS	29612 (23%)	10 (3%)	13 (4%)	7 (2%)
	Exon	19945 (15%)	37 (11%)	37 (12%)	36 (11%)
	Intron	36557 (28%)	117 (38%)	108 (35%)	126 (41%)
	Other	9564 (7%)	12 (4%)	14 (4%)	11 (3%)

Supplementary Table 3.7: Validation of RRBS results by bisulfite pyrosequencing.

Groups compared	Genic Region	Pyrosequencing Assay	CpG Coordinate	RRBS Methylation Change	Pyrosequencing Methylation Change
FCD vs. 7FD	Intron	1	Chr 5: 112467190	↓	↓ [2]
	Intron	1	Chr 5: 112467206	=	= [2]
	Intron	2	Chr 5: 112467237	↓	↓ [2]
	Intron	2	Chr 5: 112467247	↓	↓ [2]
	Intergenic	3	Chr x: 95461111	↓	↓
	Intergenic	3	Chr x: 95461115	↓	↓
	Intergenic	3	Chr x: 95461124	↓	=
	Intergenic	3	Chr x: 95461142	↓	↓
FCD vs. 20FS	Exon	4	Chr 7: 80390950	↑	↑ [1]
	Exon	4	Chr 7: 80390982	↑	↑ [1]

FCD: Folic acid control diet; **7FD**: Seven-fold folic acid deficient diet; **20FS**: Twenty-fold folic acid supplemented diet.
1: Validation performed using 5 samples from the group 20FS. **2**: Validation performed using 5 samples from groups FCD and 7FD.

Supplementary Table 3.8: Gene lists corresponding to the top 2 most statistically significantly enriched biological pathways among genic DMTs within placenta conserved in all three folic acid exposure groups identified by DAVID Bioinformatic analysis.

Multicellular organism development	Transcription
Alx3	Ccnk
Angpt2	Erbb4
Casz1	Esrrg
Dact2	Fezf2
Dbx1	Gtf2h4
Dll4	Hoxa10
Dlx4	Ncor2
Efnb3	Nfil3
En1	Onecut2
Erbb4	Park2
Esrrg	Pax2
Fezf2	Pou2f2
Hoxa10	Prdm11
Hoxc12	Stat4
Lrp1	Tbr1
Pax2	Tbx1
Six6	Tbx21
Smad5	Vax1
Tbx1	Vopp1
Unc5a	Vsx2
Vax1	Wt1
Vsx2	Ybx1
Wnt2	Zfp423
Wnt7a	Zfp710
Zfa-ps	Zfp784
Zfp423	

Supplementary Table 3.9: Corresponding genes of DMTs conserved in both placenta and cortex with >10% change in methylation. For each gene, the directionality and magnitude of change is demonstrated, along with the diet exposure.

Gene Name	Gene Description	DMT Location	Direction		Diet
			Placenta	Cortex	
Map1lc3b	microtubule-associated protein 1 light chain 3 beta	Intergenic	-21.89	-20.64	7FD
Gm6588	predicted gene 6588	intron	-27.97	-17.61	7FD
Etaa1	Ewing tumor-associated antigen 1	Intergenic	-12.43	-17.16	7FD
Fbxo4	F-box protein 4	Intergenic	-14.12	-16.08	7FD
Sntb1	syntrophin, basic 1	Intergenic	-15.33	-13.58	7FD
2200002D01Rik	RIKEN cDNA 2200002D01 gene	TTS	-21.23	-12.04	7FD
Slc25a13	solute carrier family 25 (mitochondrial carrier, adenine nucleotide translocator), member 13	Intergenic	-10.64	-11.83	7FD
Tas2r139	taste receptor, type 2, member 139	Intergenic	-14.03	-10.81	7FD
1700049E22Rik	RIKEN cDNA 1700049E22 gene	Intergenic	-16.30	-10.61	7FD
Ceacam18	carcinoembryonic antigen-related cell adhesion molecule 18	exon	-15.06	-10.36	7FD
Mtpn	myotrophin	Intergenic	-17.40	-10.11	7FD
Arhgef26	Rho guanine nucleotide exchange factor (GEF) 26	Intergenic	36.55	10.01	7FD
Ccdc166	coiled-coil domain containing 166	exon	15.21	10.07	7FD
Ybx1	Y box protein 1	exon	20.17	10.17	7FD
Fam58b	family with sequence similarity 58, member B	exon	12.31	10.20	7FD
Ckb	creatine kinase, brain	intron	15.42	10.70	7FD
Mir6943	microRNA mir-6943	intron	13.61	10.73	7FD
Slc38a1	solute carrier family 38, member 1	Intergenic	10.57	10.78	7FD
Olfr136	olfactory receptor 136	Intergenic	21.38	10.92	7FD
Gnas	GNAS (guanine nucleotide binding protein, alpha stimulating) complex locus	exon	25.01	11.17	7FD
Esrrg	estrogen-related receptor gamma	intron	10.24	11.18	7FD
1700030C10Rik	RIKEN cDNA 1700030C10 gene	Intergenic	12.44	11.36	7FD
Prok2	prokineticin 2	Intergenic	13.24	11.42	7FD
Smad1	SMAD family member 1	intron	15.48	11.63	7FD
Alkbh5	alkB, alkylation repair homolog 5 (E. coli)	Intergenic	15.27	11.64	7FD
Arhgef28	Rho guanine nucleotide exchange factor (GEF) 28	intron	11.92	12.01	7FD
Cdh15	cadherin 15	intron	14.54	12.48	7FD
Sall1	sal-like 1 (Drosophila)	Intergenic	10.26	12.62	7FD
Aif1l	allograft inflammatory factor 1-like	Intergenic	12.82	12.65	7FD
Fam19a5	family with sequence similarity 19, member A5	Intergenic	-10.81	12.67	7FD
Cd209c	CD209c antigen	Intergenic	17.80	12.80	7FD
Tmem63a	transmembrane protein 63a	3' UTR	21.12	13.20	7FD
Anks6	ankyrin repeat and sterile alpha motif domain containing 6	exon	12.37	13.38	7FD

Pex14	peroxisomal biogenesis factor 14	3' UTR	18.17	14.66	7FD
Mir378c	microRNA mir-378c	intron	12.55	14.71	7FD
Ckb	creatine kinase, brain	exon	25.05	14.76	7FD
Pcdh19	protocadherin 19	Intergenic	10.10	15.20	7FD
Scgb2b26	secretoglobin, family 2B, member 26	Intergenic	10.73	15.40	7FD
Fam58b	family with sequence similarity 58, member B	exon	23.31	15.52	7FD
Olfm1	olfactomedin 1	Intergenic	10.92	15.76	7FD
Stxbp6	syntaxin binding protein 6 (amisyn)	Intergenic	17.69	16.11	7FD
Mir7214	microRNA mir-7214	Intergenic	16.39	16.43	7FD
Ybx1	Y box protein 1	exon	17.68	17.71	7FD
Ccnk	cyclin K	intron	15.89	21.73	7FD
Mir153	microRNA 153	intron	14.09	23.15	7FD
Eltd1	EGF, latrophilin seven transmembrane domain containing 1	Intergenic	13.46	26.73	7FD

Map1lc3b	microtubule-associated protein 1 light chain 3 beta	Intergenic	-22.78	-18.38	10FS
Sntb1	syntrophin, basic 1	Intergenic	-11.09	-17.82	10FS
Six3os1	SIX homeobox 3, opposite strand 1	Intergenic	12.71	-17.43	10FS
2610316D01Rik	RIKEN cDNA 2610316D01 gene	Intergenic	-19.01	-16.24	10FS
Mif	macrophage migration inhibitory factor	intron	20.52	-15.79	10FS
Etaa1	Ewing tumor-associated antigen 1	Intergenic	-15.82	-12.35	10FS
Sh3pxd2a	SH3 and PX domains 2A	intron	-11.80	-11.46	10FS
Krtcap3	keratinocyte associated protein 3	intron	-15.99	-11.32	10FS
Gpr110	G protein-coupled receptor 110	Intergenic	-13.73	-11.28	10FS
Rps6kc1	ribosomal protein S6 kinase polypeptide 1	Intergenic	-17.30	-10.27	10FS
2200002D01Rik	RIKEN cDNA 2200002D01 gene	TTS	-15.91	-10.17	10FS
Psg16	pregnancy specific glycoprotein 16	intron	14.10	-10.12	10FS
Gnas	GNAS (guanine nucleotide binding protein, alpha stimulating) complex locus	exon	11.13	-10.02	10FS
Ino80d	INO80 complex subunit D	Intergenic	13.27	10.20	10FS
Ppp1r2-ps3	protein phosphatase 1, regulatory (inhibitor) subunit 2, pseudogene 3	promoter-TSS	33.06	10.33	10FS
Rpp21	ribonuclease P 21 subunit	Intergenic	14.19	10.43	10FS
H2-DMa	histocompatibility 2, class II, locus DMa	intron	12.24	10.58	10FS
LOC100503676	uncharacterized LOC100503676	intron	13.75	10.66	10FS
Alkbh5	alkB, alkylation repair homolog 5 (E. coli)	Intergenic	17.31	10.78	10FS
1700034G24Rik	RIKEN cDNA 1700034G24 gene	Intergenic	16.47	10.97	10FS
Anks6	ankyrin repeat and sterile alpha motif domain containing 6	exon	12.29	11.11	10FS
Olfr136	olfactory receptor 136	Intergenic	18.56	11.13	10FS
Farp1	FERM, RhoGEF (Arhgef) and pleckstrin domain protein 1 (chondrocyte-derived)	Intergenic	10.87	11.23	10FS
Hrh2	histamine receptor H2	Intergenic	13.71	11.36	10FS
Fam58b	family with sequence similarity 58, member B	exon	16.40	11.37	10FS
Rcan2	regulator of calcineurin 2	intron	16.56	11.46	10FS

Neurl1a	neuralized homolog 1A (Drosophila)	exon	11.36	11.61	10FS
Nenf	neuron derived neurotrophic factor	Intergenic	-11.22	11.85	10FS
1700030C10Rik	RIKEN cDNA 1700030C10 gene	Intergenic	13.49	11.91	10FS
Mup5	major urinary protein 5	Intergenic	18.71	12.22	10FS
Mir7032	microRNA mir-7032	intron	11.61	12.68	10FS
1700010K23Rik	RIKEN cDNA 1700010K23 gene	intron	26.82	12.97	10FS
Prok2	prokineticin 2	Intergenic	16.24	13.08	10FS
Stxbp6	syntaxin binding protein 6 (amisyn)	Intergenic	15.56	13.11	10FS
Kit	kit oncogene	intron	12.23	13.45	10FS
Ereg	epiregulin	promoter-TSS	11.23	14.18	10FS
Ckb	creatine kinase, brain	exon	24.94	14.31	10FS
Aif1l	allograft inflammatory factor 1-like	Intergenic	14.86	14.66	10FS
Mir7214	microRNA mir-7214	Intergenic	15.72	15.42	10FS
Arhgef26	Rho guanine nucleotide exchange factor (GEF) 26	Intergenic	28.44	15.50	10FS
Scgb2b26	secretoglobin, family 2B, member 26	Intergenic	15.82	15.62	10FS
Gdnf	glial cell line derived neurotrophic factor	intron	10.85	15.95	10FS
Ccnk	cyclin K	intron	15.99	15.96	10FS
Pisd-ps3	phosphatidylserine decarboxylase, pseudogene 3	promoter-TSS	10.04	16.50	10FS
Sipa1	signal-induced proliferation associated gene 1	exon	17.11	17.52	10FS
Eltd1	EGF, latrophilin seven transmembrane domain containing 1	Intergenic	12.96	17.72	10FS
Ube2v1	ubiquitin-conjugating enzyme E2 variant 1	Intergenic	13.55	18.10	10FS
Rpp21	ribonuclease P 21 subunit	Intergenic	-12.37	18.21	10FS
Arhgef26	Rho guanine nucleotide exchange factor (GEF) 26	Intergenic	17.86	18.46	10FS
Mark4	MAP/microtubule affinity-regulating kinase 4	Intergenic	17.13	19.03	10FS
Irgq	immunity-related GTPase family, Q	exon	12.04	19.19	10FS
Esp38	exocrine gland secreted peptide 38	Intergenic	14.41	21.06	10FS
Mir8110	microRNA mir-8110	Intergenic	18.39	22.40	10FS
Rab11fip3	RAB11 family interacting protein 3 (class II)	intron	17.99	28.26	10FS
Efhc2	EF-hand domain (C-terminal) containing 2	intron	13.31	29.55	10FS
Mif	macrophage migration inhibitory factor	intron	28.35	-27.45	20FS
Map1lc3b	microtubule-associated protein 1 light chain 3 beta	Intergenic	-25.52	-22.70	20FS
Six3os1	SIX homeobox 3, opposite strand 1	Intergenic	12.55	-21.17	20FS
Zc3h10	zinc finger CCCH type containing 10	promoter-TSS	-13.71	-17.57	20FS
2610316D01Rik	RIKEN cDNA 2610316D01 gene	Intergenic	-20.18	-17.44	20FS
Gm20319	predicted gene, 20319	exon	-11.45	-14.58	20FS
1700086O06Rik	RIKEN cDNA 1700086O06 gene	Intergenic	10.08	-13.84	20FS
Gpr110	G protein-coupled receptor 110	Intergenic	-15.25	-13.21	20FS
Krtcap3	keratinocyte associated protein 3	intron	-15.29	-13.13	20FS

2200002D01Rik	RIKEN cDNA 2200002D01 gene	TTS	-19.74	-12.39	20FS
Clmn	calmin	Intergenic	11.22	-12.20	20FS
Htr1a	5-hydroxytryptamine (serotonin) receptor 1A	Intergenic	14.23	-11.96	20FS
Mtpn	myotrophin	Intergenic	-15.10	-11.61	20FS
Sntb1	syntrophin, basic 1	Intergenic	-12.66	-11.59	20FS
Tas1r2	taste receptor, type 1, member 2	Intergenic	13.82	-11.52	20FS
Ear2	eosinophil-associated, ribonuclease A family, member 2	Intergenic	-11.04	-11.36	20FS
Eva1b	eva-1 homolog B (C. elegans)	TTS	12.76	-11.16	20FS
Myog	myogenin	3' UTR	17.56	-10.98	20FS
Slc25a13	solute carrier family 25 (mitochondrial carrier, adenine nucleotide translocator), member 13	Intergenic	-10.61	-10.62	20FS
Slc28a3	solute carrier family 28 (sodium-coupled nucleoside transporter), member 3	intron	12.99	-10.44	20FS
Gdf2	growth differentiation factor 2	exon	11.20	-10.38	20FS
Gm10377	predicted gene 10377	Intergenic	-13.93	-10.29	20FS
Chsy3	chondroitin sulfate synthase 3	intron	-14.35	-10.28	20FS
Gm13152	predicted gene 13152	intron	11.25	10.05	20FS
Olfr136	olfactory receptor 136	Intergenic	22.33	10.07	20FS
Tbce	tubulin-specific chaperone E	intron	25.53	10.21	20FS
Pim1	proviral integration site 1	Intergenic	14.48	10.31	20FS
Arhgef26	Rho guanine nucleotide exchange factor (GEF) 26	Intergenic	20.57	10.61	20FS
Rcan2	regulator of calcineurin 2	intron	11.33	10.77	20FS
Anks6	ankyrin repeat and sterile alpha motif domain containing 6	exon	17.85	11.03	20FS
Car10	carbonic anhydrase 10	Intergenic	17.38	11.12	20FS
Ncor2	nuclear receptor co-repressor 2	Intergenic	18.34	11.15	20FS
Ephb3	Eph receptor B3	promoter-TSS	13.32	11.16	20FS
Fbxo16	F-box protein 16	intron	-12.33	11.31	20FS
Cyp51	cytochrome P450, family 51	Intergenic	10.42	11.46	20FS
Piezo1	piezo-type mechanosensitive ion channel component 1	intron	11.88	11.53	20FS
Stc2	stanniocalcin 2	Intergenic	-23.84	11.85	20FS
Arhgef26	Rho guanine nucleotide exchange factor (GEF) 26	Intergenic	39.30	12.01	20FS
Ckb	creatine kinase, brain	exon	32.09	12.02	20FS
Stxbp6	syntaxin binding protein 6 (amisyn)	Intergenic	23.25	12.72	20FS
Ybx1	Y box protein 1	exon	16.99	12.76	20FS
Pex2	peroxisomal biogenesis factor 2	Intergenic	-14.17	12.85	20FS
Erdr1	erythroid differentiation regulator 1	Intergenic	-10.82	13.10	20FS
Ppp1r2-ps3	protein phosphatase 1, regulatory (inhibitor) subunit 2, pseudogene 3	promoter-TSS	36.40	13.22	20FS
LOC100503676	uncharacterized LOC100503676	intron	10.79	13.36	20FS
Kcns1	K+ voltage-gated channel, subfamily S, 1	5' UTR	12.58	13.41	20FS
Spred2	sprouty-related, EVH1 domain containing 2	intron	12.93	13.64	20FS
Eltd1	EGF, latrophilin seven transmembrane domain containing 1	Intergenic	18.02	13.70	20FS

Scrg1	scrapie responsive gene 1	intron	16.52	13.73	20FS
Aif1l	allograft inflammatory factor 1-like	Intergenic	12.08	14.06	20FS
Mark4	MAP/microtubule affinity-regulating kinase 4	Intergenic	25.01	14.15	20FS
Pax7	paired box 7	exon	10.88	14.28	20FS
A930016O22Rik	RIKEN cDNA A930016O22 gene	intron	14.62	14.36	20FS
Proser2	proline and serine rich 2	exon	-10.04	14.39	20FS
Scgb2b26	secretoglobin, family 2B, member 26	Intergenic	13.55	14.55	20FS
Aldh2	aldehyde dehydrogenase 2, mitochondrial	intron	12.94	14.66	20FS
Ptprtos	protein tyrosine phosphatase, receptor type T, opposite strand	intron	-18.15	15.28	20FS
Hmgn2	high mobility group nucleosomal binding domain 2	intron	12.43	15.41	20FS
Hao1	hydroxyacid oxidase 1, liver	intron	-16.85	16.22	20FS
Irgq	immunity-related GTPase family, Q	exon	14.01	16.48	20FS
4921524L21Rik	RIKEN cDNA 4921524L21 gene	Intergenic	10.97	16.80	20FS
Psg16	pregnancy specific glycoprotein 16	intron	16.75	17.59	20FS
Mir7214	microRNA mir-7214	Intergenic	18.32	18.01	20FS
Esp38	exocrine gland secreted peptide 38	Intergenic	-11.34	18.29	20FS
Sipa1	signal-induced proliferation associated gene 1	exon	20.13	18.91	20FS
Tshz3	teashirt zinc finger family member 3	Intergenic	29.74	19.27	20FS
Rab11fip3	RAB11 family interacting protein 3 (class II)	intron	17.66	21.82	20FS
Ap5z1	adaptor-related protein complex 5, zeta 1 subunit	intron	27.76	21.94	20FS
Rpp21	ribonuclease P 21 subunit	Intergenic	-22.79	22.09	20FS
Mir153	microRNA 153	intron	10.95	34.38	20FS
Rasa1	RAS p21 protein activator 1	Intergenic	-16.35	35.42	20FS

Supplementary Table 3.10: Placenta (A) and cortex (B) RRBS DMTs localized to regions in previously published sequencing data with low methylation in sperm (>10%) but moderate to high methylation in germinal vesicle oocytes (□25%) and inner cell mass (>25%)

(A)

	Placenta					
	7FD		10FS		20FS	
DMT Direction	Hyper	Hypo	Hyper	Hypo	Hyper	Hypo
Total	44	6	63	4	44	11
% Genic (# DMTs)	77.3 (34)	83.3 (5)	82.5 (52)	100 (4)	86.4 (38)	90 (10)

(B)

	Cortex					
	7FD		10FS		20FS	
DMT Direction	Hyper	Hypo	Hyper	Hypo	Hyper	Hypo
Total	16	3	17	5	13	24
% Genic (# DMTs)	81.3 (13)	66.7 (2)	58.8 (10)	80 (4)	76.9 (10)	87.5 (21)

Supplementary Table 3.11: List of DMTs with location, closest associated Ensembl gene ID, degree of alteration in DNA methylation and the level of methylation of the loci taken from the published GVO and ICMm data.

Tissue	Diet	Location (chr:start-stop)	Peak_Score	Annotation	Gene_ID	GVO	ICMm
Cortex	7FD	chr1:171287801-171287900	23.1	TTS	Usp21	69.5	33.5
		chr1:171287901-171288000	24.4	promoter-TSS	Usp21	69.5	33.5
		chr19:30539701-30539800	15.2	promoter-TSS	Ppp1r2-ps3	92.5	28.2
		chr15:72809801-72809900	10.8	non-coding	Peg13	96.8	86.0
		chr15:72809701-72809800	10.6	non-coding	Peg13	96.8	86.0
		chrX:169983701-169983800	11.0	intron	G530011O06Rik	57.0	34.9
		chr5:125612501-125612600	23.9	intron	Tmem132b	95.3	59.2
		chr18:75258801-75258900	14.2	intron	2010010A06Rik	96.5	56.9
		chr7:82771901-82772000	17.0	intron	4933406J10Rik	95.3	48.0
		chr16:89954801-89954900	18.3	intron	Tiam1	95.2	45.2
		chr3:62159001-62159100	10.0	Intergenic	Arhgef26	84.2	86.2
		chr15:84417201-84417300	12.0	exon	1810041L15Rik	90.9	57.6
		chr13:95763201-95763300	23.0	exon	F2rl2	97.6	56.8
		chr8:108956801-108956900	10.0	exon	Mir3108	96.5	49.3
		chr4:148953801-148953900	14.7	3' UTR	Pex14	74.0	29.7
		chr5:24482501-24482600	11.2	3' UTR	Agap3	90.4	41.1
		chr17:47867001-47867100	-12.1	TTS	Mdfi	79.5	37.5
		chr16:11143901-11144000	-12.0	intron	Txndc11	36.7	32.1
		chr12:24493501-24493600	-15.7	Intergenic	Taf1b	86.1	36.5
	10FS	chr7:4866401-4866500	20.4	promoter-TSS	Isoc2b	85.1	43.4
		chr19:30539701-30539800	10.3	promoter-TSS	Ppp1r2-ps3	92.5	28.2
		chr16:11144101-11144200	11.7	intron	Txndc11	36.7	32.1
		chr7:44630401-44630500	10.7	intron	Myh14	45.2	32.8
		chr10:13091001-13091100	18.5	intron	Plagl1	96.9	64.3
		chr15:80367001-80367100	10.9	intron	Cacna1i	96.7	62.4
		chr7:82771901-82772000	14.4	intron	4933406J10Rik	95.3	48.0
		chr3:62159401-62159500	18.5	Intergenic	Arhgef26	84.2	86.2

		chr3:62159001-62159100	15.5	Intergenic	Arhgef26	84.2	86.2
		chr11:116498701-116498800	13.1	Intergenic	Prpsap1	47.4	41.7
		chrX:169994101-169994200	15.6	Intergenic	G530011O06Rik	57.0	34.9
		chr14:65111001-65111100	27.9	Intergenic	Extl3	96.0	71.2
		chr8:108241501-108241600	14.4	Intergenic	3010033K07Rik	95.2	47.5
		chr8:108241401-108241500	11.4	Intergenic	3010033K07Rik	95.2	47.5
		chr19:5660901-5661000	17.5	exon	Sipa1	96.8	65.6
		chr15:84417201-84417300	14.8	exon	1810041L15Rik	90.9	57.6
		chr1:15892401-15892500	10.1	exon	Sbspon	96.2	49.3
		chr4:154087101-154087200	-11.6	intron	Trp73	97.3817838	52.5
		chr11:116991601-116991700	-19.2	Intergenic	Mgat5b	79.1751366	36.0
		chr11:101165501-101165600	-11.4	exon	Plekhh3	96.6450234	58.0
		chr11:116009901-116010000	-12.0	exon	Galk1	87.8266254	44.5
		chr16:43974001-43974100	-10.8	exon	Zdhhc23	97.1549415	51.4
	20FS	chr19:30539701-30539800	13.2	promoter-TSS	Ppp1r2-ps3	92.5	28.2
		chr16:11144101-11144200	15.7	intron	Txndc11	36.7	32.1
		chr7:44630401-44630500	13.1	intron	Myh14	45.2	32.8
		chrX:169983701-169983800	39.0	intron	G530011O06Rik	57.0	34.9
		chr15:80367001-80367100	10.1	intron	Cacna1i	96.7	62.4
		chr7:82771901-82772000	26.4	intron	4933406J10Rik	95.3	48.0
		chr16:89954801-89954900	16.3	intron	Tiam1	95.2	45.2
		chr3:62159001-62159100	12.0	Intergenic	Arhgef26	84.2	86.2
		chr3:62159401-62159500	10.6	Intergenic	Arhgef26	84.2	86.2
		chr8:108241501-108241600	11.6	Intergenic	3010033K07Rik	95.2	47.5
		chr19:5660901-5661000	18.9	exon	Sipa1	96.8	65.6
		chr15:84417201-84417300	17.9	exon	1810041L15Rik	90.9	57.6
		chr13:95763201-95763300	30.6	exon	F2rl2	97.6	56.8
		chr11:116009901-116010000	-14.5	exon	Galk1	87.8	44.5
		chr8:70392901-70393000	-10.3	exon	Comp	96.1	48.4
		chr5:115231401-115231500	-11.0	Intergenic	Pop5	95.4	53.6
		chr5:115231301-115231400	-12.7	Intergenic	Pop5	95.4	53.6
		chr5:113193901-113194000	-13.2	Intergenic	2900026A02Rik	97.0	45.5

		chr2:31495801-31495900	-11.6	intron	Gm5424	95.4	63.2
		chr5:117622301-117622400	-13.0	intron	Ksr2	96.1	62.9
		chr5:125612401-125612500	-15.0	intron	Tmem132b	95.3	59.2
		chr11:108940301-108940400	-15.8	intron	Axin2	96.7	59.7
		chr12:108259801-108259900	-12.8	intron	Ccdc85c	96.5	58.2
		chr1:36721601-36721700	-10.7	intron	4933424G06Rik	92.6	48.2
		chr8:84731601-84731700	-11.1	intron	Lyl1	87.7	42.8
		chr8:84722001-84722100	-13.8	intron	Lyl1	87.7	42.8
		chr8:108920001-108920100	-11.9	intron	Mir3108	96.5	49.3
		chr1:133068901-133069000	-10.6	intron	Pik3c2b	97.4	42.8
		chr5:113126501-113126600	-12.7	intron	2900026A02Rik	96.9	42.2
		chr5:113126601-113126700	-15.1	intron	2900026A02Rik	96.9	42.2
		chr15:55121401-55121500	-12.2	intron	Gm9920	92.6	37.5
		chr10:127653401-127653500	-12.8	intron	Stat6	86.0	30.3
		chr17:28023101-28023200	-17.0	intron	Tcp11	97.3	33.1
		chr17:63937801-63937900	-16.8	promoter-TSS	Fert2	96.3	33.4
		chr4:155740001-155740100	-11.3	TTS	Tmem240	72.8	47.6
		chr8:70331301-70331400	-11.8	TTS	Gdf1	89.3	53.1
		chr17:47867001-47867100	-16.2	TTS	Mdfi	79.5	37.5
Placenta	**7FD**	chr4:148952901-148953000	21.0	3' UTR	Pex14	74.0	29.7
		chr4:148953801-148953900	18.2	3' UTR	Pex14	74.0	29.7
		chr11:69560001-69560100	13.5	5' UTR	Efnb3	95.0	36.2
		chr15:81235701-81235800	16.3	5' UTR	Mchr1	96.8	44.2
		chr9:31349201-31349300	14.4	exon	Prdm10	96.7	31.4
		chr5:137315301-137315400	12.6	exon	Trip6	93.2	35.2
		chr10:7867601-7867700	15.0	exon	Mir5104	49.6	37.4
		chr10:7867501-7867600	12.4	exon	Mir5104	49.6	37.4
		chr8:121753501-121753600	11.0	exon	Jph3	89.1	39.1
		chr8:121753201-121753300	10.7	exon	Jph3	89.1	39.1
		chr11:101265401-101265500	14.1	exon	Wnk4	89.6	41.2
		chr5:110342201-110342300	10.5	exon	P2rx2	90.8	46.3
		chr11:116145701-116145800	14.6	exon	Mrpl38	97.2	46.3

		chr1:134987601-134987700	10.6	exon	Ube2t	95.6	53.4
		chr5:24414001-24414100	12.1	exon	Asic3	93.8	54.8
		chr10:127049701-127049800	12.0	exon	Cyp27b1	95.0	56.4
		chr4:130072801-130072900	10.0	exon	Col16a1	91.2	56.6
		chr2:162948501-162948600	10.7	exon	L3mbtl1	96.8	64.7
		chr19:5660901-5661000	17.4	exon	Sipa1	96.8	65.6
		chr19:5660801-5660900	11.5	exon	Sipa1	96.8	65.6
		chr5:114923401-114923500	10.8	exon	Oasl1	96.7	73.8
		chr6:54021401-54021500	17.7	Intergenic	Chn2	83.2	35.3
		chr2:44556901-44557000	20.2	Intergenic	Gtdc1	79.1	35.7
		chr2:44557001-44557100	11.6	Intergenic	Gtdc1	79.1	35.7
		chr11:116994801-116994900	13.4	Intergenic	Mgat5b	79.2	36.0
		chr13:65258901-65259000	10.7	Intergenic	Zfp369	51.0	37.8
		chr11:19475601-19475700	14.8	Intergenic	4933406G16Rik	93.6	51.3
		chr3:62159001-62159100	36.5	Intergenic	Arhgef26	84.2	86.2
		chr3:62159401-62159500	19.3	Intergenic	Arhgef26	84.2	86.2
		chr10:127620101-127620200	11.2	intron	Lrp1	75.6	32.9
		chr17:28013801-28013900	16.1	intron	Tcp11	97.3	33.1
		chr7:16858501-16858600	13.3	intron	Prkd2	96.2	36.3
		chr10:11449801-11449900	10.0	intron	Epm2a	94.5	39.6
		chr8:87938601-87938700	14.7	intron	Zfp423	93.5	41.2
		chr11:69399801-69399900	12.6	intron	Tmem88	96.2	44.8
		chr5:35763001-35763100	11.7	intron	Ablim2	88.8	46.1
		chr1:52009101-52009200	10.5	intron	Stat4	95.1	52.8
		chr4:148108001-148108100	13.4	intron	Agtrap	91.0	53.8
		chr5:111273701-111273800	11.8	intron	Pitpnb	95.8	62.4
		chr2:168553601-168553700	15.0	intron	Nfatc2	97.3	64.1
		chr3:93214101-93214200	10.4	intron	Flg2	57.8	71.6
		chr6:148355301-148355400	11.8	non-coding	Rps4l	94.6	32.8
		chr6:148355401-148355500	10.6	non-coding	Rps4l	94.6	32.8
		chr5:114806701-114806800	15.5	TTS	1500011B03Rik	96.6	57.4
		chr11:96712501-96712600	-13.9	intron	Snx11	95.6	65.6

		chr1:134997401-134997500	-10.9	intron	Ube2t	95.6	53.4
		chr2:20235701-20235800	-13.8	Intergenic	Etl4	30.0	33.7
		chr4:154341601-154341700	-14.5	exon	Arhgef16	94.2	56.3
		chr17:47877101-47877200	-10.1	exon	Mdfi	95.4	47.4
		chr1:34807101-34807200	-11.6	exon	Arhgef4	97.3	45.2
	10FS	chr4:148952901-148953000	20.7	3' UTR	Pex14	74.0	29.7
		chr4:148953801-148953900	12.3	3' UTR	Pex14	74.0	29.7
		chr4:148952601-148952700	11.8	3' UTR	Pex14	74.0	29.7
		chr4:133527901-133528000	10.9	5' UTR	1810019J16Rik	81.3	34.1
		chr11:69560001-69560100	21.8	5' UTR	Efnb3	95.0	36.2
		chr15:81235701-81235800	10.1	5' UTR	Mchr1	96.8	44.2
		chr9:31349201-31349300	14.8	exon	Prdm10	96.7	31.4
		chr4:148952201-148952300	15.4	exon	Pex14	74.0	29.7
		chr4:148952501-148952600	11.8	exon	Pex14	74.0	29.7
		chr4:148952101-148952200	10.3	exon	Pex14	74.0	29.7
		chr19:5660901-5661000	17.1	exon	Sipa1	96.8	65.6
		chr10:127049701-127049800	12.1	exon	Cyp27b1	95.0	56.4
		chr13:21363801-21363900	11.8	exon	Zscan12	95.0	35.2
		chr8:121753101-121753200	11.5	exon	Jph3	89.1	39.1
		chr11:117873201-117873300	19.6	exon	Tha1	88.7	45.8
		chr7:44864901-44865000	13.5	exon	Ptov1	95.9	60.4
		chr11:116145901-116146000	15.4	exon	Mrpl38	97.2	46.3
		chr10:18526901-18527000	10.4	exon	Hebp2	95.7	54.7
		chr11:101265301-101265400	10.9	exon	Wnk4	89.6	41.2
		chr7:30860701-30860800	10.2	exon	Ffar1	92.6	30.5
		chr4:134569001-134569100	14.9	exon	Mir6403	96.7	57.2
		chr6:54021401-54021500	14.1	Intergenic	Chn2	83.2	35.3
		chr2:44556901-44557000	18.6	Intergenic	Gtdc1	79.1	35.7
		chr2:44557001-44557100	17.8	Intergenic	Gtdc1	79.1	35.7
		chr5:111571001-111571100	11.0	Intergenic	C130026L21Rik	90.6	49.4
		chr1:86313701-86313800	10.8	Intergenic	B3gnt7	59.0	49.6
		chr11:19475601-19475700	10.7	Intergenic	4933406G16Rik	93.6	51.3

		chr12:12810101-12810200	11.4	Intergenic	Mycn	96.2	55.8
		chr1:6441401-6441500	12.5	Intergenic	St18	94.9	63.2
		chr3:62159001-62159100	28.4	Intergenic	Arhgef26	84.2	86.2
		chr3:62159401-62159500	17.9	Intergenic	Arhgef26	84.2	86.2
		chr2:168553601-168553700	13.6	intron	Nfatc2	97.3	64.1
		chr12:108887301-108887400	11.3	intron	Wars	96.1	58.0
		chr5:111273701-111273800	13.6	intron	Pitpnb	95.8	62.4
		chr4:136426901-136427000	12.0	intron	Htr1d	96.7	60.2
		chr5:74230201-74230300	17.4	intron	Rasl11b	95.7	53.5
		chr8:108901501-108901600	13.9	intron	Mir3108	96.2	46.7
		chr10:4709901-4710000	10.8	intron	Esr1	96.2	56.2
		chr10:127620101-127620200	12.3	intron	Lrp1	75.6	32.9
		chr5:125058001-125058100	10.7	intron	Fam101a	96.7	54.7
		chr1:52009101-52009200	10.2	intron	Stat4	95.1	52.8
		chr11:98721401-98721500	16.2	intron	Med24	91.1	41.5
		chr3:67462601-67462700	11.2	intron	Lxn	96.4	48.9
		chr11:117873101-117873200	25.2	intron	Tha1	88.7	45.8
		chr8:87938601-87938700	12.7	intron	Zfp423	93.5	41.2
		chr8:87938501-87938600	12.1	intron	Zfp423	93.5	41.2
		chr10:75667701-75667800	12.8	intron	Susd2	95.2	28.7
		chr11:119257201-119257300	12.9	intron	Gaa	78.5	43.9
		chr11:119257301-119257400	12.4	intron	Gaa	78.5	43.9
		chr5:124011001-124011100	10.6	intron	Mir7032	96.2	67.8
		chr7:16858501-16858600	18.9	intron	Prkd2	96.2	36.3
		chr10:127527501-127527600	12.1	intron	Shmt2	60.5	26.6
		chr5:119687101-119687200	12.3	intron	Tbx3os2	86.2	38.9
		chr5:119687301-119687400	11.9	intron	Tbx3os2	86.2	38.9
		chr5:119626801-119626900	11.9	intron	Gm16063	96.0	37.3
		chr11:85800001-85800100	17.9	intron	Bcas3os2	93.7	51.1
		chr6:148355201-148355300	12.5	non-coding	Rps4l	94.6	32.8
		chr10:127642901-127643000	10.2	promoter-TSS	Stat6	86.0	30.3
		chr1:171287901-171288000	15.2	promoter-TSS	Usp21	69.5	33.5

		chr19:30539601-30539700	27.7	promoter-TSS	Ppp1r2-ps3	92.5	28.2
		chr19:30539701-30539800	33.1	promoter-TSS	Ppp1r2-ps3	92.5	28.2
		chr1:171287801-171287900	19.0	TTS	Usp21	69.5	33.5
		chr5:114806601-114806700	12.9	TTS	1500011B03Rik	96.6	57.4
		chr11:96712501-96712600	-11.4	intron	Snx11	95.6203577	65.6
		chr4:136222801-136222900	-10.3	intron	Asap3	69.2940039	25.6
		chr17:3350001-3350100	-11.4	intron	Tiam2	96.15595	51.7
		chr13:45567501-45567600	-11.0	exon	Gmpr	97.0248411	49.0
	20FS	chr4:148953801-148953900	23.7	3' UTR	Pex14	74.0	29.7
		chr4:148952901-148953000	13.6	3' UTR	Pex14	74.0	29.7
		chr1:134987601-134987700	10.9	exon	Ube2t	95.6	53.4
		chr1:134987701-134987800	17.3	exon	Ube2t	95.6	53.4
		chr2:162948501-162948600	11.7	exon	L3mbtl1	96.8	64.7
		chr4:148952401-148952500	10.7	exon	Pex14	74.0	29.7
		chr4:148952301-148952400	10.2	exon	Pex14	74.0	29.7
		chr4:148952501-148952600	10.0	exon	Pex14	74.0	29.7
		chr19:5660901-5661000	20.1	exon	Sipa1	96.8	65.6
		chr19:5660801-5660900	10.2	exon	Sipa1	96.8	65.6
		chr7:44629901-44630000	16.3	exon	Myh14	45.2	32.8
		chr10:127049701-127049800	14.0	exon	Cyp27b1	95.0	56.4
		chr8:121753201-121753300	11.7	exon	Jph3	89.1	39.1
		chr8:121753501-121753600	11.0	exon	Jph3	89.1	39.1
		chr2:145860701-145860800	10.2	exon	Naa20	89.6	40.4
		chr10:7867601-7867700	13.2	exon	Mir5104	49.6	37.4
		chr11:101265401-101265500	10.6	exon	Wnk4	89.6	41.2
		chr11:101265301-101265400	10.0	exon	Wnk4	89.6	41.2
		chr4:134569001-134569100	18.9	exon	Mir6403	96.7	57.2
		chr3:62159001-62159100	39.3	Intergenic	Arhgef26	84.2	86.2
		chr3:62159401-62159500	20.6	Intergenic	Arhgef26	84.2	86.2
		chr18:83062201-83062300	11.0	Intergenic	4930592I03Rik	84.2	63.2
		chr2:44556901-44557000	14.1	Intergenic	Gtdc1	79.1	35.7
		chr6:54021401-54021500	20.5	Intergenic	Chn2	83.2	35.3

		chr11:58960301-58960400	11.4	Intergenic	Trim17	56.0	33.3
		chr11:69399801-69399900	10.8	intron	Tmem88	96.2	44.8
		chr17:28013801-28013900	10.3	intron	Tcp11	97.3	33.1
		chr10:127620101-127620200	12.5	intron	Lrp1	75.6	32.9
		chr10:127620201-127620300	12.1	intron	Lrp1	75.6	32.9
		chr10:11449801-11449900	13.1	intron	Epm2a	94.5	39.6
		chr1:52009101-52009200	10.8	intron	Stat4	95.1	52.8
		chr5:24332101-24332200	13.4	intron	Kcnh2	82.8	44.1
		chr4:148108001-148108100	11.0	intron	Agtrap	91.0	53.8
		chr8:87938601-87938700	19.8	intron	Zfp423	93.5	41.2
		chr8:87938501-87938600	14.4	intron	Zfp423	93.5	41.2
		chr11:88402501-88402600	10.9	intron	Mir378b	79.6	37.2
		chr10:75667701-75667800	18.1	intron	Susd2	95.2	28.7
		chr6:116647901-116648000	10.7	intron	Mir7043	64.9	47.8
		chr7:16858501-16858600	18.8	intron	Prkd2	96.2	36.3
		chr7:16853601-16853700	10.8	intron	Prkd2	96.2	36.3
		chr17:63937901-63938000	21.0	promoter-TSS	Fert2	96.3	33.4
		chr7:60005101-60005200	10.3	promoter-TSS	Snurf	93.8	62.3
		chr19:30539701-30539800	36.4	promoter-TSS	Ppp1r2-ps3	92.5	28.2
		chr5:114806701-114806800	10.1	TTS	1500011B03Rik	96.6	57.4
		chr19:5756701-5756800	-10.87596	intron	Scyl1	64.4	34.9
		chr11:96712501-96712600	-11.663381	intron	Snx11	95.6	65.6
		chr17:26120801-26120900	-14.717965	intron	Mrpl28	96.4	65.8
		chr5:99613501-99613600	-10.970688	intron	A930011G23Rik	95.5	55.1
		chr1:134997401-134997500	-14.558143	intron	Ube2t	95.6	53.4
		chr6:128043701-128043800	-12.529061	intron	Tspan9	93.7	43.8
		chr2:20235701-20235800	-12.803134	Intergenic	Etl4	30.0	33.7
		chr9:119338101-119338200	-10.034581	exon	Myd88	66.7	34.5
		chr4:154341701-154341800	-12.870083	exon	Arhgef16	94.2	56.3
		chr1:34807101-34807200	-11.48459	exon	Arhgef4	97.3	45.2
		chr8:84701801-84701900	-10.958275	5' UTR	Lyl1	87.3	47.1

Supplementary Table 3.12: DAVID bioinformatics analysis showing the all enriched biological pathways for all genic DMTs in placenta and cortex localized to regions in previously published sequencing data with low methylation in sperm (>10%) but moderate to high methylation in germinal vesicle oocytes and inner cell mass (>25%).

Tissue	Exposure	Term	Count	PValue	Benjamini
Cortex	7FD	Signal Transduction	3	0.060	9.91E-1
		GTPase mediated signal transduction	2	0.076	9.50E-1
	10FS	Neuronal Action Potential	2	0.019	7.70E-1
	20FS	Dorsal/ventral Axis of Specification	2	0.013	9.05E-1
		Regulation of Wnt Signalling Pathway	2	0.018	8.04E-1
		Signal Transduction	5	0.032	8.63E-1
		Neuronal Action Potential	2	0.033	7.86E-1
Placenta	7FD	Multicellular Organism Development	6	0.026	9.98E-1
		Transcription, DNA-templated	7	0.086	1.00E0
		Negative Regulation of Wnt Signalling Pathway	2	0.089	1.00E0
	10FS	Glycine Biosynthetic Process	10	0.008	9.06E-1
		Transcription, DNA-templated	2	0.010	7.73E-1
		Positive Regulation of Transcription, DNA-templated	10	0.027	9.32E-1
		Regulation of Transcription, DNA-templated	5	0.031	9.05E-1
		Intracellular Steroid Hormone Receptor Signalling Pathway	7	0.033	8.66E-1
		Signal Transduction	2	0.036	8.36E-1
		Positive Regulation of Calcium Ion Transport	2	0.058	9.21E-1
	20FS	Protein Phosphorylation	5	0.009	9.18E-1
		positive regulation of NF-kappaB transcription factor activity	3	0.011	7.82E-1
		cytokine-mediated signaling pathway	3	0.019	8.31E-1
		response to lipopolysaccharide	3	0.032	9.04E-1
		cell proliferation	3	0.040	9.00E-1

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Connecting Text – Chapter III to Chapter IV

The main objective of Chapter III was to determine whether exposures to FD and FS strictly isolated to the prenatal to early postnatal window of female germ cell development would lead to reproductive and epigenetic effects. Our female exposure paradigm was designed to target the DNA methylation erasure phase of epigenetic reprogramming but spare the DNA methylation reacquisition phase. The results in Chapter III showed that environmental exposures to either low or high folate during in utero and pre-weaning female germ cell development can compromise oocyte quality, adversely affecting the health offspring of the next generation, which showed alterations in DNA methylation patterns in the brain and placenta.

The results in Chapter III suggested that germ cells are susceptible to the induction of epigenetic defects during the prenatal DNA methylation erasure phase. These results led us to look more closely at the prenatal exposure window in the male exposure paradigm presented in Chapter II. We chose to examine the effects of male exposures as large numbers of pure populations of germ cells can be isolated for high resolution DNA methylation studies by reduced representation bisulfite sequencing (RRBS). For these studies we first targeted early spermatogonia, allowing us to assess in utero effects of the diets on the DNA methylation in male germ cells prior to the meiotic and post-meiotic phases of spermatogenesis when further epigenetic remodeling and potentially compensatory events occur. The postnatal effects of the FD and FS diets were subsequently assessed by comparing DNA methylation patterns in the spermatozoa versus those in the spermatogonia of the F1 males.

Additionally, as both Chapter II and Chapter III showed evidence for the inheritance of altered epigenetic marks following parental (F1) prenatal or lifetime exposures in the F2 progeny, we wanted to explore whether altered DNA methylation could ultimately escape

epigenetic reprogramming and be inherited through to the F3 generation. Following lifetime exposure of the F1 generation to the folate diets we performed RRBS on consecutive generations of mature sperm (F1, F2 and F3) to determine the heritability of these effects. Thus, the main objectives of Chapter IV were to distinguish effects of the FD and FS diets on DNA methylation patterning in the prenatal versus the postnatal phases of male germ cell development and determine whether altered DNA methylation is epigenetically reprogrammed or transgenerationally heritable up to the F3 population.

Chapter IV: Transgenerational Impact of Grand-paternal Lifetime Exposures to both Folic Acid Deficiency and Supplementation on Genome-wide Methylation

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Running Title: Effects of low and high doses of folic acid on male germ cells

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Abstract

Environmental insults during early germ cell development coinciding with DNA methylation erasure and epigenetic reprogramming present a potential mechanism for the transmission of environmental exposures across multiple generations. We investigated whether the prenatal window in male germ cell development is epigenetically distinctively susceptible to environmental influences and whether lifetime exposures to folate deficient (FD) and folic acid supplemented (FS) diets including this early window of germ cell development can alter germ cell epigenetics and result in adverse transgenerational effects. Female BALB/c mice (F0) were placed on one of four amino acid defined diets for 4 weeks before pregnancy, and throughout pregnancy and lactation: folic acid control (Ctrl), 7−fold folic acid deficient (7FD), 10−fold high folic acid supplemented (10FS) or 20−fold folic acid supplemented (20FS) diets. F1 males were weaned at 8 weeks onto their respective prenatal diets to allow for lifetime diet exposure during all windows of germline epigenetic reprogramming: the erasure, re-establishment and maintenance phases and subsequently mated to produce up to the F3 generation. Genome-wide DNA methylation in early F1 spermatogonia, mature F1 spermatozoa, F2 spermatozoa, and F3 spermatozoa was assessed by reduced representation bisulfite sequencing (RRBS). Nucleotide resolution DNA methylation analysis yielded genome-wide DNA methylation changes resulting from all three diet exposures identified as differentially methylated tiles (DMTs). All three diet exposures resulted in both hyper- and hypomethylated DMTs, with the majority being hypomethylated. The number of DMTs present was most dramatic in early F1 spermatogonia, with a reduction across all three groups following spermatogenesis in mature F1 spermatozoa. DMTs remained present in the spermatozoa of the F2 generation. Broadly, the number of DMTs continued to decrease in F2 spermatozoa, but surprisingly rose in the F3 spermatozoa of the 7FD and 10FS exposure groups. The findings of this study suggest that prenatal development

coinciding with *de novo* DNA methylation is a key window that is susceptible to environmental exposures. The results indicate that both FD and high FS can perturb DNA methylation in male germ cells, which can maintain epigenetic memory for up to three generations.

Key words: folic acid, paternal effects, DNA methylation, transgenerational effects, developmental programming, epigenetics, intergenerational effects, sperm, male germ cells

Introduction

Increasing evidence continues to emerge supporting the role of paternal environmental exposures in offspring health. Epidemiological studies have linked environmental factors to increased risk of metabolic disturbances in the offspring [1, 2]. Animal models have further emphasized the heritability of effects from paternal environmental exposures such as diet, cigarette smoke, pesticides, and herbicides. These studies all demonstrated a range of offspring phenotypes and cellular pathway changes [3-6]. The study of paternal transmission of environmental effects is unique as exposures are clearly limited to the germ cells, sparing the offspring direct exposure and uterine environment from any downstream effects. Observations in the offspring are thus a consequence of inherited alterations in the paternal sperm genome and/or epigenome. Indeed, epigenetic changes in both sperm and/or progeny epigenetics have been identified and associated with paternal environmental exposures.

Epigenetics include a slew of modifications to DNA that modulate gene expression without altering the genetic code. DNA methylation (DNAme) is the most well understood epigenetic mechanism. It plays a critical role in gene expression regulation, genomic imprinting, X chromosome inactivation (XCI) and genome stability [7]. Key windows of DNA methylation dynamics exist. In male germ cells, the prenatal period is critical for epigenetic reprogramming as male germ cells largely acquire their DNA methylation during gestation [8]. Additionally, two distinct waves of epigenetic reprogramming occur in males - during the preimplantation period and during gametogenesis [9-11]. Although two waves of DNA methylation erasure take place, certain sequences escape epigenetic reprogramming [7]. Environmental exposures, which disrupt the pathways involved in DNA methylation, have the potential to alter DNA methylation establishment leading to gene expression alterations and abnormal developmental outcomes;

thus, disruptions of epigenetic modifications that escape epigenetic reprogramming have the opportunity to be inherited across multiple generations.

Folic acid is a water-soluble vitamin required for various biochemical reactions. The folate cycle plays a major role in the generation of S-adenosyl methionine (SAM), the universal methyl donor in methylation reactions including DNA methylation. Folic acid supplementation is recommended to women of child-bearing age and has become widespread due to food fortification, and self-medication [12]. High dose folic acid treatments are also used in infertility treatment and for high risk pregnancies. The great success of folic acid fortification measures across North America however, has recently been recognized to be accompanied with 90.4% of surveyed pregnant women exceeding the upper limit of 1 mg, causing some concern [13]. Despite widespread dietary folate fortification, deficiencies in the universal methyl donor SAM can still occur. Mutations to genes of the folic acid pathway, such as 5,10-methylenetetrahydrofolic acid reductase (*MTHFR*), that cause lower enzyme activity can lead to a decrease in available SAM pools [14, 15]. Furthermore, high folic acid supplementation has been shown to induce a "pseudo-MTHFR deficiency" associated with abnormal SAM levels and SAM/SAH ratios in the liver in mice, introducing the possibility for induced folate deficiency but over-supplementation [15].

Fluctuations in SAM availability during the previously mentioned key developmental windows have been implicated to have multigenerational consequences. Deleterious effects from high gestational folic acid supplementation have been described in mouse models. Maternal supplementation during gestation with 10-fold and 20-fold recommended doses of folic acid resulted in embryonic loss and delay, growth retardation and birth defects [16, 17]. High dose paternal postnatal folic acid supplementation was also shown to result in sperm DNA

hypomethylation [18]. Meanwhile, paternal lifetime folate deficiency resulted in an aberrant DNA methylation in sperm associated with an increase in birth defect rates in the next generation [3]. These studies highlight the potential for environmental exposures, specifically folate deficiency and folic acid supplementation, to alter epigenetic profiles in germ cells and affect future offspring health.

We previously studied reproductive effects of lifetime FS and FD in males, and showed that lifetime (prenatal and postnatal) FS and FD altered DNA methylation in sperm with decreased sperm counts, had negative reproductive effects, and that paternal lifetime FS and FD resulted in DNA methylation perturbations in offspring [19]. We also previously identified that prenatal FS and FD of female mice also resulted in negative reproductive outcome in the next generation associated with genome wide DNA methylation alterations in embryonic and extraembryonic tissue (Ly et al., 2020). In this current study, we hypothesized that the prenatal window of development, where male germ cells undergo two distinct waves of epigenetic reprogramming, is a period particularly susceptible to environmental exposures which disrupt epigenetic pathways. As such, we explored the effects of prenatal exposures to FS and FD in F1 early spermatogonia, and investigated whether aberrant DNA methylation was transmitted through to the F3 generation.

Materials and Methods

Mice and diets

Experiments and procedures were carried out in conformity with the Canadian Council on Animal Care guidelines and the study was approved by the McGill University Animal Care Committee. Mice were maintained under a 12 hour light: 12 hour dark cycle in a temperature and humidity controlled environment at the Montreal Children's Hospital Research Institute's pathogen-free animal facility with food and water ad libitum.

The study design and breeding scheme are outlined in Fig. 1. Eight-week old (F0) female BALB/c mice (n = 15/group) (Charles River, Canada) were sorted onto one of four folate defined diets (Harlan Teklad, USA) at least four weeks before mating: folic acid control (Ctrl; 2 mg/kg diet) with the recommended daily intake of folic acid for mice [20], 7-fold folic acid deficient (7FD; 0.3 mg/kg diet), 10-fold folic acid supplemented (10FS; 20 mg/kg diet) or 20-fold folic acid supplemented (20FS; 40 mg/kg diet) diets. The diets were supplemented with 1% succinylsulfathiazole to inhibit *de novo* synthesis of folate by gut flora. Previously published studies have explored the developmental and epigenetic effects of these diets [3, 15-17, 19]. After four weeks of maintenance on the defined diets, females were mated with ten-week-old (F0) male BALB/c mice fed with regular mouse regular 18% protein rodent chow diets (Harlan Teklad, USA). F0 females were fed their respective folate defined diets through mating, gestation and lactation, and the F1 male offspring were weaned at postnatal day (PND) 20 onto their respective maternal folate defined diets.

F1 males representative of 10 Ctrl, 11 FD, 10 10FS, and 9 20FS different original F0 litters, at 18 weeks of age, were mated with 10 week old female BALB/c mice fed regular mouse chow. Throughout mating, gestation and lactation, the females remained on their regular 18% protein rodent chow diets. The presence of a vaginal plug on the morning after mating was designated as 0.5 days post coitum (dpc). Subsequently, F2 males representative of the 10 Ctrl, 11 FD, 10 10FS, and 9 20FS different original F0 litters per diet group were mated with 10 week old female BALB/c mice fed regular mouse chow to produce F3 litters.

GOF/deltaPE-Oct4/GFP transgenic mice were a kind gift from Dr. Makoto Nagano ,(RI-MUHC, Montreal, Canada) and have been used in previous studies by our group. Oct4/GFP mice were back-crossed onto the BALB/c model. Genomic DNA isolation was performed using

Qiagen DNeasy Blood & Tissue Kits (Cat No./ID: 69504) using neonatal mouse tail snips. Genotyping of *Oct4/GFP* transgene was done by polymerase chain reaction (PCR), using specific primers: 5′-CTT CTT CAA GGA CGA CGG CAA CTA-3′ (forward) and 5′-ATC GCG CTT CTC GTT GGG GTC TTT-3′ (reverse) for *eGFP* gene detection.

Sperm and tissue collection

Tissue collection was conducted as previously described [19]. Upon successful generation of the following generation of males, F1, F2 and F3 male mice were sacrificed at PND ~200 and weighed. Paired testes, epididymides and emptied seminal vesicles were removed and weighed. Mature spermatozoa from paired cauda epididymides were collected as previously described [21] and kept frozen at -80^{o}C until use.

Cell preparation and FACS of spermatogonia

Isolation of Oct4/GFP male germ cells was done as previously described in Niles et al., 2013 [22]. In brief, postnatal Oct4/GFP testes were collected from animals at 6 (n = 5/diet) days of age. Male mice were sacrificed by decapitation (pups). Decapsulated testes were digested in 0.25% trypsin-EDTA (Gibco-BRL/Invitrogen, Burlington, ON, Canada) for 10 minutes at 37°C, dispersed and digested for another 10 minutes. Dnase was added for the final 5 minutes of digestion. The resulting cell suspension was washed twice and resuspended in sterile phosphate buffer solution (PBS) with Dnase. The testicular cell suspension was centrifuged, resuspended in PBS-EDTA-2% BSA and filtered through a 50 μm pore size Nylon mesh. The resultant single cell suspension was used for flow cytometry analysis.

Flow cytometry analysis was performed in FACSAria Fusions equipped with blue (488nm), red (633nm), violet (405nm), yellow-green (561nm) and UV lasers (BDBiosciences, CA, USA). Data was acquired with FACSDiva™ software v6.1.3 software (BDBiosciences). For

each sample, the following parameters were studied: forward scatter versus side scatter to define the acquisition gates for intact cells, forward scatter versus Pulse Width dot plot for doublet discrimination and side scatter versus FL1 channel (530/40 band pass) dot plot for GFP detection. A minimum of 10,000 single intact cells were collected for analysis in each sample.

DNA methylation analyses

Imprinted gene DNA methylation was done as previously described [19]. Frozen tissues were homogenized and aliquots were used to isolate ~10mg of DNA using the Dneasy Blood and Tissue kit (Qiagen, Germany). The EpiTect Bisulfite kit (Qiagen, Germany) was used for bisulfite treatment of genomic DNA prior to imprinted gene differentially methylated regions (gDMRs) amplification with pyrosequencing specific primers. PyroMark Q24 kit (Qiagen) with PyroMark Q24 Vacuum Workstation (Qiagen) were used for sequencing. Primers used for assessment of *H19* [23], *Snrpn* [23], *Kcnq1ot1* [24], *Peg1* [25] and *Peg3* [24] are listed in Supplementary Table 3.3.

Previously published protocols of the gel-free technique reduced representation bisulfite sequencing (RRBS) were used [26-28]. Libraries were generated using spermatogonia and spermatozoa DNA Samples of 500 ng (n = 6/diet). DNA samples were digested, followed by end repair and A-tailing. Fragment size filtration was performed with AMPure XP beads (Beckman Coulter, Brea, CA, USA). Fragments were ligated to methylated adapters (Illumina), bisulfite converted twice, followed by a AMPure bead clean-up. RRBS libraries were prepared by large scale PCR. Spermatogonia and spermatozoa DNA libraries were then multiplexed for paired-end sequencing with twelve samples per lane of a HiSeq 2000 sequencer (Illumina) followed by initial data processing and alignment of reads by the software pipeline bsmap version 2.6 [29]. MethylKit software (version 0.5.3) was used for identification of differentially methylated tiles

(DMTs). Tiles were defined as 100 hundred bp step-wise tiling windows, containing ≥2 CpG per tile and ≥10 × CpG coverage of each tile per sample. The methylation level of a 100-bp tile was calculated by the average of all CpGs within the tile. A Benjamini-Hochberg false discovery (FDR)-based method for P-value correction was used and only DMTs passing the q-value threshold (q = 0.01) were considered [30]. If significant changes of DNA methylation after folate deficiency or folic acid supplementation exceeded 10%, the tile was designated as a DMT; HOMER software version 3.51 was used to further annotate DMTs.

Intersections

We examined whether alterations in DNA methylation persisted through germ cell development (F1 spermatogonia vs. F1 sperm) or across generations (F1, F2, F3 sperm) by looking for common DMTs between the different endpoints. To accomplish this, DMT lists were intersected using the intersectBed function of bedtools (version 2.26.0) to obtain the overlapping 100-bp tiles between the different comparisons. As well, we re-analyzed data from our previously published Aarabi et al. paper, where mice were exposed to a 20FS-diet for 12 months post-weaning and compared these results this with our F1 spermatogonia data (strictly pre-natal vs. strictly post-natal exposures) [18]. RRBS data from that study were analyzed using the same criteria as above (≥2 CpG per tile and ≥10 × CpG coverage) with a minimum of 10% change in methylation.

Statistical analysis

Results are expressed as the mean ± SEM, unless stated otherwise. Data were graphed and analyzed with Prism 5 (GraphPad Software Incorporated, USA). Comparisons and statistical

calculations were made by Fisher's exact test, ANOVA followed by the Dunnett's multiple comparison test compared to control, or 1-way ANOVA. A level of significance for the analyses was set at $p<0.05$.

Results

Dose-dependent effects in spermatogonia (F1) genome-wide DNA methylation following prenatal folic acid supplementation and folate deficiency

We previously demonstrated that lifetime folate deficiency and high folic acid supplementation resulted in subtle changes in spermatozoa imprinted gene methylation associated with negative offspring outcomes and altered extraembryonic and embryonic DNA methylation [19]. We have also shown that altered methylation is seen in the offspring and placenta of the female mice exposed during prenatal exposure which encompasses early female germ cell development (Ly et al., 2020). In this study, we sought to understand the effects of these exposures on early male germ cell development and hypothesized that the prenatal window is most susceptible to perturbation by environmental exposures. Using RRBS on FACS isolated PND6 spermatogonia, we confirmed that prenatal folate deficiency and folic acid supplementation cause significant numbers of genome-wide DNA methylation changes in spermatogonia that occur in all three exposures, with a dose-dependent effect regarding supplementation (2225, 1983, and 4810 DMTs in 7FD, 10FS and 20FS exposure groups, respectively; Fig. 2B). Compared to our results from the previously mentioned prenatal female exposure study, spermatogonia display remarkably higher numbers of DMTs overall (9018 total DMTs across all diet groups to 3187 total DMTs; Ly et al., 2020).

Spermatogonia displayed both hypermethylation and hypomethylation regions in all three exposure groups. Both folate deficiency and high folic acid supplementation generated statistically significant changes in methylation of intergenic, intron and exon regions (Fig 2A). High folic acid supplementation (20-fold) also resulted in statistically significant altered levels of methylation of tiles in promoter regions. Intergenic regions appeared more susceptible to dietary folate DNA methylation (DNAme) disruptions (Fig 2A and Fig 2C). A bias for hypomethylated differentially methylated tiles was observed in all diet exposures groups (60.7%, 67%, and 96.8% in 7FD, 10FS and 20FS respectively; Fig 2B). Changes in DNAme identified were largely in the 10-15% change range (Fig 2D). A dose-dependent effect was observed in the 20FS group however, as it was observed to not only result in more than double the number of DMTs in comparison to the 10FS group, but also have increased proportions of higher degree methylation change (>15%) DMTs in comparison to the 7FD and 10FS groups (Fig 2B and 2D).

Gene ontology analysis was performed to assess whether folate deficiency or folic acid supplementation affected DNA methylation at regions of biological significance amongst genic DMTs. Recurring biological pathways involved in embryonic and nervous system development were identified in the DMTs of 7FD and 20FS groups (Table 1). Statistically significant enrichment of pathways only occurred within 20FS DMTs, after correction. These pathways were: axon guidance, nervous system development, positive regulation of transcription from RNA PII promoters, and cell adhesion.

Thus, we found that folate deficiency and high folic acid supplementation exposure during the prenatal development of male germ cells resulted in notable numbers DMTs in neonatal spermatogonia, which eclipsed the number of DMTs found in the offspring of prenatally exposed mothers, with a propensity for hypomethylated DMTs, and significant

enrichment was identified in pathways of embryo development and nervous system development.

Genome-wide DNA methylation effects observed in spermatozoa (F1) following lifetime folic acid supplementation and folate deficiency

Having discovered large numbers of DMTs in early spermatogonia, we investigated if DNA methylation perturbations persisted in fully mature spermatozoa. Statistically significant changes in DNAme were also observed in intergenic, intronic and exonic regions in F1 spermatozoa of all three diet groups (Fig 3A). However, no significant change in methylation of tiles in promoter regions was identified. In all, 2044, 1552, and 1541 DMTs were identified in the 7FD, 10FS and 20FS exposure groups respectively – representing a reduction in the number of DMTs from spermatogonia to spermatozoa by 8.1%, 21.7%, and 68.0%, respectively. The majority of DMTs remained in intergenic regions, consistent to spermatogonia DMTs (Fig 3C). The bias for hypomethylated differentially methylated tiles remained in all diet exposures group (65.1%, 57.9%, and 55.4% in 7FD, 10FS and 20FS respectively; Fig 3B). No dose-dependent effect remained in 10FS to 20FS exposures. Similar distributions in affected genomic elements were observed across all three exposures with a preponderance in the 10-15% change in methylation range (Fig 3D).

Gene ontology analysis was performed once more to assess whether regions of altered methylation in mature spermatozoa enriched for the pathways mirrored those in neonatal spermatogonia. Biological pathways thematic to embryo and nervous system development were identified in all three diet exposure groups when restricting to genic DMTs in F1 spermatozoa. However, statistically significant enrichment was only achieved in 10FS DMTs, after correction. These pathways were: aorta morphogenesis, positive regulation of synapse assembly (Table 2).

Thus, we found that lifetime folate deficiency and high dose folic acid supplementation exposure resulted in DMTs which remained present in adult fully mature spermatozoa after spermatogenesis. The number of DMTs found in the spermatozoa after lifetime exposures were reduced in comparison to the number originally found prenatally exposed spermatogonia, maintained their propensity for hypomethylated DMTs, and also maintained their significant enrichment for pathways of embryo development and nervous system development.

Persistent DMTs in F1 neonatal spermatogonia and F1 mature spermatozoa

To determine whether DMTs in spermatozoa localized to the same regions as DMTs originally found in spermatogonia, we identified whether DMTs intersected to the same 100-bp tiles in both tissues. Cross-matching the DMT list in spermatozoa to the DMT list in spermatogonia, 108, 80 and 172 one-hundred bp tiles were found to be differentially methylated in both spermatogonia and spermatozoa in the 7FD, 10FS and 20FS exposure groups respectively (5.2-11.2% of total spermatozoa list; average of 7.2% DMTs intersecting across all three groups; Table 3). More than half of the persistent DMTs maintained their directionality of change (hypermethylated or hypomethylated in both tissues) (59 DMTs, 52 DMTs, and 108 DMTs or 54.6%, 65.0%, and 62.8% in 7FD, 10FS and 20FS, respectively). In large, most of persistent DMTs which maintained directionality of change were hypomethylated DMTs (35/59, 39/52, and 104/108 in 7FD, 10FS and 20FS, respectively) In genic DMTs alone, 38, 27 and 48 DMTs (27.9-35.2% of persistent DMTs) were found to be differentially methylated in both tissues in 7FD, 10FS, and 20FS exposure groups.

As sperm that survive through the steps of spermatogenesis escape normal germ cell degeneration of incompetent germ cells, we performed gene ontology analysis to verify whether relevant biological pathways were implicated among genic DMTs which originate in

spermatogonia and persist in mature spermatozoa. No statistically significant biological pathways enriched in persisting genic DMTs were identified (Table 4). We did identify two genes which persisted in spermatogonia and in spermatozoa in all three diet exposure groups. One, *Stxbp3a*, codes for microRNA-15b which was newly described to be expressed in the brain and suggested to be an epigenetic regulator involved in mood and behaviour [31]. MicroRNA-15b was found to be hypomethylated in all three diet groups in spermatogonia, but hypermethylated in all three diet groups in spermatozoa. The other gene which was identified to be persistent in all three exposure groups was *Bahcc1*. BAH Domain And Coiled-Coil Containing 1 was found to be consistently among the most hypomethylated DMTs in both spermatogonia and spermatozoa in the 7FD and 20FS exposure groups. Interestingly, BAHCC1 has been identified to be susceptible for epigenetic perturbation in a brain specific deletion of a histone methyltransferase, KMT2A [32].

Our group previously investigated the effects of postnatal high folate exposure alone on genome wide methylation using the same diets [18]. A re-analysis was done of the published data to reflect DMTs with identical thresholds of 10x sequencing depth and 10% methylation change. When cross-matching DMTs between spermatogonia from the present study and the re-analysed Aarabi et al., study, an astonishing number of DMTs identified to both exposures (1474 DMTs (21.1%); Table 5). Notably, 84.5% of these DMTs common to both prenatal-only exposure effects and postnatal-only exposure effects maintain the same directionality. Genic DMTs which overlapped both prenatal and postnatal exposure DMT lists were strikingly associated with nervous system function and diseases for 15 of 18 DMTs. These included: *Efhc1* (associated with epilepsy), *Plcl1* (associated with speech development and autoimmune disease), *Sphkap* (associated with anencephalopathy and cognitive function), *Katna1 (*associated with

neurogenesis), *Rnf217* (associated with retinal function), *Col18a1* (associated with Knobloch syndrome, a rare condition associated with severe vision problems and skull defects), *Nol7* (a candidate tumor suppressor gene;), *Dlg1* (associated with dendritic function), *Ttc21b* (associated with nephropathy;), *Nbea* (associated with epilepsy), *Ccdc24* (associated with ADHD), *Tshz3* (associated with neuronal function and linked to autism spectrum disorder), *Art5* (found to be preferentially expressed in the brain), and lastly, *Scn10a* (associated with the heart condition – Brugada syndrome), while no known published functional data exists on Mir8103 and Mir1963. [9, 33-44]

Thus, we found that folate deficiency and high folic acid supplementation exposure resulted in a subset of DMTs which remained consistently altered in both prenatally exposed spermatogonia and lifetime exposed mature spermatozoa. The number of specific DMTs found in both spermatogonia and spermatozoa however was only a minor subset of the DMT lists in each separate tissue. Furthermore, some affected genes identified to consistently be altered in both tissues were involved in nervous system function, but also previously identified to be vulnerable to epigenetic alteration. Finally, a large number of DMTs resulting from prenatal folate diet exposures, overlap with DMTs which resulted from strictly postnatal folate diet exposures, of which nearly all the genic DMTs consistent to both prenatal and postnatal high folate diet supplementation were associated with nervous system function and development.

Effects of paternal lifetime folic acid supplementation and folate deficiency on genome-wide DNA methylation in offspring spermatozoa (F2)

We previously identified increased variance of imprinted gene methylation in the F1 spermatozoa and F2 E18.5 brain cortex following these folic acid diet exposures [19]. As such, bisulfite pyrosequencing was used to examine the methylation of the same DMRs of the three

maternally methylated imprinted genes *Snrpn, Kcnq1ot1* and *Peg1* and the paternally methylated *H19* (Fig 4). The paternally methylated imprinted gene *H19* had the expected high levels of DNA methylation (89–100%) in F2 spermatozoa in all exposure groups (Fig 4A). All three maternally methylated imprinted loci possessed normal low levels of DNA methylation (2–8%) in the F2 spermatozoa of all four diet groups (Fig 4B-D). No alteration of variances at individual sites was observed in the sperm from F2 males.

To determine if the presence of DMTs observed in F1 spermatozoa persist following fertilization and epigenetic re-programming, spermatozoa from diet-unexposed F2 males using RRBS. Once more, statistically significant changes in DNA methylation were observed in intergenic, intronic and exonic regions in F2 spermatozoa of all three diet groups (Fig 5A). Significant change in methylation of tiles in promoter regions was identified only in the 20FS group. In all, 903, 609, and 1911 DMTs were identified in the 7FD, 10FS and 20FS exposure groups respectively – representing a reduction of DMTs from F1 spermatogonia to F2 by 55.8% in the 7FD group and 60.8% in the 10FS group, and an increase of 24.0% in the 20FS group. The bias for hypomethylated differentially methylated tiles remained in all diet exposures group with the most marked effect in the 20FS group (57.5%, 64.9%, and 79.6% in 7FD, 10FS and 20FS respectively; Fig 5B). Similar distributions in affected genomic elements were observed across all three exposures (Fig 5C). The majority of DMTs were in intergenic regions, with some exon and intron effects. Very few DMTs were observed in promoter regions. Most changes in DNA methylation were identified in the 10-15% change range, with similar distribution of magnitude in changed tiles (Fig 5D).

Gene ontology analysis was performed once more to assess whether regions of altered methylation in mature F2 spermatozoa enriched for the pathways similar to those in F1 sperm.

Biological pathways thematic to embryo and nervous system development were identified in all three diet exposure groups when restricting to genic DMTs in F2 spermatozoa. However, statistically significant enrichment was only achieved in 10FS and 20FS exposure group DMTs, after correction. These pathways were: nervous system development in the 10FS group and neuron migration and regulation of Rho protein signal transduction (Table 6).

We also determined whether DMTs in F2 spermatozoa localized to the same regions as DMTs originally found in F1 spermatozoa. Cross-matching the DMTs list in F2 spermatozoa to the DMT list in F1 spermatozoa, 96, 70 and 143 one-hundred bp tiles were found to be differentially methylated in both the cross-matched father-son pairs in 7FD, 10FS and 20FS exposure groups respectively (7.5-11.5% of total F2 spermatozoa list; average of 9.9% DMTs intersecting across all three groups; Table 7). Nearly half of the persistent DMTs in the father-son pairs maintained their directionality of change (hypermethylated or hypomethylated in both tissues; 44 DMTs, 32 DMTs, and 72 DMTs or 45.8%, 45.7%, and 50.3% in 7FD, 10FS and 20FS, respectively). In large, most of persistent DMTs which maintained directionality of change were hypomethylated DMTs (28/44, 23/32, and 52/72 in 7FD, 10FS and 20FS, respectively). In genic DMTs alone, 43, 23 and 56 DMTs (32.9-44.8% of persistent DMTs) were found to be persistently differentially methylated in both generations in 7FD, 10FS, and 20FS exposure groups. Amongst the genic DMTs which were identified in both F1 and F2 spermatozoa, *Tet1* was identified in all three diet groups.

Thus, we found that folate deficiency and high folic acid supplementation exposure during the prenatal development of male germ cells resulted in a number of DMTs which remained present in F2 mature spermatozoa. Intriguingly, the number of DMTs decreased in the 7FD and 10FS group, between the F1 to F2 generation, but increased in the 20FS group. The

DMTs maintained their propensity for hypomethylated DMTs, and also maintained their significant enrichment for pathways of nervous system development. A small number of DMTs resulting from prenatal folate diet exposures were found to be consistent from the F1 DMT list, but the majority were unique to F2 spermatozoa.

Health and reproductive effects of grandpaternal (F1) lifetime exposure to folic acid deficient or supplemented diets

We next assessed whether grandpaternal (F1) in utero and postnatal exposures to folate deficiency and folic acid supplementation, and as such, whether the DMTs identified in F2 spermatozoa, affected the general health and reproductive outcome two generations down in the F3 litters. F3 litter sizes at birth did not differ between folic acid control and supplementation diet groups (Fig 6A). Adult F3 male body weights at necropsy were similar among the exposure groups (Fig 6B). In addition, there were no differences in reproductive organ weights (paired epididymides, paired testes and seminal vesicle weights) among the groups (Fig 6C-E). Thus, no reproductive effects of grandpaternal (F1) lifetime exposures to the folate diets were observed.

Effects of paternal lifetime folic acid supplementation and folate deficiency on genome-wide DNA methylation in offspring spermatozoa (F3)

Methylation of the same DMRs of the three maternally methylated imprinted genes *Snrpn*, *Kcnq1ot1* and *Peg1* and the paternally methylated *H19* was also investigated in F3 spermatozoa using bisulphite pyrosequencing (Fig 7). No alteration of mean methylation or variances at individual sites were observed in the sperm from F3 males.

To determine if the presence of DMTs observed in F1 and F2 spermatozoa persisted to the F3 generation, causing a transgenerational effect, DNA methylation was examined in the

spermatozoa from diet-unexposed F3 males. Once more, statistically significant changes in DNA methylation were observed in intergenic, intronic, and exonic regions in F3 spermatozoa of all three diet groups (Fig 8A). Significant change in methylation of tiles in promoter regions was identified only in the 7FD and 10FS groups. In all, 3650, 4376, and 1082 DMTs were identified in the 7FD, 10FS and 20FS exposure groups respectively – representing an increase of DMTs from F2 spermatozoa to F3 by 403% in the 7FD group and 719% in the 10FS group, and a reduction of 56.6% in the 20FS group. The bias for hypomethylated differentially methylated tiles remained in all diet exposures group with the most marked effect in the 7FD and 10FS groups (97.2%, 97.4%, and 82.3% in 7FD, 10FS and 20FS respectively; Fig 8B). Similar distributions in affected genomic elements were observed across all three exposures (Fig 8C). The majority of DMTs were in intergenic regions, with some exon and intron effects. Very few DMTs were observed in promoter regions. Most changes in DNA methylation were identified in the 10-15% change range, with similar distribution of magnitude in changed tiles (Fig 8D).

Gene ontology analysis was performed to assess whether regions of altered methylation in mature F3 spermatozoa enriched for the specific biological pathways. Biological pathways thematic to embryo and nervous system development were identified in all three diet exposure groups when restricting to genic DMTs in F3 spermatozoa. However, statistically significant enrichment was only achieved in the 7FD exposure group DMTs, after correction in the pathway for cell adhesion (Table 8).

We also determined whether DMTs in F3 spermatozoa localized to the same regions as DMTs originally found in F2 spermatozoa. Cross-matching the DMTs list in F3 spermatozoa to the DMT list in F2 spermatozoa, 105, 49 and 68 one-hundred bp tiles were found to be differentially methylated in both the cross-matched pairs in 7FD, 10FS and 20FS exposure

groups respectively (1.1-6.3% of total F3 spermatozoa list; Table 9. Again, about half of the persistent DMTs in the father-son pairs maintained their directionality of change (hypermethylated or hypomethylated in both tissues) (61 DMTs, 26 DMTs, and 41 DMTs or 58.1%, 53.1%, and 60.3% in 7FD, 10FS and 20FS, respectively). In large, most of persistent DMTs which maintained directionality of change were hypomethylated DMTs (59/61, 24/26, and 35/41 in 7FD, 10FS and 20FS, respectively). In genic DMTs alone, 39, 18 and 25 DMTs (36.7-37.1% of persistent DMTs) were found to persistently differentially methylated in both generations in 7FD, 10FS, and 20FS exposure groups.

Finally, we investigated whether DMTs localized to the same regions in the spermatozoa of all three generations. Cross-matching the DMTs list in F1, F2, and F3 spermatozoa, a mere 16, 8 and 12 one-hundred bp tiles were found to be differentially methylated in both the cross-matched pairs in 7FD, 10FS and 20FS exposure groups respectively.

Discussion

In this study, we provide evidence that the prenatal window of development is particularly susceptible to environmental exposures that disrupt pathways involved in epigenetic reprogramming. Notably, we found that in utero exposure to folate deficiency and folic acid supplementation both led to large numbers of alterations in DNA methylation within the developing spermatogonia. The prenatal window of development in the male germ cell is epigenetically distinct from that of the female germ cell, as male germ cell *de novo* methylation largely occurs prior to birth, while female germ cells undergo *de novo* methylation after birth, during oocyte growth. From this, one would presume that DNA methylation in male germ cells would be relatively vulnerable to prenatal folate deficiency and folic acid supplementation compared to female germ cells. Although definite conclusions cannot be drawn from

comparisons between separate RRBS runs, and even more so since they are of different tissues, our previous prenatal female exposure study results were indeed consistent with this hypothesis, as DMT numbers in prenatally exposed spermatogonia are considerably higher than the number observed in the offspring of prenatally exposed females (Ly et al., 2020). These observations further support the idea that key windows which encompass epigenetic events during development may be more susceptible to perturbations.

By targeting early spermatogonia in our study, we investigated the effects of the diet exposures during a key window of *de novo* DNA methylation. Furthermore, this germ cell population precedes spermatogenesis and spermiogenesis, where abnormal germ cells can be lost at check points of degeneration by apoptosis [45, 46]. Indeed, the RRBS data revealed that the number of DMTs in spermatogonia from the diet exposures, exceeded the number of DMTs in spermatozoa. This suggests that a subpopulation of severely affected germ cells may be lost during the process of spermatogenesis, possibly explaining previously associated decreased sperm counts in the high dose supplementation group following lifetime exposure [19]. The 20FS group showed the highest degree of DNA methylation alteration, with more than two-fold the DMTs compared to the 7FD and 10FS group. This may be an indication that excessively high folic acid supplementation may be especially detrimental to early germ cell development. This is in accordance with our previous work which identified that lifetime high dose folic acid supplementation is associated with decreased sperm counts and decreased progeny litter sizes [19].

In large, DMTs following the diet exposures demonstrated hypomethylation, even in the case of supplementation. A decreased level of the methyl-donor SAM, along with a “pseudo-MTHFR deficiency” in mice has been shown to result from such high-dose folic acid

supplementation diets [15]. Decreased availability of methyl-donors resulting from MTHFR deficiency could explain how excessive supplementation of folic acid can have the same deleterious results as folate deficiency. Interestingly, very high folic acid supplementation in mice has been shown to be associated with global hypomethylation in sperm and associated with decreased MTHFR expression in the testis [18].

Another goal of this study was to also identify whether alterations in DNA methylation from dietary exposures can be inherited across multiple generations. Indeed, we identified disruptions of DNA methylation in spermatogonia following lifetime exposure, beginning in utero, to folate deficiency or folic acid supplementation which persist through to mature spermatozoa for up to three generations. The genome-wide RRBS data demonstrated a sharp decrease in DMTs in the transition from spermatogonia to spermatozoa. These findings are consistent with a studyshowing that epigenetic aberrations from a mutation in a folate pathway enzyme could exhibit epigenetic memory for up to three generations [47]. Supposedly, this decrease may be an indication of compensatory mechanisms aside from epigenetic reprogramming. It would be conceivable that such mechanisms are in place during spermatogenesis to identify and correct abnormalities in DNA methylation caused by the diet exposures. Yet within the F1 spermatogonia to F1 spermatozoa transition, we found on average across the diets that 7.2% of DMTs are persistent in both spermatogonia and mature sperm, the majority (60.8%) maintaining their directionality of DNA methylation change. These observations suggest that, although compensatory mechanisms may exist to eradicate germ cells with excessively perturbed DNA methylation, some germ cells with alterations survive.

The father-son F1-F2 spermatozoa sample pairings allowed us to investigate the heritability of DNA methylation alterations following the diet exposures. In fact, a greater

proportion of persistent DMTs was identified in the cross-analysis between these two data sets (9.9% of DMTs are persistent across the two generations on average amongst the diets). While it is not surprising that epigenetic reprogramming successfully further reduces the number of DMTs that endure from F1 to F2 spermatozoa, such low proportions of persistent DMTs between the two generations suggests that more stochastic mechanisms to epigenetic alterations may cause lingering effects of environmental exposures. This is further supported by the even lower proportion (47.3%) of maintained directionality amongst the already few persisting DMTs and more so, the number of DMTs persisting across all three generations F1-F3 spermatozoa being near negligible.

Statistically significant enrichment of biological pathways was found in genic DMTs of both spermatogonia and spermatozoa following the diet exposures. Despite the lowered number of DMTs in spermatozoa, the propensity for these DMTs to be enriched in pathways involved in nervous system development and embryogenesis remained consistent in both tissue stages of germ cell development. Interestingly, the detrimental effects of folic acid supplementation and folate deficiency on neurodevelopment have been postulated in various other studies [48-50]. Furthermore, our previous studies also uncovered perturbations of DNA methylation in the brain cortex and placenta imprinted gene methylation in the F2 males after paternal lifetime exposures to the folate diets [19]. Our group also previously demonstrated that postnatal high folate exposure alone was sufficient to alter genome-wide DNA methylation with similar effect sizes to that of lifetime exposures [18]. Interestingly, our cross-matching of DMTs between the present study and the Aarabi et al., study revealed a proportion of DMTs identified to both exposures with maintained directionality of methylation change. These observations reinforce the hypothesis that certain genomic regions are more susceptible to environmental perturbations.

Intriguingly, one of the two DMTs identified to be persistent from spermatogonia to spermatozoa in all three diets, had been previously described to be susceptible to epigenetic perturbation by tissue-specific deletion of a histone methyltransferase, *Kmt2a* [32]. Altogether, our data suggest that a subset of regions of increased susceptibility to the diet exposures in the F1 sperm may exist, that genic regions pertaining to nervous system development and embryogenesis may be of increased representation in this subset, and that abnormalepigenetic marks in these regions may have the potential tocause abnormalities in the development of the F2 progeny.

Analogous characteristics in the DMTs extended beyond the genic regions that were affected in the two germ cell types. The majority of the DMTs localized to intergenic regions in both F1 spermatogonia and F1 spermatozoa. Intergenic regions are important as they host not only regulatory elements, but also transposable elements [51-56]. Transposable element methylation has been shown to be crucial to germ cell survival and their improper methylation has been demonstrated to cause germ cell death [57-60]. We have previously shown decreased sperm counts following the lifetime exposure to the 20FS diet. Our current F1 data suggests that there may be intergenic regions that are more susceptible to environmental influences and insults. It is conceivable that disrupted methylation of transposable elements results in increased germ cell degeneration during germ cell development.

With alterations in DNA methylation discovered within F1 sperm following lifetime folate diet exposures, we investigated whether proper DNA methylation profiles are established in the absence of the exposures in F2 spermatozoa. Our previous studies found that paternal lifetime folate diets lead to disturbances of imprint methylation in F2 brain cortex and placenta [19]. In the present study, imprinted genes showed no methylation abnormalities in F2 spermatozoa. However, DMTs were identified to persist in F2 sperm suggesting that more subtle

genome-wide effects can occur as a result of environmental exposures. Conflicting data exist in the literature regarding inheritance of epimutations following paternal exposures. One study demonstrated that prenatal paternal undernutrition caused adverse methylation in F1 spermatozoa, but these were not found to carry through to F2 tissues and instead, were associated with perturbed locus specific expression [61]. Meanwhile, studies of toxicants and endocrine disruptors have implicated epimutations to be inherited transgenerationally past the F2 generation, up to the F4 generation [62-64]. The lack of inherited DNA methylation changes in some studies may be attributable to higher threshold for selection. For example, Radford et al. set a minimal change of 1.5-fold cut-off for DMT inclusion, whereas other studies using similar methylation techniques do not set this limitation in their analysis [64]. Coupled with recent studies of epigenetic reprogramming which have identified genomic regions that retain memory it is highly plausible that environmental exposures, which induce epigenetic perturbations, can be transgenerationally inherited. When these epimutations such as DMTs occur in biologically active sites, progeny health can be compromised.

Indeed, somatic and germline tissues undergo epigenetic reprogramming which in theory should protect the epigenome from transmission of deleterious epimutations [65, 66]. Yet, the evidence of epigenetic memory is increasing with studies identifying a surprising range of genomic regions which resist complete reprogramming during germline development [67, 68]. These regions have been proposed to be protected from demethylation at least in part by the presence of factors including RNAs and transcription factors, which very well add to the plausibility of transgenerational inheritance of epigenetic information [69, 70]. Together, these data support a mechanistic model for the transgenerational epigenetic inheritance.

Surprisingly, our study uncovered an increase in DMTs in the 7FD and 10FS diet groups at the F3 generation, in the complete absence of exposure in this generation. It has been proposed that epigenetic mechanisms exist to bestow organisms a plasticity to allow for adaptive responses to the environment akin to the "thrifty phenotype" [71]. We speculate that by changing the environment within tolerable levels in the 7FD and 10FS diets, minimal phenotypic abnormalities are observed in the F2 or F3. Instead, the change in environments between the F2 and F3 generations (exposure diets to chow diets) is reflected in the degree of DMT numbers within the F3 generation, representing an adaptive response in the 7FD and 10FS groups. In contrast, the harshness of very high folic acid levels in the 20FS group may lead to increased losses and phenotypes in the F2, as seen in decreased sperm counts in the F1 and decreased F2 litter sizes. In turn, the F3 generation is lacking an adaptive epigenetic response, as any affected F1 sperm does not propagate. Interestingly, a recent study of epigenetic memory resulting from DNA methylation disruption in a grandpaternal genetic mutation in a key folate pathway enzyme also noted inheritance of epigenetically instability [47]. The inherited epigenetic changes in F2 and F3 did not mirror the original DMRs in the F1 and the authors suggest that epigenetic reprogramming may lead to re-methylation/maintenance in other genomic regions in a cell-specific manner. Such a stochastic mechanism could lead to overcompensation, creating new DMTs or overshooting previously identified DMTs, causing hypomethylated DMTs to become hypermethylated or vice versa.

A major criticism of epigenetic inheritance studies is the significance of environmentally induced heterogeneity of methylation within tissues. That is to ask, what degree of methylation changes reflect physiological changes? Do DMTs in the 10-15% methylation change range alter tissue and organ phenotypes? Are DMTs with greater degree changes in methylation more

significant? Questions remain regarding whether the number of DMTs reflects abnormal outcomes. Furthermore, the connection between methylation and transcription is questioned given the multiple levels of epigenetic regulation and as global changes in methylation don't necessarily induce global changes in expression [72]. Our study focused on the environmental effects on DNA methylation, yet the interplay between epigenetic factors, crosstalk between DNA methylation and histone modifications in particular, is becoming increasingly important [73, 74].

In summary, our study demonstrated the effects of lifetime folate deficiency and folic acid supplementation on transgenerational health. We show evidence supporting the existence of key windows of development which are of particular susceptibility to epigenetic disruption from environmental exposures, specifically in male germ cells. Furthermore, we suggest that a subset of genic regions may be epigenetically more vulnerable to external influence. DNA methylation of genes enriched in pathways involved in embryo and nervous system development may be specifically susceptible to the folate diet exposures. Ultimately, we demonstrated that these epigenetic changes have the potential to propagate for at least three generations. Many avenues of investigation can be derived from these findings including the histone changes that occur from these exposures, especially at early germ line stages, and how they interact with the rest of the epigenome. Altogether, these results implicate the role of DNA methylation in transgenerationally propagating these dietary effects.

Figures and Figure Legends

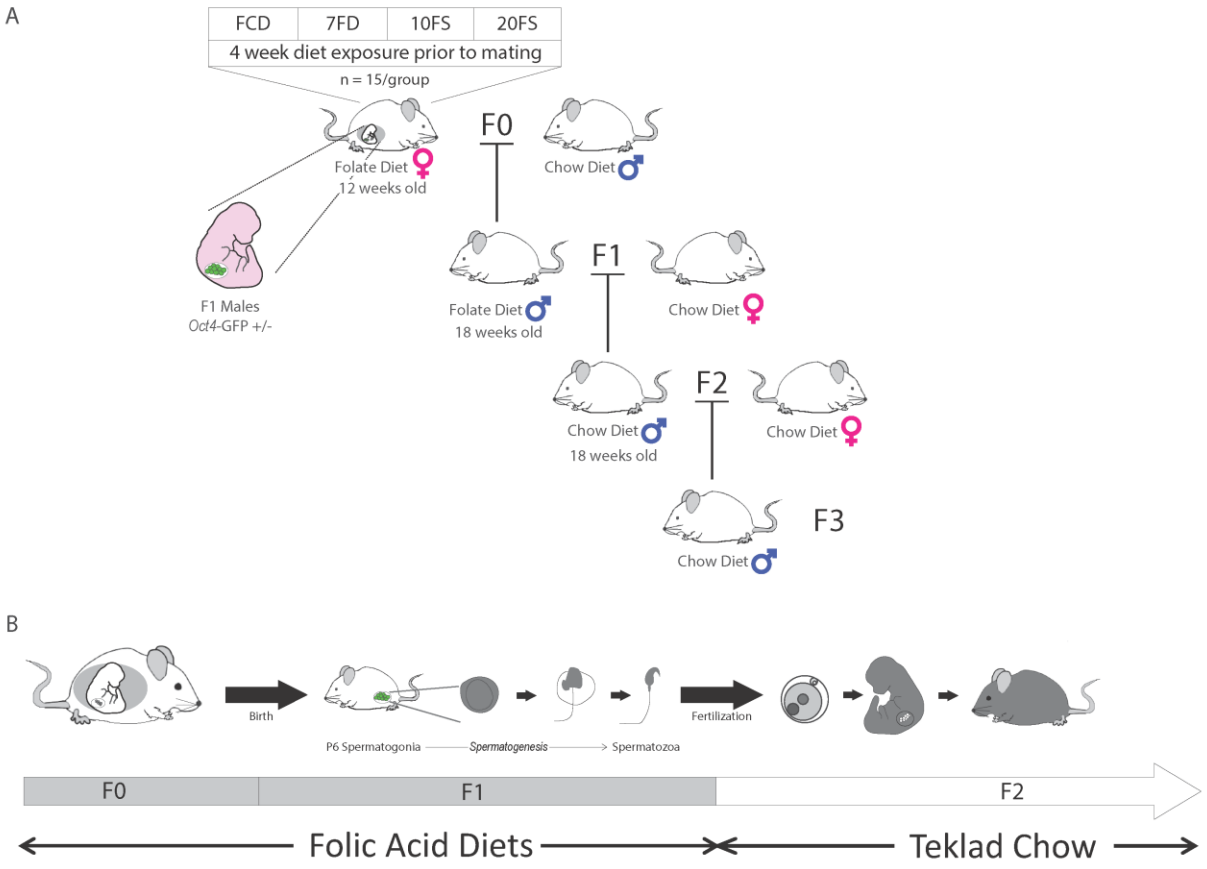

Fig 4.1. A) Mating scheme of grandpaternal lifetime exposure of diets up to unexposed F3 progeny.

Eight-week-old BALB/c F0 females were fed either a Ctrl, 7FD, 10FS or 20FS diet, (n = 15/group) from 4 weeks prior to breeding with BALB/c males fed with regular rodent chow and through gestation until weaning. F1 male pups were weaned onto the same prenatal experimental diet. At 18 weeks of age, F1 males were mated with rodent chow fed females. Females were maintained on the rodent chow through gestation and lactation. F2 male pups were maintained on rodent chow and bred with rodent chow females at 18 weeks to produce unexposed F3 litters. Separate eight-week-old BALB/c F0 females were similarly fed the diets (n = 5/group) from 4 weeks prior to breeding with Oct4-GFP +/- regular rodent chow fed BALB/c males through

gestation until weaning. F1 Oct4-GFP +/- males were sacrified at PND6 for spermatogonia isolation via fluorescent activated cell sorting. B) Time line of exposure to folic acid defined diets. (Ctrl, folic acid control diet; 7FD, 7× folate deficient diet; 10FS, 10× folic acid supplemented diet; 20FS, 20× folic acid supplemented diet).

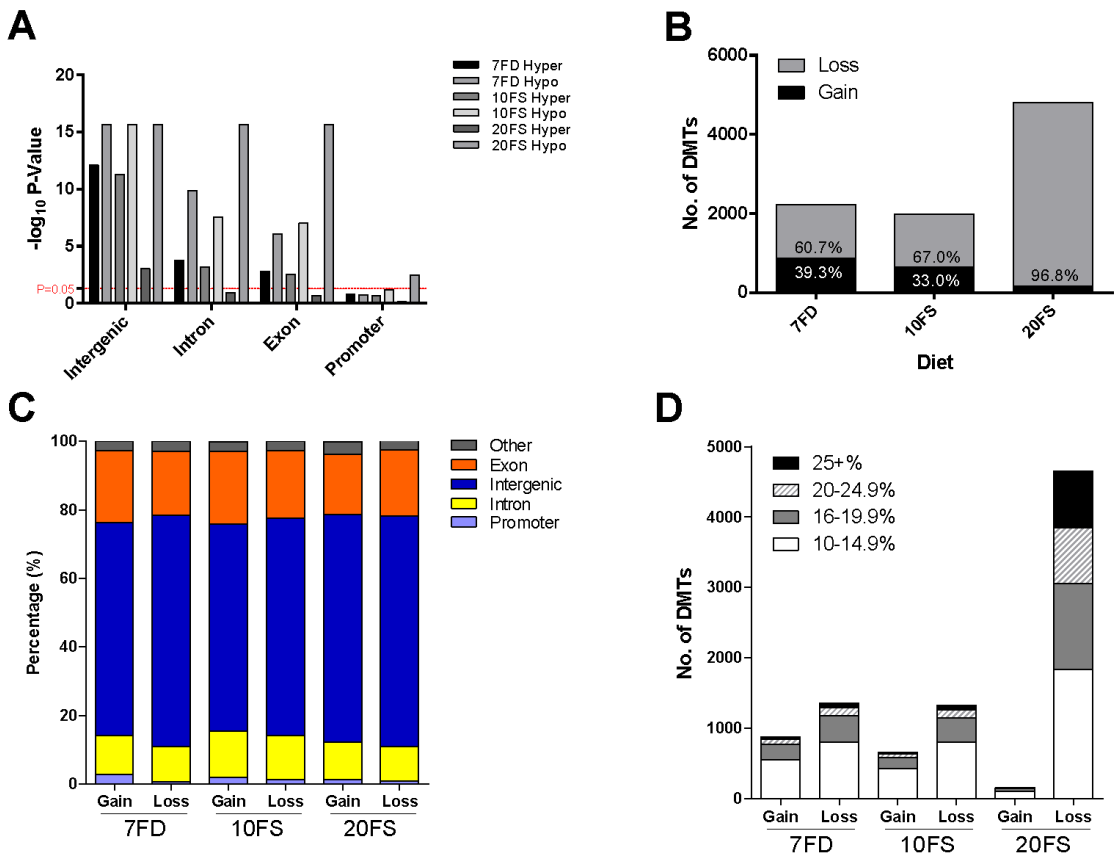

Figure 4.2. Reduced representation bisulfite sequencing data of effects in genome-wide DNA methylation in spermatogonia (F1) following perinatal folic acid supplementation and folate deficiency (n = 5/group).

A) Statistical significance (-log(PVal)) of difference in methylation of DMTs between diet groups compared to Ctrl. B) Prevalence of loss of methylation and gain of methylation DMTs. C) Localization of DMTs by genomic region. D) Categorization of DMTs by magnitude of methylation change. (7FD = 7x Folic Acid Deficient, 10FS = 10x Folic Acid Supplemented, 20FS = 20x Folic Acid Supplemented).

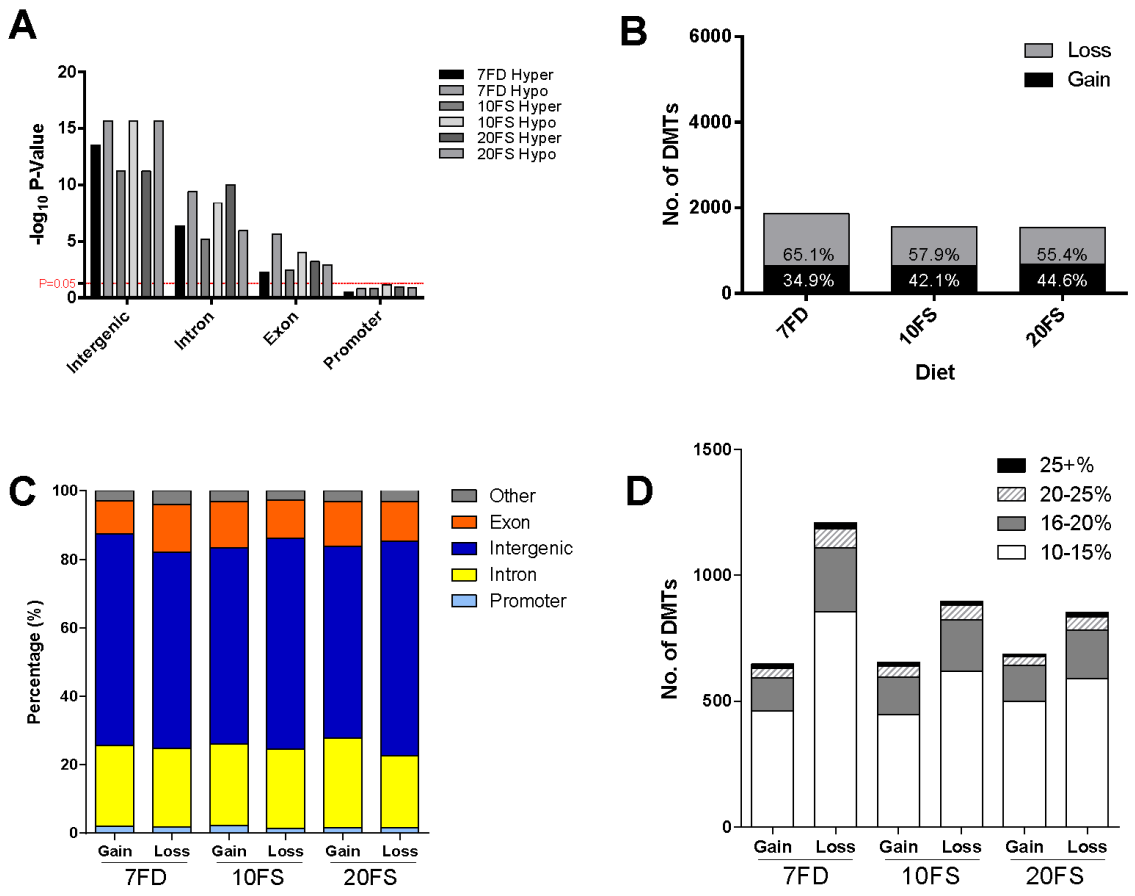

Figure 4.3. Reduced representation bisulfite sequencing data of effects in genome-wide DNA methylation in spermatozoa (F1) following lifetime folic acid supplementation and folate deficiency (n = 5/group).

A) Statistical significance (-log(PVal)) of difference in methylation of DMTs between diet groups compared to Ctrl. B) Prevalence of loss of methylation and gain of methylation DMTs. C) Localization of DMTs by genomic region. D) Categorization of DMTs by magnitude of methylation change. (7FD = 7x Folic Acid Deficient, 10FS = 10x Folic Acid Supplemented, 20FS = 20x Folic Acid Supplemented).

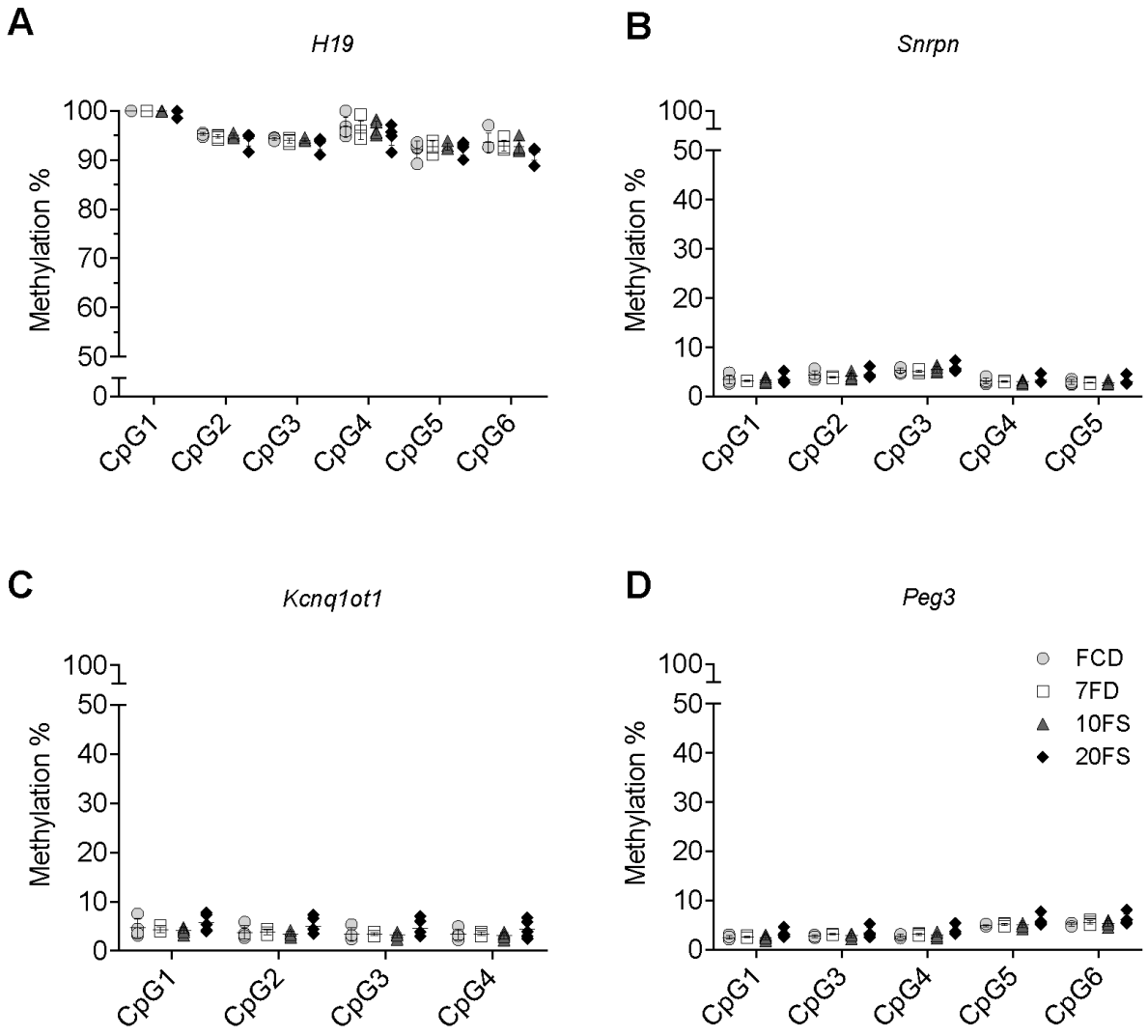

Figure 4.4. F2 spermatozoa DMR methylation at imprinted genes.

Loci of paternally methylated gene *H19* (n = 5/group) and maternally methylated genes *Snrpn*, *Kcnq1ot1*, *Peg1* and *Peg3* (n = 5/group) methylation levels were quantified by bisulfite pyrosequencing. (Ctrl = Folic Acid Control Diet, 7FD = 7x Folic Acid Deficient, 10FS = 10x Folic Acid Supplemented, 20FS = 20x Folic Acid Supplemented).

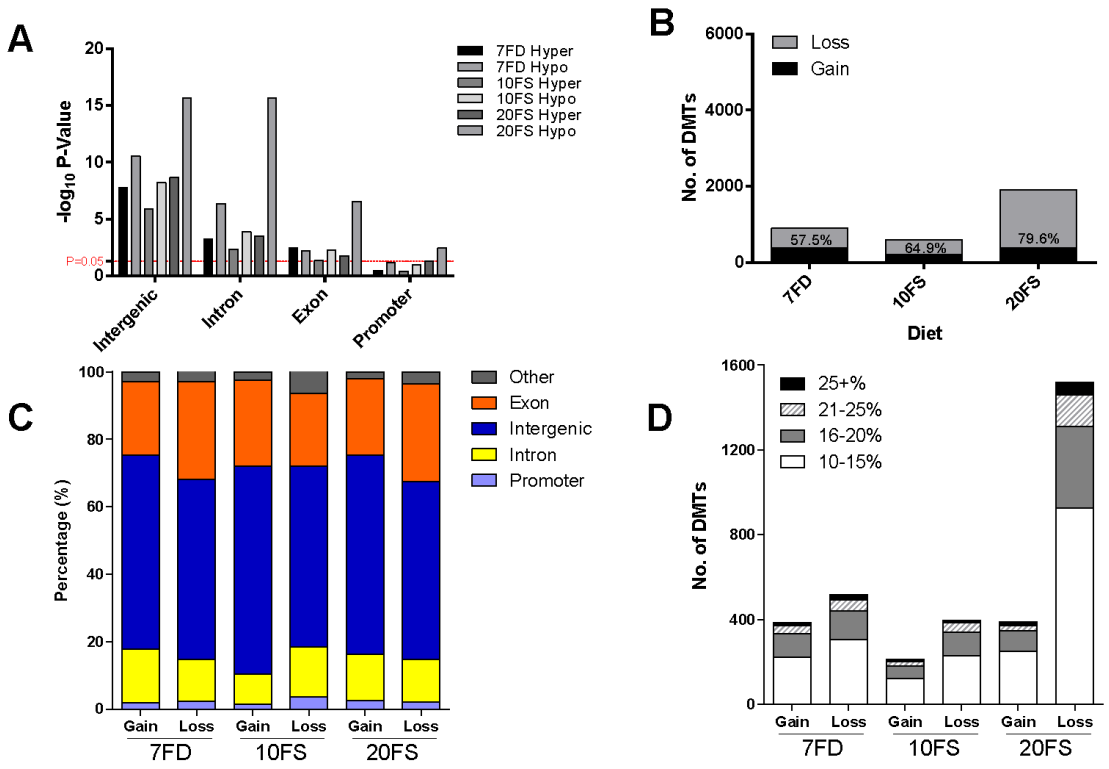

Figure 4.5. Reduced representation bisulfite sequencing data of effects in genome-wide DNA methylation in spermatozoa (F2) following lifetime paternal folic acid supplementation and folate deficiency (n = 5/group).

A) Statistical significance (-log(PVal)) of difference in methylation of DMTs between diet groups compared to Ctrl. B) Prevalence of loss of methylation and gain of methylation DMTs. C) Localization of DMTs by genomic region. D) Categorization of DMTs by magnitude of methylation change. (7FD = 7x Folic Acid Deficient, 10FS = 10x Folic Acid Supplemented, 20FS = 20x Folic Acid Supplemented).

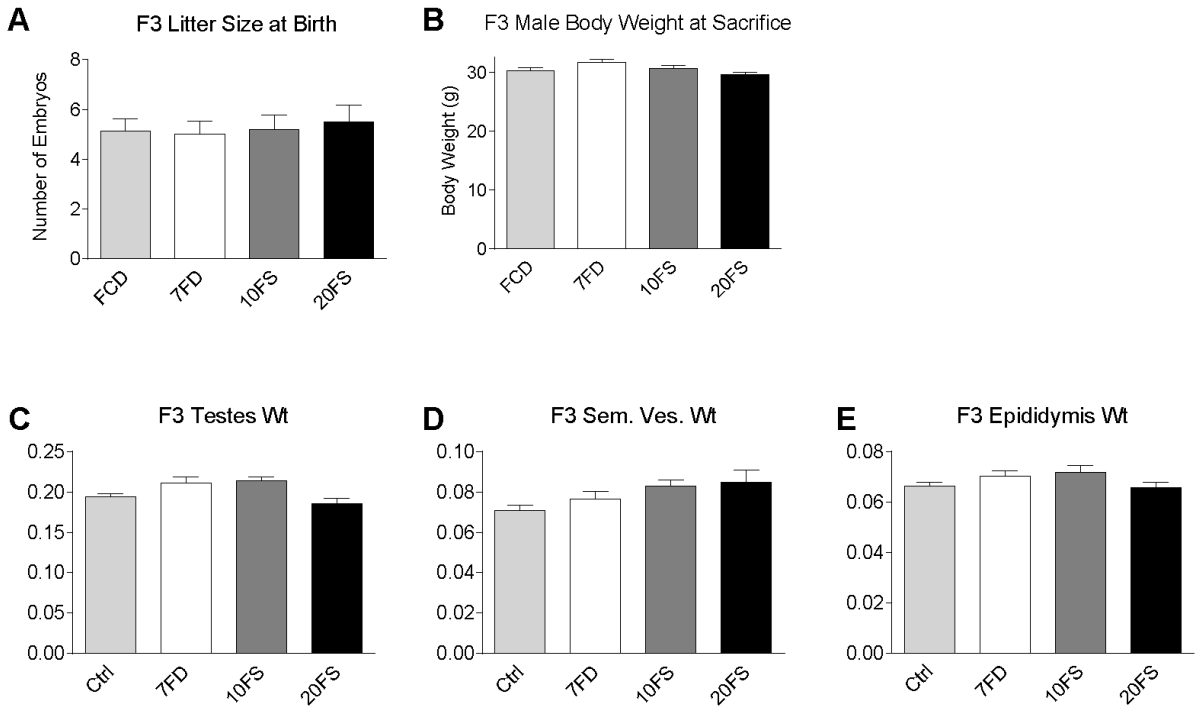

Figure 4.6. Health and reproductive effects of grandpaternal (F1) lifetime exposure to folic acid deficient or supplemented diets.

A) F3 litter sizes at birth (n = 13-15 litters/group) B) F3 adult male body weight (n = 25-32/group) C-E) F3 reproductive organ weights at sacrifice (n = 3-14 litters/group). (7FD = 7x Folic Acid Deficient, 10FS = 10x Folic Acid Supplemented, 20FS = 20x Folic Acid Supplemented).

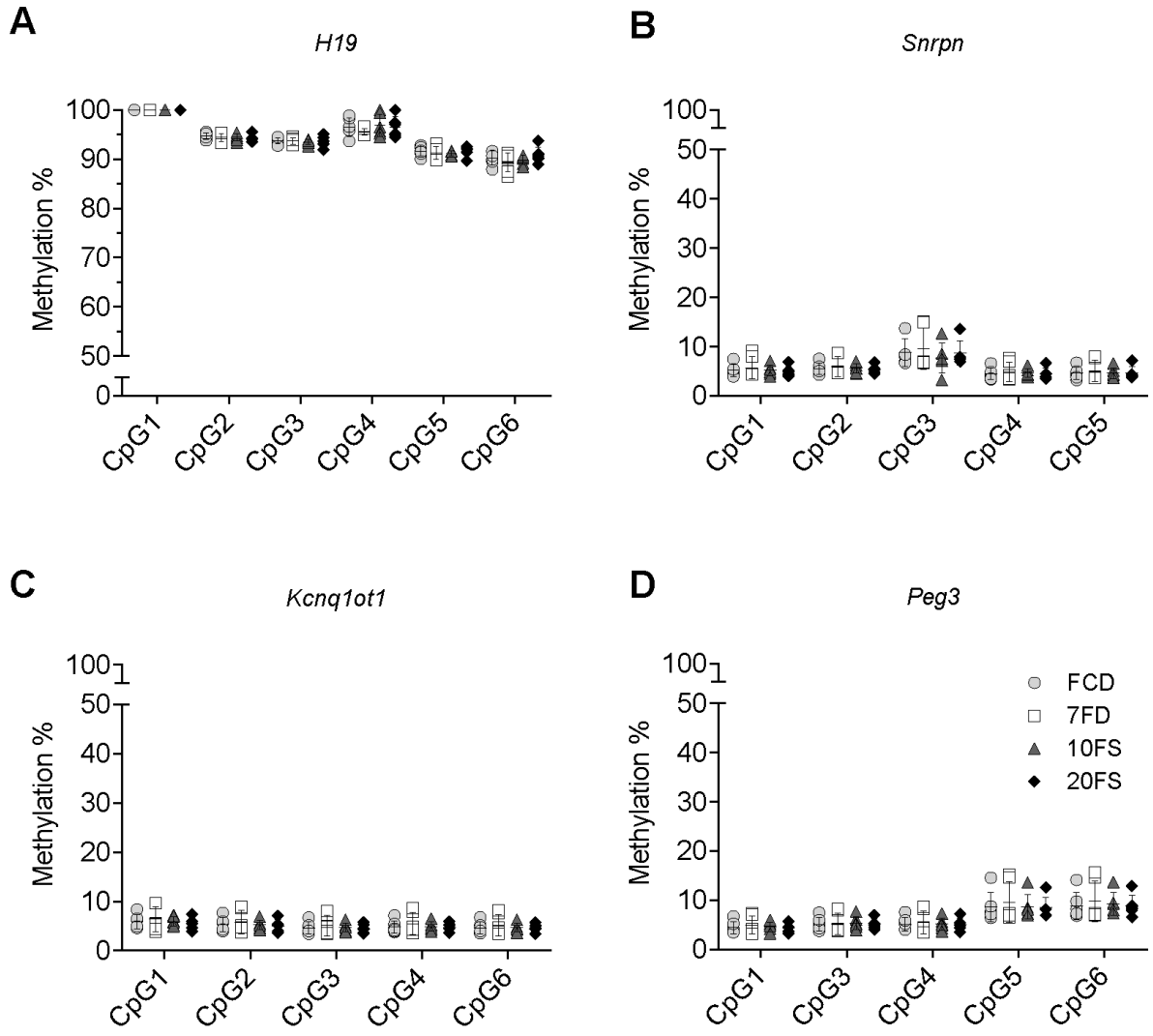

Figure 4.7. F3 spermatozoa DMR methylation at imprinted genes.

Loci of paternally methylated gene H19 (n =5/group) and maternally methylated genes *Snrpn*, *Kcnq1ot1*, *Peg1* and *Peg3* (n = 5/group) methylation levels were quantified by bisulfite pyrosequencing. (Ctrl = Folic Acid Control Diet, 7FD = 7x Folic Acid Deficient, 10FS = 10x Folic Acid Supplemented, 20FS = 20x Folic Acid Supplemented).

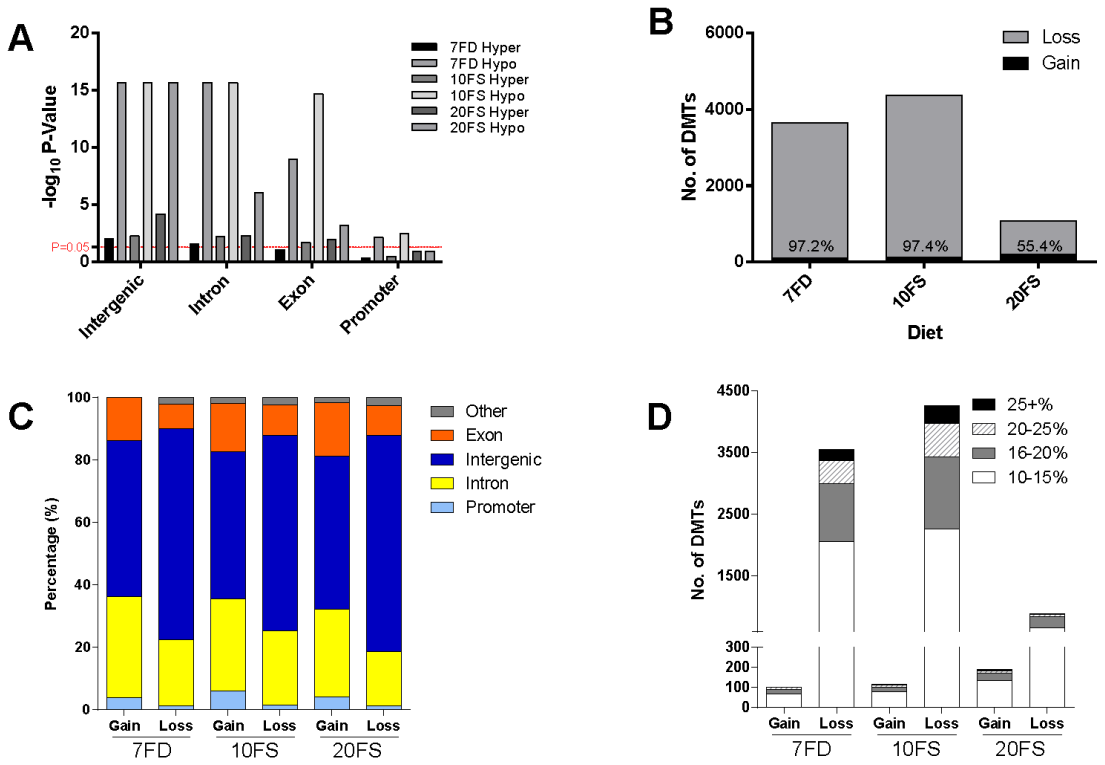

Figure 4.8. Reduced representation bisulfite sequencing data of effects in genome-wide DNA methylation in spermatozoa (F3) following grandpaternal (F1) lifetime folic acid supplementation and folate deficiency.

A) Statistical significance (-log(PVal)) of difference in methylation of DMTs between diet groups compared to Ctrl. B) Prevalence of loss of methylation and gain of methylation DMTs. C) Localization of DMTs by genomic region. D) Categorization of DMTs by magnitude of methylation change. (7FD = 7x Folic Acid Deficient, 10FS = 10x Folic Acid Supplemented, 20FS = 20x Folic Acid Supplemented).

Table 4.1. (A) 7FD (B) 10FS and (C) 20FS Top Three Enriched and Statistically Significant DAVID Enriched Pathways In DAVID Enriched Pathways In Spermatogonia In Genic DMTs

A

Term	Count	PValue	Benjamini
Protein phosphorylation	26	3.92E-04	5.17E-01
Phosphorylation	27	4.12E-04	3.17E-01
Extracellular matrix organization	10	5.82E-04	3.02E-01

B

Term	Count	PValue	Benjamini
Cell adhesion	24	1.60E-03	9.68E-01
Inflammatory response	19	1.78E-03	8.53E-01
Negative regulation of blood coagulation	4	3.30E-03	9.07E-01

C

Term	Count	PValue	Benjamini
Axon guidance	23	3.00E-07	9.32E-04
Nervous system development	36	1.12E-05	1.72E-02
Positive regulation of transcription from RNA PolII promoter	71	1.78E-05	1.82E-02
Cell adhesion	42	1.96E-05	1.51E-02

Table 4.2. (A) 7FD (B) 10FS and (C) 20FS Top Three Enriched and Statistically Significant DAVID Enriched Pathways In F1 Mature Spermatozoa In Genic DMTs

A

Term	Count	PValue	Benjamini
Regulation of Rho protein signal transduction	10	3.82E-04	6.13E-01
Axon guidance	14	4.12E-04	4.00E-01
Embryonic cranial skeleton morphogenesis	7	8.41E-04	5.02E-01

B

Term	Count	PValue	Benjamini
Aorta morphogenesis	7	1.33E-05	3.55E-02
Positive regulation of synapse assembly	13	1.61E-05	2.17E-02
Neuron migration	15	3.66E-04	2.83E-01

C

Term	Count	PValue	Benjamini
Positive regulation of gene expression	23	1.52E-04	2.71E-01
Positive regulation of transcription, DNA-templated	28	4.01E-04	3.40E-01
Positive regulation of transcription from RNA polymerase II promoter	41	4.40E-04	2.62E-01

Table 4.3. Intersection of differentially methylated tiles (DMTs) lists between Spermatogonia RRBS and F1 sperm RRBS

Diet	Total Persisting DMT (% All DMTs)	Same Direction (%)	Both Hyper	Both Hypo	Opposite Direction	Genic Persisting DMTs
7FD	108 (5.3)	59 (54.6)	24	35	49	38
10FS	80 (5.2)	52 (65.0)	13	39	28	27
20FS	172 (11.2)	108 (62.8)	4	104	64	48

Table 4.4. (A) 7FD (B) 10FS and (C) 20FS Enriched DAVID Enriched Pathways In Persistent DMTs between F1 Spermatogonia and F1 Mature Spermatozoa In Genic DMTs

A

Term	Count	PValue	Benjamini
Response to insulin	2	7.9E-02	1.0

B

Term	Count	PValue	Benjamini
Proteolysis	4	2.5E-02	9.9E-01

C

Term	Count	PValue	Benjamini
Cortical microtubule formation	2	7.9E-03	8.9E-01
Protein localization	3	1,1E-02	7.7E-01
Negative regulation of calcium ion dependent exocytosis	2	1.8E-02	8.0E-01

Table 4.5. Intersection of differentially methylated tiles (DMTs) lists between F1 spermatogonia RRBS and Aarabi et al. spermatozoa RRBS

Diet	Total Persisting DMT (% All DMTs)	Same Direction (%)	Both Hyper	Both Hypo	Opposite Direction	Genic Persisting DMTs
10FS	57 (4.6)	17 (29.8)	13	4	40	33
20FS	1474 (21.1)	1277 (86.6)	7	1270	197	345

Table 4.6. (A) 7FD (B) 10FS and (C) 20FS Top Three Enriched and Statistically Significant DAVID Enriched Pathways In F2 Mature Spermatozoa In Genic DMTs

A

Term	Count	PValue	Benjamini
Multicellular organism development	33	5.65E-05	7.71E-02
Cell adhesion	17	2.37E-03	8.15E-01
Positive regulation of MAPK cascade	7	4.45E-03	8.79E-01

B

Term	Count	PValue	Benjamini
Nervous system development	16	4.72E-06	4.42E-03
Dendrite morphogenesis	5	8.13E-04	3.17E-01
Multicellular organism development	22	1.14E-03	3.00E-01

C

Term	Count	PValue	Benjamini
Neuron migration	15	2.16E-05	2.48E-02
Regulation of Rho protein signal transduction	12	1.80E-05	4.10E-02
Multicellular organism development	54	9.06E-05	6.79E-02

Table 4.7. Intersection of differentially methylated tiles (DMTs) lists between F1 spermatozoa RRBS and F2 spermatozoa RRBS

Diet	Total Persisting DMT (% All DMTs)	Same Direction (%)	Both Hyper	Both Hypo	Opposite Direction	Genic Persisting DMTs
7FD	96 (10.6)	44 (45.8)	16	28	52	43
10FS	70 (11.5)	32 (45.7)	9	23	38	23
20FS	143 (7.5)	72 (50.3)	20	52	71	56

Table 4.8. (A) 7FD (B) 10FS and (C) 20FS Top Three Enriched and Statistically Significant DAVID Enriched Pathways In F3 Mature Spermatozoa In Genic DMTs

A

Term	Count	PValue	Benjamini
Cell adhesion	38	4.62E-06	1.20E-02
Neural crest cell migration involved in autonomic nervous system development	4	1.25E-03	8.04E-01
Positive regulation of cell migration	17	1.66E-03	7.65E-01

B

Term	Count	PValue	Benjamini
Nervous system development	35	6.0E-05	6.4E-02
Learning	12	1.3E-04	8.1E-02
Multicellular organism development	73	6.0E-05	9.4E-02

C

Term	Count	PValue	Benjamini
Cell adhesion	16	1.5E-03	4.9E-01
Organ morphogenesis	7	2.8E-03	6.1E-01
Negative regulation of cell proliferation	14	1.4E-03	6.1E-01

Table 4.9. Intersection of differentially methylated tiles (DMTs) lists between F2 spermatozoa RRBS and F3 spermatozoa RRBS

Diet	Total Persisting DMT (% All DMTs)	Same Direction (%)	Both Hyper	Both Hypo	Opposite Direction	Genic Persisting DMTs
7FD	105 (2.9)	61 (58.1)	2	59	44	39
10FS	49 (1.1)	26 (53.1)	2	24	23	18
20FS	68 (6.3)	41 (60.3)	6	35	27	25

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Chapter V: General Discussion

5.1. Summary

The advent of affordable single base resolution sequencing ushered in a new era in epigenomics. It became economically feasible to investigate the effects of environmental exposures on epigenetics that possibly underlie the resulting physiological abnormalities. The folate pathway plays a major role in supplying methyl donors required for DNA methylation, a keystone of epigenetic modifications. This makes folic acid an ideal candidate for investigating whether this common diet sourced nutrient can cause epigenetic perturbations that could be transmitted across multiple generations and affect reproductive fitness.

This thesis focused on investigating the reproductive effects of dietary folate exposures during various key windows of development, along with the use of genome-wide approaches to explore the heritability of underlying epigenetic changes in DNA methylation across generations. In **Chapter II**, we investigated whether folate deficiency or folic acid supplementation, encompassing the entirety of male germ cell development, had reproductive consequences and altered DNA methylation at a global imprinted gene level in mature sperm and future progeny. In **Chapter III**, we targeted exposures to the prenatal and early postnatal windows of development of female germ cells, which excludes the time of DNA methylation re-establishment. We therefore explored whether exposures in a phase, which avoided *de novo* methylation, resulted in future reproductive consequences associated with epigenetic alterations. In **Chapter IV**, we sought to contrast the effects on DNA methylation between exposures isolated to the prenatal window of development of male germ cells and exposures spanning complete male germ cell development, while characterizing the transgenerational heritability of DNA methylation changes from such exposures.

5.2 Intergenerational Heritability Associated with Paternal Folate Exposures

In Chapter II, we demonstrated that lifetime FD and FS in males both result in reproductive effects with decreased sperm counts and increased abnormal offspring outcomes. It has been established that direct FD and FS during embryonic development is harmful to embryo health and is associated with the development of adult-onset disease in mouse models (Pickell et al., 2009, Pickell et al., 2011, Mikael et al., 2013). Amongst these effects, both FD and FS diets have been identified to decrease SAM levels in mice (Devlin et al., 2004, Christensen et al., 2015l, Bahous et al., 2019). While these studies focussed on the direct effects of in utero FD and FS exposures to embryonic development, demonstrating defects in placenta, cardiac, and neural tube formation, we demonstrated that the effects extended to reproductive organs and tissues. The manuscript forming this chapter is the first to discover that high parental FS can have detrimental effects on germ cell fitness and future progeny development. The implications of these findings are widespread, in particular considering current guidelines to supplement women of child-bearing age (Wilson et al., 2015). Excessive supplementation could lead to nocuous effects through existing negative feedback mechanisms and hard cut-offs need to be established. Additionally, this chapter identifies that both FD and FS diets are associated with increased variance in the DNA methylation of imprinted genes. While Lambrot et al. 2013 showed that FD exposure in male mice altered DNA methylation in sperm, our study is novel in demonstrating that DNA methylation alterations in sperm persist in the offspring with abnormal imprinted gene methylation in both embryonic and extra-embryonic tissue.

Genomic imprinting contributes significantly to embryogenesis, reproduction, and gametogenesis. Abnormal imprinted gene methylation is associated with various syndromes, notably neurodevelopmental disorders (reviewed in Elhamamsy, 2017). Changes in the variance of imprinted gene methylation between the tissue of non-directly exposed progeny suggests the

possibility of the inheritance of DNA methylation alterations in sperm, potentially causing expression dysregulation in progeny. With such critical roles in embryonic development and other postnatal developmental processes, imprinted genes have evolved to be tightly regulated by multiple mechanisms through epigenetic reprogramming (Kalish and Bartolomei, 2014, Keverne, 2015). In light of the methylation changes found at imprinted genes, it is plausible that broader genome-wide effects of the diets existed.

Chapter II applied a widespread lifetime exposure to the diets, in order to elicit any epigenetic effects which were posited to be likely to be subtle. Numerous windows of epigenetic events occur through development (Ly et al., 2015, Clarke and Vieux, 2015). While our study emphasises the detrimental effects of both in utero FD and FS, other studies have replicated the detrimental effects of high FS but also highlighted the protective effects of FS when in conjunction with other environmental exposures such as assisted reproductive therapy techniques and organic pollutants (Rahimi et al., 2019, Herst et al., 2019). Exploring the windows of exposure specific to key epigenetic events would benefit the understanding of epigenetic adaptability and vulnerability to environmental factors.

5.3 FD and FS during *De Novo* Methylation are not the Sole Cause of Epigenetic Alterations from Exposures to Developing Germ Cells

In Chapter III, our diet exposures were restricted to the prenatal window of development. In doing so, FD and FS exposures of fetal germ cells excluded the *de novo* methylation phase of epigenetic reprogramming that would occur in postnatal growing oocytes (Clarke and Vieux, 2015). We showed that in utero FD and high FS in females resulted in impaired reproductive fitness with increased abnormal offspring outcomes in the litters of progeny. In addition, these reproductive effects were also associated with genome-wide DNA methylation alterations in

placentas and brains. Alterations in DNA methylation, as quantified in units of differentially methylated tiles (DMTs), were found scattered through genic and non-genic regions. Amongst genic DMTs, we identified enrichment of genes involved in embryo and nervous system development.

Rigorous research has implicated the role of folates in neurophysiology, a fundamental component of the 'Developmental Origins of Behaviour, Health, and Disease' concept (Van den Bergh, 2011). Starting from its widespread use in national dietary fortification in grain products in order to reduce neural tube defect incidence, the mechanistic role of this B vitamin, has been enigmatic (Lee and Gleeson, 2020). Concomitantly, in utero FD and FS, and postnatal FD have been described to result in abnormal cognitive dysfunction and brain development (Jadavji et al., 2015, Bahous et al., 2017, Bahous et al., 2019, Cosin-Tomas, et al., 2020). Both FD and high FS were associated with epigenetic changes including abnormal DNA methylation and altered expression of genes encoding DNA methylation enzymes. Combined with other disturbances in choline metabolism, the abnormal neurophysiology from folate exposures is proposed to derive from SAM availability, and further downstream through epigenetic aberrations disrupting gene expression.

We originally hypothesized that excluding the window of DNA methylation re-establishment in germ cell development would minimize the epigenetic effects of the FD and FS diet exposures. It was thus somewhat surprising that we found extensive genome-wide DNA methylation alterations. This suggests that other events and windows of epigenetic reprogramming are equally important for the appropriate epigenetic modifications. Although the *de novo* DNA methylation wave in germ cell development was spared from the exposure in our study design, another wave of *de novo* DNA methylation occurs earlier in embryo development.

Perturbation of DNA methylation re-establishment in embryonic cells, prior to gonocyte lineage specification, would have the potential to transmit altered marks that resist erasure in germ cell epigenetic reprogramming. It is not inconceivable that DNA methylation erasure could be impacted by the folate diet exposures. As FD and FS have been identified to alter *Dnmt1* expression, these compensatory mechanisms may lead to inappropriate maintenance of DNA methylation marks (Bahous et al., 2019). Furthermore, other epigenetic modifications have been characterized to undergo dynamic changes during prenatal development of the oocyte and embryo such as histone methylation (Andreu-Vieyra., 2010, Brind'amour and Lorincz, 2020). Histone modifications are unquestionably linked to the DNA methylation. Thus, altered histone methylation patterns from FD and FS fluctuations in SAM availability could be reflected in the DNA methylation landscape (Ooi et al., 2007, Law and Jacobsen, 2010, Zhang et al., 2010, Guo et al., 2015, Au Yeung et al., 2019).

A major contribution of Chapter III to the field of epigenetics is to provide evidence that the *de novo* methylation window of epigenetic reprogramming in germ cells is not the sole window of development that is susceptible to influences from environmental factors. This chapter also demonstrated that, while known to cause abnormal neurophysiology to a directly exposed developing embryo, in utero FD and FS exposure extends via female germ cells to future progeny reflected in brain and placenta DNA methylation alterations. Notably, DNA methylation changes in genic regions were enriched for genes relevant to nervous system development. Thus, with such broad effects of environmental exposures on reproductive fitness despite excluding *de novo* methylation in germ cell epigenetic reprogramming, investigating the exposures directly after *de novo* re-methylation could further help characterize the underlying mechanism behind susceptible epigenetic marks.

5.4 Neonatal Spermatogonia Carry an Increased Burden of Altered DNA Methylation Immediately after In Utero FD and FS Exposure

To complement Chapters II and III, we investigated the effects of FD and FS on DNA methylation in early postnatal day 6 spermatogonia, before the completion of spermatocytogenesis and spermiogenesis. As the major wave of *de novo* methylation occurs prior to birth, DNA methylation patterns in neonatal spermatogonia would exhibit any epigenetic effects due to direct exposures during methylation re-establishment. As mentioned previously, this population of cells precedes spermatogenesis, where there is potential loss of severely affected germ cells to apoptosis, and spermiogenesis, during which chromatin remodeling may also induce further DNA methylation changes.

As Chapter II showed that decreased sperm counts resulted from FD and high FS diets, it is reasonable to postulate that a significant portion of severely affected germ cells from the diets do not make it to mature spermatozoa stages in adulthood. Our results from Chapter IV showed that the most dramatic effect of the diets, in terms of numbers of DMTs, was found in neonatal F1 spermatogonia. This supports our original hypothesis of spermatogenesis acting as a checkpoint for identifying severely affected germ cells, leading to these unfit germ cells being filtered out by apoptosis (Kim et al., 2002, Hikim et a., 2003). As such, exposures that occur later in postnatal spermatogenesis, although not impacting the major *de novo* events, would bypass this quality assurance phase and possibly lead to other exposure window unique epigenetic changes.

Previous work by our group has demonstrated that postnatal FD and FS results in altered DNA methylation in sperm (Aarabi et al., 2018). A postnatal exposure in male germ cells would occur after DNA methylation re-establishment. This implicates other epigenetic events in being vulnerable to environmental exposures, another reason which compelled us to explore the early

spermatogonia population. Indeed, cross-matching our RRBS data to the Aarabi et al. RRBS data yielded approximately one-fifth of DMTs being present in germ cells from both prenatal and postnatal FS exposures. These results suggest that postnatal male germ cell development is also susceptible to influence from FS and possibly FD. Notably, postnatal male germ cell development includes key epigenetic events such chromatin remodeling during spermiogenesis. Interestingly, retained histones from chromatin remodeling have been shown to be critical for the reproductive capacity of the sperm, and future embryonic development. As such, these altered marks in sperm from prenatal or postnatal exposures carry a lot of potential for harm in the next generation, and if not properly reprogrammed in the offspring, may be inherited through multiple future generations.

5.5 Epigenetic Reprogramming is Insufficient in Erasing Altered DNA methylation from Folate Environmental Exposures after Three Generations.

Ultimately, a key question emerges from Chapters II and III regarding the heritability of an altered epigenetic landscape, and specifically perturbed DNA methylation. Theoretically, epigenetic reprogramming allows for the regulation of improper epigenetic patterns. Therefore, is altered DNA methylation from folate exposures propagated to future generations? To determine if the aberrant epigenetic effects resulting from the folate diets were resistant to epigenetic reprogramming, the second objective in Chapter IV assessed the DNA methylome in the sperm of up to three generations following the original F0 exposure. All three diet exposures resulted in both hyper- and hypomethylated DMTs in the sperm, which remained present up to the F3 generation. While abnormal methylation in F2 sperm did not correlate with any signs of reproductive effects seen in the F3, the presence of DMTs in all three generations of sperm showed that altered DNA methylation can resist multiple waves of epigenetic reprogramming.

A recurrence of both hypo- and hyper-methylation resulting from FD and FS was seen in all exposure groups, across all three generations. Hypomethylation can result from FD through decreased SAM reserves. Similarly, high FS has been shown to induce a feedback response that reduces MTHFR activity and thereby reduces SAM generation (Christensen et al., 2015). However, the presence of both hypo- and hypermethylated tiles is slightly perplexing. One possibility is that reduced SAM pools induce methylation errors resulting in stochastic genome-wide hypermethylation despite a reduced availability of SAM. However, it has also been shown that environmental exposures can alter histone retention sites. Indeed, disrupted H3K27 methylation, a repressive histone mark, in sperm has been reported from environmental exposures up to two generations later (Skinner et al., 2018). As repressive histone marks can promote DNA methylation, differential histone retention and alterations in histone marks may be, in part, driving the hypermethylation seen from these diets, in spite of decreases in SAM availability. Altogether, the literature suggests that the environment, which includes diet, can have long lasting consequences on male reproductive fitness, that can linger in future generations (Bailey et al., 2020).

As expected, the largest exposure effect, with regards to the number of DMTs, was in early F1 spermatogonia, followed by a reduction of DMTs across all three groups following spermatogenesis in mature F1 spermatozoa. While a trend of decreased aberrant DNA methylation was observed in F2 sperm, DMTs inexplicably rose in the 7FD and 10FS exposure group F3 spermatozoa. Another study similarly found that epigenetic changes are not faithfully transmitted across generations, but instead may translate to a stochastic cascade of changes from one generation to the next (Blake et al., 2021). It is proposed that the altered methylation in one generation may result in physiological changes that activate compensatory mechanisms within

that generation's somatic tissue and germ line. These compensatory mechanisms in the germ line subsequently direct gene expression changes in the next generation, that are reflected in their own epigenetic changes in that respective generation, which are distinct from the original changes due to the exposure.

The enigmatic concept of epigenetic memory is further explored in a recent study that showed that environmental pollutants could result in epigenetic perturbations in miRNA across up to four generations (Herst et al., 2019). Yet, while the degree of epigenetic perturbation fluctuated in later generations, reproductive competence remained compromised despite the absence of visible sperm parameter abnormalities (Lessard et al., 2019). Thus, the oscillation in DNA methylation changes across the three sperm generations we observe in Chapter IV has been seen in other studies of environmental exposures and transgenerational exposures. The non-congruency in the number of DMTs across generations, we postulate, is possibly produced by the effects from accrued epimutations such as perturbed histone modifications, non-coding RNAs, and DNA methylation, which create a cascade of epigenetic effects across generations. Accordingly, to truly grasp the epigenetic effects of folate diets or any exposure, parallel studies on all the integrated components forming the epigenome are required.

Chapter VI: Future Directions and Conclusions

6.1. Future directions

6.1.1 Epigenetic Landscape

While we have looked at DNA methylation in neonatal spermatogonia, we have retained samples and are currently working towards collaborating with another group in order to investigate the histone marks in these early germ cells. As previously stated, spermatogonia have not undergone chromatin remodeling. Therefore, looking at histone modifications, with emphasis

on methylation marks that have been shown to have roles in development such as H3K4, H3K9, and H3K27 methylation, may help elucidate the true impact of the folate exposures on the epigenome, and reveal the underlying mechanism of FD and FS related perturbations in reproductive outcomes. A transgenic mouse model showed that disruption of histone methylation during spermatogenesis can result in transgenerational inheritance of histone methylation changes (Siklenka et al., 2015). Moreover, investigating the effects of FD and FS on non-coding RNA expression may similarly help ascertain folate effects on reproductive fitness. For instance, small non-coding RNAs in sperm have been linked to intergenerational inheritance of metabolic disorders (Zhang et al., 2018). As discussed, both in Chapter IV's discussion and Chapter V, the incongruency in sperm DMTs across generations may heavily be driven by histone modifications and altered histone marks directing DMTs in divergent regions in the genome. Assessing histone marks across all three generations in sperm will allow for an improved understanding of epigenetic memory from the diet effects.

Decreases in SAM and MTHFR expression have previously been shown to result from FD and FS respectively, suggesting that methylation reactions can be impacted by these diets (Devlin et al., 2004, Aarabi et al., 2018). Determining the effects of FD and FS on the expression of DNA methyltransferases would further our understanding of the epigenetic effects and the underlying mechanism. *Dnmt1* and *Dnmt3a* have been shown to have altered expression levels in mouse brain following high folate diets (10-fold folate supplemented; Bahous et al., 2017, Bahous et al., 2019). Investigating the expression and protein level of DNMTs may shed light on the degree to which reduced SAM impairs *de novo* and maintenance DNA methylation through the methylating machinery, rather than SAM availability. The assessment of target windows of development include the early embryo and various stages of testis development including ~E9.5

after PGC DNA methylation erasure, and from E13.5 through E18.5 where *de novo* methylation takes place. In addition, investigating apoptosis levels in the postnatal testis during spermatogenesis should be considered.

6.1.2 Prenatal spermatogonia

We investigated neonatal spermatogonia in Chapter IV, in order to assess the effects of the FD and FS diet exposures directly on the *de novo* methylation phase in germ cells. As a primary wave of epigenetic reprogramming occurs upon fertilization, targeting primordial germ cells, either before the second-wave, at ~E9.5, or after the second wave of DNA methylation erasure in PGCs, at ~E13.5, would differentiate the effects of the folate exposures on the first wave from the second wave of *de novo* DNA methylation. The first wave of epigenetic reprogramming is particularly interesting as much of the methylation lost and regained is at repeat sequences. Inappropriate methylation at repeat sequences could result in activation of transposable elements and create genomic instability. While our studies showed large numbers of DMTs in intergenic with some localization to repeat sequences, there remains a need to assess repeat activity. Additionally, we identified in Chapter II increased variance in imprinted gene methylation. As imprinting is acquired early in male germ cells, prior to the onset of meiosis, investigating sites subject to early methylation may be worthwhile. While we hypothesized in Chapter II that decreased sperm counts following in utero FD and FS may have been the result of increased apoptosis during spermatogenesis, this remains to be experimentally confirmed. Immunohistological studies in post-pubertal F1 males aimed at identifying markers of apoptosis, such as caspase protease family enzymes and BCL2 family proteins, would reveal increased levels of apoptosis during spermatogenesis.

6.1.3 Gene Expression in the Context of Altered DNA Methylation

Altered methylation of repeat sequences can result in aberrant repeat sequence activation. As such, it would be of interest to investigate whether the 30-50% of DMTs we identified as being in repeat regions translate into activation of repeat expression, and ultimately, retrotransposition.

We identified an enrichment of genes related to pathways involved in nervous system development. Examining if altered expression of these genes, and ultimately protein level, results from the perturbation in DNA methylation may further our understanding of how reproductive outcomes are impacted. Doing so may strengthen the evidence in the literature that these diets have dramatic neurophysiology effects in mice.

6.1.4 Limitations of RRBS

To date, whole genome bisulfite sequencing remains the gold standard for DNA methylation analysis, with advantages of low-input and single-nucleotide resolution (Rauluseviciute et al., 2019, Gouil, 2019). RRBS incorporates these advantages, while however, introducing several drawbacks which create limitations to the technique. RRBS is susceptible to bias from incomplete bisulfite conversion and PCR amplification, and selects for CpG dense regions, therefore focuses on only 10% of the CpG sites in the mouse genome. As sequencing technologies continue to be developed, possible solutions are emerging. For example, lower input and single cell sequencing decreases the PCR amplification bias, long-read sequencing by Nanopore technology erases the mappability issue of short reads, and affinity enrichment-based methods such as MeDIP bypass the need for harsh bisulfite treatments to DNA (Singer 2019, Gigante et al., 2019). Until breakthroughs of these technologies and the reduction in costs of their applications occur however, DNA methylation analyses will remain bound to the limitations of these current methods.

6.2. Conclusions

In this thesis, we have shown that both FD and high FS exposure during various windows of epigenetic reprogramming result in decreased reproductive fitness associated with genome-wide perturbations in DNA methylation of germ cells that can escape epigenetic reprogramming. These perturbations can persist up to the F2 generation for in utero and early postnatal female exposures, and up to the F3 generation for in utero and postnatally exposed males. Genic regions associated with pathways related to embryo and nervous system development appeared to be at increased susceptibility to DNA methylation perturbations. Therefore, this thesis adds to the growing body of literature emphasizing plasticity of the epigenome, and how key windows of development are vulnerable to environmental influence causing epigenetic changes that can be disseminated across multiple generations with potentially detrimental effects. This further reinforces the need for establishing a safe upper limit of folate supplementation in order to prevent the deleterious effects of over-dosing.

Chapter VII: References

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