FOLATE DEFICIENCY IN UTERO AND POSTNATALLY IMPAIRS SPERMATOGENESIS, FERTILITY, EPIGENETIC PROGRAMMING AND OFFSPRING HEALTH IN A MOUSE MODEL

by

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This thesis is dedicated to…

…My parents, who have always encouraged and supported me with unconditional love. I could not have accomplished any of this without their guidance.

…My grandparents, for your trust in my ability, which has always motivated me throughout my life.

…My boyfriend, for always being there for me, and whose patience and optimism were essential elements through every step of the way.

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ABSTRACT

Previous studies have shown that folate is a determinant of male reproductive health but the underlying molecular mechanisms are unknown. The objective of this study was to determine the impact of a low folate diet, during embryonic development and into adulthood on histone methylation, DNA integrity, spermatogenesis, fertility and offspring health. C57/BL6 females were fed either a folate-sufficient (FS, 2 mg of folate/kg of diet) or a folate-deficient diet (FD, 0.3 mg of folate/kg of diet) two weeks prior to breeding, through pregnancy and lactation. Weaned male pups received the same diet as their mother until sacrifice. Histological analysis of postnatal testis revealed a delay in meiotic onset in FD males. While germ cells were affected by reduced folate, Sertoli and Leydig cells were not. In breeding trials, FD males had reduced fertility in comparison to FS males. Remarkably, increased DNA double strand breaks were found in pachytene spermatocytes from FD males. However, these DNA breaks were repaired in later stages of spermatogenesis, as no difference of DNA breaks was detected by COMET assay in spermatozoa. Epigenetic programming was disturbed in the sperm of FD mice with a reduction in histone H3-lysine4 mono-methylation and in histone H3-lysine9 mono-methylation. The pregnancy outcomes were compromised by folate deficiency, as evidenced by increased occurrence of resorption and abnormalities of placental and embryonic development in the litters sired by FD males. These results suggest adequate folate intake is required for normal spermatogenesis, histone methylation and offspring health.

Résumé

Les recherches précédentes ont montrée que la folacine est un élément important requis dans la santé reproductive des mâles. Pourtant, les mécanismes moléculaires restent inconnus. L'objectif de cette recherche était de déterminer l'influence exercée par un régime alimentaire faible en folacine sur la méthylation de l'histone, l'intégration d'ADN, la spermatogenèse, la fertilité et la santé des progénitures pendant le développement embryonnaire jusqu'à l'âge adulte. Les femelles C57⁄BL6 ont été offertes une diète contenant de la folacine suffisante (FS, 2 mg folacine/kg), ou de la folacine déficiente (FD, 0.3 mg folacine/kg), durant les deux dernières semaines avant l'accouplement, pendant toute la période de la gestation et ainsi que la lactation. Les progénitures mâles sevrés ont été nourris d'un régime semblable à celui de leurs mères jusqu'à leurs sacrifices. L'analyse histologique des testicules postnatals indique un retard dans le processus de la méiose dans les mâles FD. Tandis que les cellules germinales ont été influées par la réduction de la folacine, celles de Sertoli et Leydig ne l'étaient pas. Selon les essais de reproduction, les mâles FS, avaient une baisse de fertilité en comparaison à des mâles FD. Remarquablement, une augmentation dans les cassures double brins d'ADN ont étaient trouvées dans les pachytènes des spermatocytes dans les mâles FD. Mais ces cassures double brin d'ADN ont été réparés dans les spermatozoïdes car aucun différence n'avait été détecté dans les cassures double brins d'ADN par l'analyse de COMET. La programmation épigénétique a été perturbée dans les spermes des souris FD accompagner par une réduction de l'histone H3-lysine 4 mono-méthylation et l'histone H3-lysine 9 mono-méthylation. Les résultats des grossesses ont été compromis par une déficience de folacine comme démontré par l'augmentation dans le taux de la résorption et des anomalies dans le développement des placentas et des

embryons chez les progénitures fécondés par des mâles FD. Ces résultats suggèrent qu'une consommation suffisante de la folacine est nécessaire à la spermatogénèse normale et l'intégrité de l'ADN.

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LIST OF ABBREVIATIONS

5, 10-MethyleneTHF: 5,10-methylene-tetrahydrofolate
5-MTHF: 5- Methyltetrahydrofolate
ALD: Adult Leydig cell
DHF: Dihydrofolate
DHFR: Dihydrofolate reductase
DNMT: DNA methyltransferases
DSB: DNA double stand breaks
E18.5: Embryo day 18.5
FD: Folate-deficient
FD12.5: Fetal day 12.5
FLD: Fetal Leydig cell
FS: Folate-sufficient
FSH: Follicle stimulating hormone
H3K4: Histone 3 at lysine 4
H3K9: Histone 3 at lysine 9
HE: Hematoxylin-eosin
HKDMs: Histone lysine demethylases
HKMTs: Histone lysine methyltransferases
HMTs: Histone methyltransferases
IAP: Intracisternal A particle
IHC: Immunohistochemistry
ILC : Immature Leydig cell
LH: Luteinizing hormone
me1: Monomethylation
me2: Dimethylation
me3: Trimethylation
MTFs: Methyltransferases
MTHFR: Methyl-tetrahydrofolate reductase
MTR: Methionine synthase
PGC: Primordial germ cell
PND: Postnatal day
PTM: Post-translational modification
SAH: S-adenosylhomocysteine
SAM: S-adenosylmethionine
SCP3: Synaptonemal complex protein 3
THF: Tetrahydrofolate
TUNEL: Terminal deoxynucleotidyl transferase-mediated dUTP Nick End Labeling

Chapter 1

Introduction

Up to 10-15% of couples in North America are infertile and one third of the cases are due to men. Even thought the reproductive health of men is as important as women, since they contribute to half of the offspring's genome and epigenome, less is known about optimizing pre-conception health for males. There is mounting evidence that human semen quality has been deteriorating over the past few decades. Understanding how environmental factors impact male reproductive health is crucial not only for improving fertility but also for the health and wellbeing of next generation.

It is estimated that large numbers (10-70%) of populations in developed countries are not consuming the recommended dietary amounts of folate. Folate supplementation is recommended for all females of reproductive age, in order to improve pregnancy outcomes by preventing neural tube defects. However, the role of folate in male reproductive health is less investigated. Several studies both from clinical trials and animal models have shown that folate is a determinant of male reproductive health but the underlying molecular mechanisms linking spermatogenesis, fertility and folate are unknown.

Folates act as mediators for the transfer of one-carbon moieties in the pathways of nucleotide synthesis/repair and methylation cycle. Reduced folate availability is related to increased DNA breaks. Moreover, dietary folate level largely decides the supply of methyl donors, and subsequently has consequences for DNA methylation and probably also histone methylation. Epigenetics refers to heritable changes in gene expression that are not caused by an altered DNA sequence and include DNA and histone methylation. Spermatogenesis is

characterized by massive alterations to the epigenome and our lab's previous work has described a tightly regulated pattern of histone H3 methylation in germ cell development that coincides with highly regulated transcription and cell differentiation. Epigenetic errors in the male germ line could become a source of paternal transmitted disease.

We hypothesized that male germ cells may be sensitive to dietary folate and that this may manifest as altered epigenetic programming and impaired DNA integrity, which would have downstream consequences on spermatogenesis and fertility. The objective of this study was to determine the impact of exposure to a low folate diet, during embryonic development and into adulthood on histone methylation, spermatogenesis, fertility and offspring health.

Chapter 2

Literature Review

1.1 Spermatogenesis and somatic cells of the testis: development from the uterus to adulthood

1.1.1 Spermatogenesis

Spermatogenesis is the biological process of gradual transformation of spermatogonia to spermatozoa within the seminiferous epithelium in the testis. The testis is covered by a tough fibrous capsule and contains two major compartments: the intertubular tissue and the seminiferous tubules (Russell et al. 1990). The interstitial compartment contains blood vessels, lymphatic vessels, and Leydig cells (Fig 1.1). Seminiferous tubules, which are bounded by lymphatic endothelium, myoid cells, and acellular elements, are convoluted loops with two ends connected to the beginning of the excurrent duct system.

In mice, germ cells originate from primordial germ cells (PGCs), which migrate to the genital ridge at embryonic day 10.5 (E10.5) (de Rooij and Russell 2000). When enclosed by precursor Sertoli cells, the PGCs become gonocytes, which undergo several rounds of proliferation followed by a mitotic arrest until a few days after birth, when they resume proliferation to give rise to spermatogonia, from which spermatogenesis starts (de Rooij and Grootegoed, 1998).

Spermatogenesis can be divided into three phases: the proliferative, meiotic, and spermiogenic phases (de Kretster 2007) (Fig 1.2).

1. Proliferation phase:

Spermatogonia carry out several mitotic divisions and give rise to primary spermatocytes. According to the presence of nuclear heterochromatin, spermatogonia can be subdivided; Type A spermatogonia are without heterochromatin, as they differentiate to intermediate spermatogonia, and in the following type B spermatogonia, heterochromatin is increased (de Rooij and Russell 2000). However, more generations of spermatogonia are discovered beyond this category. The most primitive generation of type A spermatogonia is type A_{single}, which is known to be the stem cell. As A_{single} spermatogonia divide to form A_{paired} spermatogonia, the destination is irreversibly committed to further differentiate toward spermatocytes. Except the stem cell A_{single}, spermatogonia divisions are incompletely separated and daughter cells of a clone are joined by intercellular bridges, which insure the synchronized development by sharing gene products (Hess, 1999). Further division of A_{paired} form chains of $A_{aligned}$, which then differentiate into six generation of differentiating spermatogonia A1, A2, A3, A4, In, and B spermatogonia (de Rooij and Russell 2000). The appearance of A2 marks the beginning of spermatogenesis. $A_{aligned}$ is not the only sperm stem cell. A1- A4 are considered the renewing stem-cell spermatogonia. A4 has the capacity to give rise to A1 when more cells are needed for spermatogenesis (Clermont 1968; Dym 1994).

2. Meiotic phase

   The meiotic phase is the process where primary spermatocytes duplicate the genetic material and subsequently undergo the first and second meiotic divisions to form haploid spermatids (Cobb and Handel 1998).

   Type B spermatogonia lose their contact with the basement membrane and divide to form the preleptotene stage of primary spermatocytes (de Kretster 2007). Preleptotene spermatocyte then undergo DNA replications

right before the onset of meiotic prophase (Anderson et al. 2008). Meiotic prophase is a long-lasting gradual differentiation process with sequential steps: leptotene, zygotene, pachytene, and diplotene (Russell et al. 1990). The chromatin in leptotene spermatocyte looks like fine filaments because of condensation (Clermont 1972). In zygotene spermatocytes, the chromatin is further condensed and the homologous chromosomes start to pair along their length (Russell et al. 1990; Clermont 1972). The pairing is facilitated by a proteinaceous structure called synaptonemal complex (SC), lying between the axes of the paired homologous chromosomes (Cobb and Handel 1998). Proteins that are functional in DNA repair and recombination are located on the SC. When the chromosomes are fully paired and become shorter and thicker, the pachytene step begins (Clermont 1972). In pachytene spermatocyte, a process known as crossing over, which is marked by the appearance of chiasmata, allows exchanging of genetic materials between the homologous chromosomes (de Kretster 2007). The last prophase stage – diplotene -- is very short, during which the homologous chromosomes are partially separate but the chiasmata remain.

Following diplotene is metaphase I, anaphase, and telophase. From anaphase, each member of the homologous pair begins to move to the opposite poles, resulting in the formation of the secondary spermatocytes. The secondary spermatocytes enter into the meiosis II without additional duplicating of the DNA and finally yield haploid spermatids (Clermont 1972).

3. Spermiogenesis

The post-meiotic male germ cell development, which is characterized by a transition from the spermatids to the mature spermatozoa, is referred to as spermiogenesis. This process involves several morphological changes (Hess

1999; de Kretster 2007). Including:

(a) Formation of the acrosome

Elongation and formation of the acrosome lasts throughout spermiogenesis. In the round spermatid, the acrosome is derived form the Golgi apparatus. It applies a cap-like structure and covers 30%-50% of the nucleus surface (de Kretster 2007; Toshimori and Ito, 2003). The acrosome is a sac of secreted enzymes used for penetrating the oocyte vestment during the fertilization (Russell et al. 1990).

(b) Nuclear changes

During spermiogenesis, the round nuclei of the spermatid undergoes dramatic changes and turns into the spatulate head in mammals and the falciform head in rodents (Jean-Pierre 2003). As a consequence of the replacement of the majority of histones by sperm-specific nucleoproteins, the nuclei of the spermatozoon is highly compacted and condensed (Jean-Pierre 2003). This replacement can protect the DNA and ensure the spermatozoon a safe travel in the female reproductive tract. The decreased nuclear volume is also contributed by elimination of fluid from the nuclei.

(c) Shedding of the cytoplasm and cell organelles

Only a small amount of cytoplasm is kept for fertilization. The reduced nuclear volume is crucial for fertility since the spermatozoa will suffer a dramatic and rapid change of osmotic environment in the female reproductive tract (Cooper and Yeung 2003). At least two mechanisms are involved in cytoplasmic elimination. One is by generation of tubulobular complex which may account for 70% reduction. Another is by discarding the cytoplasmic remnant as the “residual body” (Sprando and Russell 1987).

(d) Formation of the flagellum

The formation of the flagellum commences early in spermiogenesis at the

opposite pole to the acrosome (de Kretster 2007). The motor apparatus of the flagellum is the axoneme, which consists of a 9X2 pattern of microtubules. This basic structure is surrounded by a dense outer fiber, fibrous sheath, and a mitochondrial sheath. These additional structures can provide structural stability and function as scaffold to position critical enzymes for sperm motility, and energy for sperm motion.

1.1.2 The cell associations and stages of spermatogenesis

There are two important concepts related to the association of germ cells in the seminiferous epithelium: the cycle describing the evolution of germ cells in time; the wave illustrating the arrangement of germ cells along the length of seminiferous tubules (Perey 1961).

Within the seminiferous tubules, the occurrence of germ cell types is not random as there is fixed composition (Berndston 1977) (Fig 1.3). That is to say, the appearance of a specific spermatogonia type is always associated with a specific stage of meiosis and spermatid development. There are several certain and distinct cellular associations, each consisting of one or two generations of spermatogonia, spermatocytes and spermatids. The cellular association is explained by: (1) synchronous division of cells of the same type in the same area; (2) each germ cell, except certain spermatogonia, follows a precise time interval to yield the next generation

Each period of a certain cellular association is a stage. There are 6 stages in human, 14 in rat and 12 in mouse (Russell et al. 1990). Because stages follow each other in a fixed order and with precise time intervals, the disappearance and reappearance of a stage constitutes one cycle of the seminiferous epithelium (Berndston 1977). In many mammals, the stages usually successively follow one

another along the length of tubules. The sequential order of stages and their repetition along the length of the tubules constitutes the “wave” in the seminiferous epithelium (Russell et al. 1990) (Fig 1.4).

1.1.3 Somatic cells in Testis

1. Leydig cell:

In the intertubular space, the prominent cells are the Leydig cells. The principle function of the Leydig cells is to produce testosterone, which is essential for the reproductive tissues (Holstein et al. 2003). There are two generations of Leydig cells, fetal Leydig cells (FLDs) and adult Leydig cells (ALDs) (Habert et al. 2001). The FLDs are required for the masculinization of the male urogenital system *in utero*, while the ALDs are responsible for the male reproductive function.

From fetal day 12.5 (FD12.5) in mice, the mesenchymal-like stem cells start to differentiate into FLDs, which are round- to oval- shaped with a round conspicuous nucleus, and they are found exclusively in clusters. Because of lacking in mitotic activity, the increasing of FLDs depends upon differentiation of stem cells. Post-natally, FLDs start to regress, which is marked by the scattering of clusters and the decreasing number. However a few FLD cells can persist at least until PND90 in rats (Haider et al. 2007; Habert et al. 2001).

ALDs also originate from mesenchymal-like stem cells, and their development can be divided into three stages. The stage of progenitor Leydig cells (PLCs) initiates from PND10-13. PLCs have thin and slender-shaped cytoplasm with an elongated nucleus, locating singly in the peritubular or perivascular area. The proliferation rate in this stage is quite high. Around PND25, they transit to round-shaped immature Leydig cells (ILCs) which locate closely to blood capillaries in the central intersitium. ILCs possess more lipid droplets and

have acquired the ability of producing testosterone, but they mainly secret 5-reduced androgen. ILCs only double once from PND28 to PND56. Around PND 90, they differentiate to mature Leydig cells (MLCs), which have larger cell sizes, increased volume of smooth endoplasmic reticulum, and decreased lipid droplets. Testosterone is abundantly produced at this stage. MLCs are in cessation of proliferation, but they can be compensated from stem cells when being destroyed (Haider et al. 2007; Habert et al. 2001; Benton et al. 1995; Mendis-Handagama and Ariyaratne 2001.).

2. Sertoli cell:

Sertoli cells are found in seminiferous tubules. In the mouse, they are multinucleated, stellate, nondividing somatic cells. Many factors responsible for the control of testicular function exert their effects through Sertoli cells (Johnson et al. 2008). In adults, Sertoli cells attach to the basal lamina and reach to the lumen, extending between germ cells in order to keep intimate contacts with a variety of germ cells (Guraya 1987). Sertoli cells proliferate in fetal/neonatal period but become quiescent in peripubertal period. The total number of Sertoli cells determines the efficiency of spermatogenesis (Brehm 2005). The regulation between germ cells and Sertoli cells are reciprocal. Germ cell plays important role in the maturation of Sertoli cell, while the adult Sertoli cells have multiple functions in spermatogenesis, such as (Mruk and Cheng 2004):

1. Providing structural support for germ cells
2. Creating an impermeable and immunological barrier;
3. Participating in germ cell movement and spermiation;
4. Secretory functions and provision of nutrients;
5. Pinocytosis and receptor-mediated endocytosis;
6. Phagocytosis;

7. Targets for FSH, androgens and other hormones.

8. Form a complex intercellular junctions with adjacent germ cells.

1.2 Epigenetic mechanisms

1.2.1 Epigenetics

Epigenetics refers to the heritable changes in gene expression that occur without changes in the DNA sequence (Delcuve et al. 2009). Epigenetic mechanisms involve several layers of regulation of gene expression, such as DNA methylation, histone modifications, chromatin remodeling, histone variant composition and noncoding RNA (Allis et al. 2006).

It is well recognized that gene expression is not only determined by the DNA sequence but also by the epigenetic information marked on the gene. In a cell, the pattern of epigenetic marks determines which genes should be turned on or off at a given moment of time. As a result, although the genetic sequence is identical in all cells of an individual, different cells can perform distinctive functions due to the differences in their epigenetic profiles (Szyf et al. 2007).

Epigenetics plays an important role in linking environment and gene expression. Environmental factors, such as nutrition, chemical, physical and even psychosocial factors, can impact the epigenome, especially during fetal development and early life, when the DNA synthetic rate is high and epigenetic patterns are generated to determine cell fates (Szyf et al. 2007; Dolinoy et al. 2007). Since epigenetic programming is considered stable and inheritable through mitotic or meiotic divisions, epigenetic changes resulting from environmental exposure *in utero* and postnatally could have a long-term effect on phenotypes and susceptibilities to diseases (Szyf et al. 2008; Jirtle and Skinner

2007). The environmental exposure in early life may predict adult risk of chronic diseases such as cancer, cardiovascular disease, diabetes, obesity, etc (Dolinoy et al. 2007). Moreover, inter-individual phenotypic variation should not only be attributed to genetic sequence polymorphisms but also to epigenetic variations.

Recently it has been proposed that epigenetic programming is also dynamic throughout life, well after the cells have established their epigenetic information and differentiated (McGowan et al. 2008). One representative example is monozygotic twins. Although they are genetically identical and epigenetically indistinguishable in their early years of life, remarkable differences in epigenetic marks emerge as they grow older, which explains the occurrence of more phenotypic discordances in their later years of life (Fraga et al. 2005). Both external factors and internal factors are attributed to the alterations. External factors, such as habits, diet, or physical activity are suggested as having effects on epigenetic information. Intrinsically, small defects could be accumulated in transmitting epigenetic information during cell divisions or maintaining it in differentiated cells. Unlike genetic sequence, epigenetic mechanisms are intrinsically dynamic and potentially reversible. Therefore, researchers have been finding drugs to reverse deleterious epigenetic disorders (Szyf et al. 2007).

1.2.1.1 DNA methylation

DNA methylation refers to a methyl group added to the carbon 5 position of the cytosine ring (Fig 1.5 A). The majority of DNA methylation occurs in 5'CpG3' dinucleotide (Costello and Plass 2001).

In 98% of the genome, CpGs present once per 80 dinucleotides and approximately 70% to 80% are methylated (Singal and Ginder 1999). However, the CpG islands, which are 200bp to several kb in length, with a frequency of G + C more than 50%, are usually unmethylated, even when their associated genes

are silent. About 70% of CpG islands are located in the promoter region and about 50-60% of all genes contain CpG islands, suggesting their importance in gene regulation (Singal and Ginder 1999; Costello and Plass 2001; Bird 2002; Tang and Ho, 2007).

DNA methylation is catalyzed and actively maintained by DNA methyltransferases (DNMTs) (Turek-Plewa and Jagodzinski 2005). There are two kinds of DNMTs in mammalians. One kind of DNMT, such as DNMT3a, DNMT3b and DNMT3L, is responsible for the *de novo* acquisition of methylation during embryogenesis. The other kind of DNMT is required for maintaining the methylation pattern during replication to ensure the methylation pattern is accurately passed to the next generation of cells. One important member of this category is DNMT1, which is capable of locating at the replication fork and methylate the newly synthesized DNA (Razin and Riggs 1980).

DNA methylation is a critical mechanism in chromatin remodeling and gene expression regulation. The presence of methylated CpG in the first exon or promoter may have an effect on gene silencing (Turek-Plewa and Jagodzinski 2005) (Fig 1.5 B). In addition, DNA methylation is also important to maintain the conformation and integrity of the chromosome. 35% of the human genome is constituted of repetitive mobile elements- transposons, including retrotransposons and DNA transposons. Retrotransposons can replicate themselves through transcription and then be inserted into other positions in the genome, resulting in various diseases when the insertion disrupts adjacent gene expression. DNA methylation is responsible for preventing most transposons from mobilizing by silencing their transcriptional activity (Waterland and Jirtle 2003; Whitelaw 2001; Kazazian 1998).

The gene silencing brought by DNA methylation occurs through the mechanisms of 1) direct interference with binding of specific transcription factors

to promoters 2) recruiting specific transcriptional repressors like MeCP-1 and MeCP-2 to prevent binding of transcription factors or 3) altering chromatin structures (Singal and Ginder 1999). Although DNA methylation is an extremely stable epigenetic mark, it is also reversible (Razin and Kafti 1994). However while it is known that there can be active demetylation, the proteins involved and the mechanisms are still not clear.

1.2.1.2 Histone post-translational modifications

The building block of chromatin is the fundamental repeating unit called the nucleosome, which is constituted by a protein octamer containing two molecules each of core histone (H2A, H2B, H3, and H4) and 147 bp of DNA wrapped around the octamer core (Allis, Jenuwein et al. 2006) (Fig 1.6 a b). Histones undergo a variety of post-translational modifications (PTMs), including methylation, acetylation, phosphorylation, ubiquitination, sumoylation, ADP-ribosylation, biotinylation, etc. Histone PTMs occur at specific residues, mainly on the flexible histone tails protruding from the nucleosome core (Fig 1.6 c). These modifications are catalyzed by specific enzymes and are all considered reversible (Shilatifard 2008). Histone acetylases (HATs) acetylate specific lysine residues and are reversed by histone deacetylases (HDACs) (Allis et al. 2006). Methylations occur on specific arginine or lysine residues, predominantly on histone H3 and H4, by protein arginine methyltransferases (PRMTs) or histone lysine methyltransferases (HKMTs). The methylation of lysines can be either mono-, di-, or trimethylated. Various histone demethylases have been identified, such as Lysine specific demethylase 1 (KDM1, also known as AOF2, BHC110 and LSD1) targeting at the H3K4me and H3K4me2.

It is proposed that histone PTMs are acting in a combinational manner (Jenuwein and Allis 2001). Modifications with the same effect are recruited to

cooperate synergistically, while simultaneously counteracting modifications are inhibited. For example, the repressive mark H3K9 methylation antagonizes active marks, such as acetylation of H3 and methylation of H3K4. Conversely, H3K4 methylation inhibits H3K9 methylation but increases H3 acetylation, leading to transcriptional activation (Fischle et al. 2003). Histone deacetylation and H3K9 methylation have a synergistic effect in heterochromatic silencing (Richards and Elgin 2002). It is believed that histone PTMs can transmit epigenetic information in the cell from one generation to the next (Shilatifard 2006).

Histone PTMs are involved in altering chromatin structure, regulating gene transcription, and DNA damage repair mechanisms (Bannister and Kouzarides 2005; Shailatifard, 2006):

1. Chromatin status and transcriptional activity

   Various histone modifications have been shown to direct the formation of specific chromatin states and to be responsible for transcriptional activity (Fig 1.7). Generally, there are two classes of chromatin structure. The highly condensed chromatin is called heterochromatin, and the loose chromatin structure which allows for transcription is euchromatin. Although in a "locked-down" state, heterochromain could serve a critical role in the organization and proper functioning of the genome, such as constituting centromeres and telomeres and facilitating chromosome segregation (Allis et al. 2006). Several histone methylation marks are found in heterochromatin, such as H3K9, H3K27, H3K79 and H4K20 (Bannister and Kouzarides 2005). Euchromatin is associated with acetylation marks and methylated H3K4 and H3K36, which can facilitate the engagement of transcription machinery, and setting it up for gene expression (Allis et al. 2006).

   Acetylation always indicates transcriptional permissive status, while methylation on different sites suggest different chromatin status (Allis et al.

2006). For example, H3K4me3 is associated with active transcription, whereas H3K9me3 is a repressive mark. H3K4me3 is exclusively present at active genes (Santos-Rosa et al. 2002). In human ES cells, a whole-genome mapping reveals that potentially over 13,000 genes are occupied by H3K4me3. The vast majority of the H3K4me3 is located within 1 kb of transcription start sites and over 80% of marked genes are transcribed (Zgao 2007). Histone H3K4me2 occurs both at active and inactive genes and is thought to indicate a transcriptional poised state whereby the gene has a potential to be rapidly activated (Santos-Rosa et al. 2002). In addition, histone H3K4me is enriched in the methylation-resistant CpG islands and an elevated level of H3K4me2 is found in CpG-rich promoters in human genome, implying the importance of histone methylation in affecting DNA methylation and gene expression (Fan et al. 2008).

2. DNA damage repair

Histone H2AX is a histone H2A variant that is involved in repair activities and targeting of DNA double stand breaks (DSBs). H2AXs is located in the damaged sites in response to DNA DSBs and it is phosphorylated to γ-H2AX. Around 2000 γ-H2AX molecules are accumulated to form a γ-H2AX focus on one DSB site (McManus and Hendzel 2005). γ-H2AX then recruits several chromatin remodelers and promotes effective repair in multiple ways (Vidanes et al. 2005). In addition, it is evident that histone methylation is involved in the DNA damage checkpoint pathway by directly interacting with checkpoint components.

1.2.1.3 Cooperation between DNA methylation and Histone PTMs

DNA methylation and histone PTMs are mutually regulated. DNA methylation is crucial in initiating chromatin remodeling and gene expression regulation. DNMT1 recruits HDACs and an H3K9 trimethylase *Suv39h1*. (Turek-Plewa and Jagodzinski 2005). The Methy1-CpG-binding protein MeCP2 recruits H3K9 methylation to reinforce gene silencing (Fuks et al. 2003). On the other hand while DNA methylation can influence histone modifications, histone modifications can also influence DNA methylation. For example, histone methylation is required for DNA methylation (Li 2002). In *Neurospora crassa*, the disruption of H3K9 methyltransferase results in a complete loss of DNA methylation (Bannister and Kouzarides 2005; Li 2002). In mouse embryonic stem cells, the double mutation of *Suv39h* is associated to reduced DNA methylation in major satellite DNA in pericentric regions (Freitag and Selker 2005).

1.2.2 Epigenetics and the relationship to diet

Methyl donors such as folic acid, choline, Vitamin B12, and betaine can influence DNA methylation levels and consequently gene expression (Dolinoy et al. 2007). Studies on the model of viable yellow agouti (A^{vy}/a) mice provided the first evidence linking the effect of maternal diet during pregnancy to the phenotype of her offspring, which was mediated by epigenetic changes (Wolff et al. 1998; Waterland and Jirtle, 2003). Transient expression of the mouse *agouti* (*A*) allele produces a subapical yellow band on hair, resulting in the brown (agouti) coat color of wild-type mice. The A^{vy} allele results from the insertion of an intracisternal A particle (IAP) transposable element upstream of the *A* allele. A cryptic promoter in the A^{vy} IAP allows ectopic agouti expression, leading to a

yellow coat color. However, if the IAP promoter is inactivated by methylation, *agouti* gene expression is blocked. The *nonagouti* (*a*) allele is a loss-of-function mutation in *A*; it is used in studying the dominant A^{vy} allele. The A^{vy} */a* strain exhibits a wide variation in coat color, raging from yellow, through to different extents of mottling, to brown, depending on the various level of IAP methylation. Dietary supplementation with folate, vitamin B12, choline, and betaine to the *a/a* dam during pregnancy can shift the coat color distribution of their A^{vy} */a* offspring towards the brown pseudoagouti phenotype; and this shift is demonstrated to be caused by increased DNA methylation in the IAP transposable element in the offspring (Wolff et al. 1998; Waterland and Jirtle, 2003). Moreover, IAP hypomethylation is related to obesity, diabetes and tumorigenesis. Therefore, this study also implies the role of environmental epigenomic changes during early development in determining adult diseases (reviewed in Jirtle and Skinner, 2007).

Another example of folate moderating gene expression involves the imprinted gene *Igf2*. Mouse fed a diet restricted in methyl donors induces IGF2 loss of imprinting. However, the DNA methylation of differentially methylated regions known to regulate *Igf2* imprinting is not affected, indicating other mechanisms, such as histone modifications may be involved in mediating the diet-induced changes (Waterland et al. 2006).

1.2.3 Role of Epigenetics in Spermatogenesis

1.2.3.1 Epigenetic reprogramming

Since epigenetic mechanisms are dynamic in response to environment, harmful environmental exposure may lead to a failure of reprogramming and

have a life long impact (Szyf 2008). Although to date this remains a theory and has not been definitively shown. There are two periods in lifetime that epigenetic information undergoes a dramatic reprogramming process – gametogenesis and early embryogenesis. Male germ cell development is characterized by extensive epigenetic resetting, in order to erase epimutations and establish the epigenetic pattern required for giving rise to a totipotent zygote (reviewed in Kimmins and Sassone-Corsi 2005; Sasaki and Matsui 2008). Importantly this indicates that there is a potential for the environment to act on the sperm epigenome and to potentially induce epigenetic errors that might lead to paternal-transmitted disease in offspring (Godmann et al. 2009).

Firstly, DNA methylation in male germ cells undergoes a substantial genome-wide erasure and rebuilding (Allegrucci et al. 2005). The DNA demethylation occurs as the primodial germ cells arrive in the genital ridge at E11.5 and completes by E13-14. The DNA *de novo* methylation then takes place at E15.5 in prospermatogonia and is completed prior to the end of pachytene after birth in the first wave of spermatogenesis (Sasaki and Matsui 2008). Mature spermatozoa are globally hypomethylated compared with somatic cells and have a unique DNA methylation pattern. An investigation of DNA methylation by restriction landmark genomic scanning of 1500 CpG island regions shows 30 loci are methylated in spermatozoa but not in embryonic germ cells (Kunio et al. 2002). However, nothing is known about the erasure of histone modifications in primodial germ cells (Godmann et al. 2009).

Genomic imprinting is a phenomenon that leads to the exclusive expression of paternally and maternally inherited alleles of a subset of genes (Trasler 2006; Allegrucci et al. 2005). Paternally imprinted genes, such as H19, Ras Grf1, and Igf2r, are methylated on the paternal-origin allele and are expressed from the maternal-origin allele. Imprinting has an important role in growth of embryos,

placental function and neurobehavioural processes. Abnormal expression of imprinted genes has been linked with cancer and many severe developmental disorders, such as Prader-Willi syndrome, Angelman syndrome, and Beckwith-Wiedemann syndrome (Dolinoy et al. 2007). Epigenetic alteration is an important contributor to these defects. DNMT3a and DNMT3L are predominant enzymes in the initial methylation of imprinted genes (Kaneda et al. 2004; Bourc'his and Bestor 2004). Imprinted genes also acquire histone modifications. It is found that the active allele is associated with H3K4 dimethylation and H3 acetylation, while the silent allele is marked by H3K9 trimethylation (Delaval et al. 2007). To ensure parent-of-origin-specific expression in the offspring, imprints have to undergo reprogramming to be erased and rebuilt before the germ cells become haploid (Allegrucci et al. 2005). The second epigenetic reprogramming occurs in the preimplantation embryo, but imprinted genes are protected from this erasure and their methylation patterns persist throughout development, indicating imprinting errors in gametes could be an origin of disease in offspring.

Strikingly, during spermiogenesis most somatic histones are sequentially replaced by transition proteins and then protamines, both of which are unique in male germ cells (Kimmins and Sassone-Corsi 2005). Transition proteins serve to prepare the chromatin for protamine incorporation by influencing DNA condensation. Protamines are small proteins with up to 50% arginine content, which permits a high DNA-binding affinity. The haploid male genome is then compacted into a sperm head that is only 5% of the size of a somatic cell nucleus (Sassone-Corsi 2002). The distinct chromatin architecture enables the sperm a safe travel in female reproductive tract to achieve fertilization and a specific transcription schedule after fertilization (Jean-Pierre 2003).

However, approximately up to 15% of histones are retained in human spermatozoa (Ooi and Henikoff 2007). Recently, a remarkable paper published in

Nature by Hammound, et al. (2009) suggests that histone methylation in sperm is important for embryo development (Hammound et al. 2009). In human sperm, there is significant histone retention at many loci important for embryo development, including embryonic transcription factors, signally pathway components, the promoters of miRNAs and imprinted genes. Interestingly, the gene active mark, H3K4me3, is retained in sperm and is localized to developmental promoters, regions in HOX clusters, noncoding RNAs, and imprinted genes, supporting a role in embryo development (Hammound et al. 2009). Whether histone retention can mediate germline epigenetic inheritance has yet to be demonstrated. It is postulated that the histone retention may contribute to the germline epigenetic inheritance, such as preventing DNA methylation on imprinted genes from being erased during embryonic reprogramming (Ooi and Henikoff 2007).

1.2.3.2 Epigenetic implications for male reproductive health and offspring health

Male germ cell maturation is a complex and remarkable cell differentiation process governed by a unique genetic and epigenetic program. The fidelity of the epigenome is required for male reproductive health and prevention of paternal disease transmission (Godmann et al. 2009). The epigenetic regulation of spermatogenesis is beginning to be investigated. Expression of DNMTs is precisely regulated during male gametogenesis and is associated with specific DNA methylation events, such as imprinting, X inactivation, *de novo* methylation, etc. (Trasler 2009). Also, H3K4 methylation is under a tight regulation in spermatogenesis (Godmann et al., 2007). A high level of H3K4 methylation

appears in spermatogonia through to leptotene, followed by downregulation in pachytene and reappearance in elongating spermatids, suggesting its role in transcription regulation and chromatin remodeling events (Godmann et al. 2009).

Disturbance of epigenetic information in male germ cells may lead to failure of spermatogenesis and impaired fertility. Several mouse models of DNA hypomethylation by knockout of DNMTs result in infertility. For example, male mice that lack Dnmt3L are sterile, with complete failure of meiosis progression in young adult and aspermatogonia in older mice (Bourc'his and Bestor 2004). Dnmt3L is expressed in the precursors of spermatogonial stem cells mainly around the time of birth. This study also showed that Dnmt3L deprivation prevents the *de novo* methylation of retrotransposons and that the defects in epigenetic methylation can be transmitted by mitosis, resulting in death of spermatogonia in adult males. In addition, male mice with disrupted Dnmt3a in germ cells display similar defects as Dnmt3L knockout mice (Kaneda et al. 2004). The impaired spermatogenesis may be due to lack methylation at paternally imprinted genes when Dnmt3a is depleted.

Not only DNA methylation but also histone methylation is critical in male fertility. Meisetz is a meiosis specific histone methyltransferase for H3K4me3 and is demonstrated to be important in mediating meiosis progression. Meisetz$^{-/-}$ mice show sterility and arrested meiosis at the pachytene stage (Hayashi et al. 2005). Suv39h1 and Suv39h2 are murine histone methyltransferases that govern H3K9 methylation at pericentric heterochromatin. Suv39h double null male mice show severely impaired viability and complete spermatogenic failure (Peters et al. 2001). The pachytene spermatocytes are almost absent due to perturbed chromosome interactions during meiosis.

Most strikingly, aberrant epigenetic information carried by gametes might be

transmitted to the next generation and affect offspring health. 5-aza-2'-deoxycytidine acts as a cytidine analogue to incorporate into DNA and decrease DNA methylation. Mice on the treatment of 5-aza-2'-deoxycytidine have abnormal spermatogenesis, reduced fertility and elevated preimplantation loss, probably as a consequence of the observed altered DNA methylation in the male germ cells (Kelly et al. 2003). Moreover, a recent study shows that aberrant DNA methylation at imprinting genes can be transmitted by male germline for at least four generations (Lee et al. 2009). This result suggests that false imprinting in male germline is a potential source of paternal transmitted disease.

1.3 Folate and Male Reproductive Health

1.3.1 Folate is a determinant of Male Reproductive Health

It is estimated that large proportions (10%-70%) of the population in developed countries are not meeting the recommended dietary folate level (Beck and Olek, 2003). Folate deficiency has been associated with slowed growth, anaemia, weight loss and colon carcinogenesis. The role of folate in male reproductive health has not been well investigated and is an open area in need of study. In clinical trials, it has been found that folate supplementation had a beneficial effect on spermatogenesis of infertile men and could increase fertility (Forges et al. 2007). High intakes of fruits and vegetables are inversely associated with sperm DNA damage, while high intakes of meat, potatoes and whole grains are positively associated with red blood cell folate level and an increase in sperm concentration (Vujkovic et al. 2010). Several experiments on animal models have further demonstrated the importance of folate in male reproductive health. Rats fed a folate-free diet showed a reduction in testes and epididymides masses, a

drastic decline in sperm counts, and higher uracil content in testis DNA (Mayr et al. 1999). In a micropigs model, folate-depleted diets were associated to reduced testis and epididymis weights, lower sperm counts and altered sex hormone profiles. The impaired spermatogenesis is also evidenced by testicular lesions in histological analysis (Wallock-Montelius et al. 2007).

MTHFR is an enzyme involved in the metabolism of folate. The Mthfr double knockout in mice resulted in a compromised survival, abnormal spermatogenesis and infertility (Kelly et al. 2005). Germ cells in these mice failed to resume mitosis postnatally and have increased apoptosis, causing dramatically reduced germ cell numbers. Clinical evidence suggests that 20% of men going to infertility clinics have a MTHFR mutation (677 C-->T) (Bezold et al. 2001).

Despite the fact that it has been clarified that folate is a determinant of male reproductive health, the underlying molecular and cellular mechanisms of folate and its role in the testis are still unknown. Folate availability can affect DNA integrity, gene expression, and perhaps the epigenetic layer which until now has not been investigated. Thus in a folate deficient setting the sperm may be impaired and contain aberrant epigenetic information and DNA damage. This compromised sperm may put the offspring's health at risk. Thus deeper study should be conducted to look into the role of folate in reproduction and its effect on further generations.

1.3.2 Folate metabolism

Folates, a group of inter-convertible co-enzymes, belong to vitamin B and act as mediators for the transfer of one-carbon moieties in the pathways of nucleotide synthesis/repair and methylation cycle which has broad substrates, such as DNA and proteins, etc. The histone methyl transferase enzymes also use

SAM (Van den Veyver 2002).

The synthetic form of folate is folic acid, which is more stable. Folic acid enters the metabolic pathways via dihydrofolate (DHF) and tetrahydrofolate (THF), which is then methylated to 5,10-methylene-tetrahydrofolate (5, 10-MethyleneTHF) when serine is converted to glycine (Fig 1.8) (Van den Veyver 2002). 5, 10-MethyleneTHF, as a one-carbon donor, is important in DNA synthesis through conversion of dUMP in dTMP and purine biosynthesis. Besides, 5, 10-MethyleneTHF can be converted to 5- Methyltetrahydrofolate (5-MTHF), which is the primary methyl donor in the homosysteine remethylation pathway, by methylene tetrahydrofolate reductase (MTHFR) (Choi et al. 2009).

From gaining a methyl group by reducing 5-MTHF to THF, methionine synthase (MTR) can methylate homosysteine to methionine. MTR is ubiquitously expressed and active in all tissues. Betaine homocysteine methyltransferases (BHMT) provides an alternative homosysteine remethylation pathway is some tissues, by obtaining methyl groups from betaine. This pathway is suggested to be operating in testis (Kelly et al. 2005).

Methionine is an essential amino acid, which can be recycled from homocysteine and taken from food. Methionine is required not only for protein synthesis but also for methylation reactions as it is converted to S-adenosylmethionine (SAM), the common methyl donor with broad substrates, including DNA, RNA, proteins, and phospholipids, etc. Adequate SAM inhibits MTHFR activity, promoting the use of 5, 10-MethyleneTHF for thymidylate synthesis. Conversely, insufficient SAM increase MTHFR activity (Wallock-Montelius et al. 2007). Because MTHFR catalyzed formation of 5-MTHF is irreversible, loss of MTR activity could result in “Methyl trap”, which is the condition that an accumulation of cellular folate as 5-MTHF and inhibited use of 5, 10-MethyleneTHF for the purine and thymidylate synthesis (Stover 2004).

When SAM donates the methyl group, it is converted to S-adenosylhomocysteine (SAH). Then homocysteine can then be recycled by the hydrolyzing of SAH. However, this step is reversible and has negative effects on the methylation reaction, since SAH can bind to methyltransferases with higher affinity than SAM.

After releasing the one-carbon unit for DNA synthesis and the methylation pathway, all the folate co-enzymes are converted back to THF, which can then be reused. However, a small amount of THF is lost and must be replenished in the cell by taking up circulating serum folate (Van den Veyver 2002).

Seminal plasma folate concentration is higher than blood plasma folate concentrations in males, indicating it has an important role in spermatogenesis. In particular, the non-methyl THF level correlate with sperm density and sperm count (Wallock et al. 2001). Also, MTHFR is required for spermatogenesis and its level is high in mice testis (Chen, Karaplis et al. 2001). These evidences suggest folate is required in testis. In rats, it has been shown that folate contents of individual tissues, including testis, correlate with dietary folate level (Clifford et al. 1990). Thus, adequate folate intake should be needed to guarantee the normal testicular function. However, folate metabolism and its role in male reproduction are largely unknown. A high-affinity folate receptor was found in human testicular tissue (Holm et al. 1999). The reduced folate carrier(RFC1) functions to transport 5-MTHF into mammalian cells (Zhao et al. 2001). Mice with double knockout of RFC1 died in the embryo stage. With folate supplementation, some of these mice could go on to live birth, however, with pathological changes in many tissues, including seminiferous tubule, and died within 12 days. These results indicate male reproductive tissues are sensitive to folate availability.

1.3.3 Impact of Folate Deficiency

Nearly one-quarter of adult Canadians are obese, which means they have the body mass index of 30 or more (Tjepkema and Shields 2005). Obesity increases the chances of developing various diseases, such as heart disease, type 2 diabetes, certain cancer, and osteoarthritis (Haslam and James 2005). Overweight and obese people have a higher level of homocysteine (Vincernt et al. 2006) and lower serum folate concentrations (Mahabir and Ettinger 2007). Some studies showed folate supplementation may be an important part of managing obesity and reducing body fat (Meshkin and Blum 2007). It is well known that obesity and being overweight is associated with reduced fertility and perhaps folate supplementation could enhance fertility by altering the sperm epigenome. Before these more complex relationships between folate, body weight and epigenetics are investigated in humans we must define underlying molecular mechanisms in animal models such as the one described here.

Folate is essential for the biosynthesis of purines and thymidylate and hence is critical in DNA synthesis, stability and integrity. A reduced cell division rate is observed when cells are cultured in folate deficient medium (Stempak et al. 2005). Moreover, several *in vitro* and *in vivo* studies have correlated folate deficiency with increased DNA breaks (Blount et al. 1997; Beck and Olek, 2003; Linhart, Troen et al., 2009). The most plausible explanation is excessive uracil misincorporation into DNA (Fenech 2001). When folate is deficient, dUMP/dTMP ratio increases and lead to massive misincorporation of dUTP into DNA. During repair of uracil in DNA, transient single strand breaks (nicks) are formed. Two opposing nicks can result in a less repairable and more dangerous double strand break (DSB). Of all types of DNA damage, DSBs are a great threat to genomic integrity (Ahmed et al. 2007). Although not demonstrated in mammals, in

bacteria when SAM levels are low, cytosine methyltransferases can deaminate cytosine to uracil at methylation sites (Linhart et al. 2009). Thus, it suggests that folate deficiency could lead to cytosine deamination and enhance the rate of C:G to A:T transition at CpG site.

It has been well documented that folate deficiency causes an accumulation of homocysteine; and folate supplementation is effective in lowing plasma homocysteine level in the person of hyperhomocysteinemia (Selhub 1999). Hyperhomocysteinemia is associated with oxidative stress (Loscalzo 1996), which is believed to cause spermatozoa DNA damage and apoptosis (Agarwal et al. 2006). Damaged DNA originating in sperm could lead to low fertilization rates, impaired preimplantation development, increased abortion and may even lead to a long-term effect in the offspring's health, including childhood cancer (Lewis and Aitken, 2005). Several animal studies have demonstrated that spermatozoa damaged by paternal exposure to anticancer agents could have adverse effects on the offspring (Trasler et al. 1986; Lewis and Aitken, 2005). Also epidemiological data showed that parental cigarette smoking is responsible for 15% of childhood cancers (Sorahan and Lancashire 2004). Moreover, preconception diet with high intakes of vegetable oils, vegetables, fish, and legumes and low intakes of snacks by couples taking in citro fertilization/intracytoplasmic sperm injection treatment are positively correlated with red blood cell folate and increased probability of pregnancy (Vujkovic et al. 2010).

Since folate-derived methyl groups are the main methyl donor for homocysteine remethylation, DNA methylation should be largely determined by folate (Choi et al. 2009). Several studies showed that folate status can alter DNA methylation. In cell culture studies, folate deficiency can induce DNA hypomethylation in normal human colonic epithelial cells, mouse fibroblast cell

line, NIH/3T3, and a Chinese hamster ovarian cell line (Stempak et al. 2005; Kim 2005). In human trials, methylation of leukocyte DNA is reduced in response to inadequate folate intake in postmenopausal women; the hypomethylation status can be reversed with sufficient folate repletion (Jacob et al, 1998; Rampersaud et al. 2000). However, results of folate deficiency effects on rodent liver are not consistent (Kim 2005). Although several studies showed folate deficiency can decrease genomic DNA methylation in rodent liver, short duration or moderate degree of folate depletion is reported to result in DNA hypermethylation by other studies. It is assumed that these contradictory results are because of different experimental design, such as folate depletion level, exposure time, and tissue analyzed (Kim 2005). Nevertheless, these studies collectively suggest that there are effects of folate deficiency on DNA methylation that is gene and tissue specific (Kim 2005). Taking into consideration of the gene silencing function of DNA methylation, folate deficiency could contribute to marked changes in chromatin organization and the loss of chromosome segregation (Lamprecht and Lipkin 2003). In considering that the global decrease in DNA methylation is a passive process requiring multiple cell divisions, one can presume the supply of dietary methyl donor could have a considerable impact in testis, where dramatic cell divisions occur, producing approximately 1000 sperm per second.

Whether folate level can determine histone methylation is still unknown. No one has showed that changing the methyl donor pool will affect the level of histone methylaiton. It is also probable that folate depletion will increase DNA damage in developing germ cells that may carry over into sperm. No studies have examined these possibilities or the consequences for offspring.

Figures

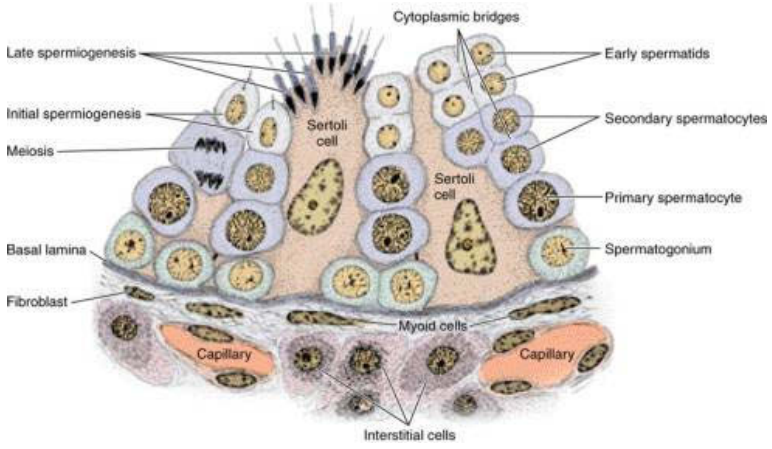

Figure 1.1: Organization of the testis. This is a schematic depiction of the cross section of the testis showing the seminiferous tubule with its surrounding tissue, including peritubular myoid cells. the Leydig cells and blood vessels lie within the intertubular tissue. Taken from Junqueira and Carneiro (2002) "Basic Histology: a text and atlas", 10th Edition, McGraw-Hill/Appleton & Lange.

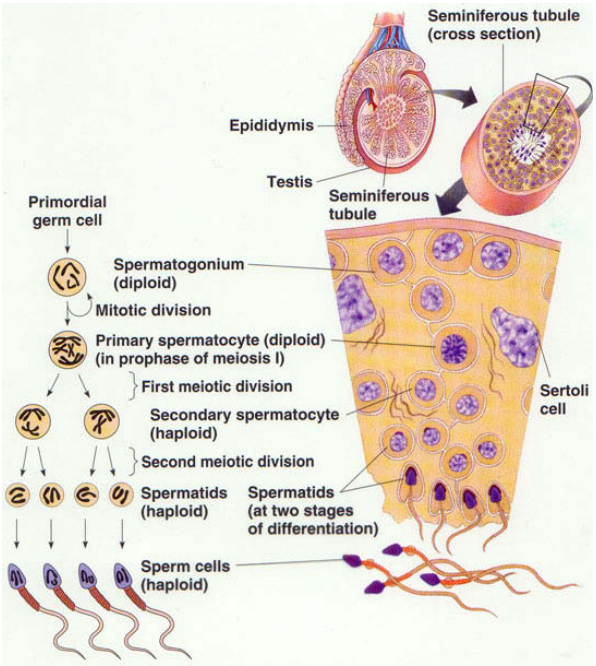

Figure 1.2: Spermatogenesis. The proliferation, meiotic and differentiation phases of spermatogenesis and their associated germ cells, spermatogonia, spermatocytes and spermatids are shown. Taken form Campbell N. A. and J. B. Reece. (2005) "Biology", 6th Edition. Pearson Education: 973-1010.

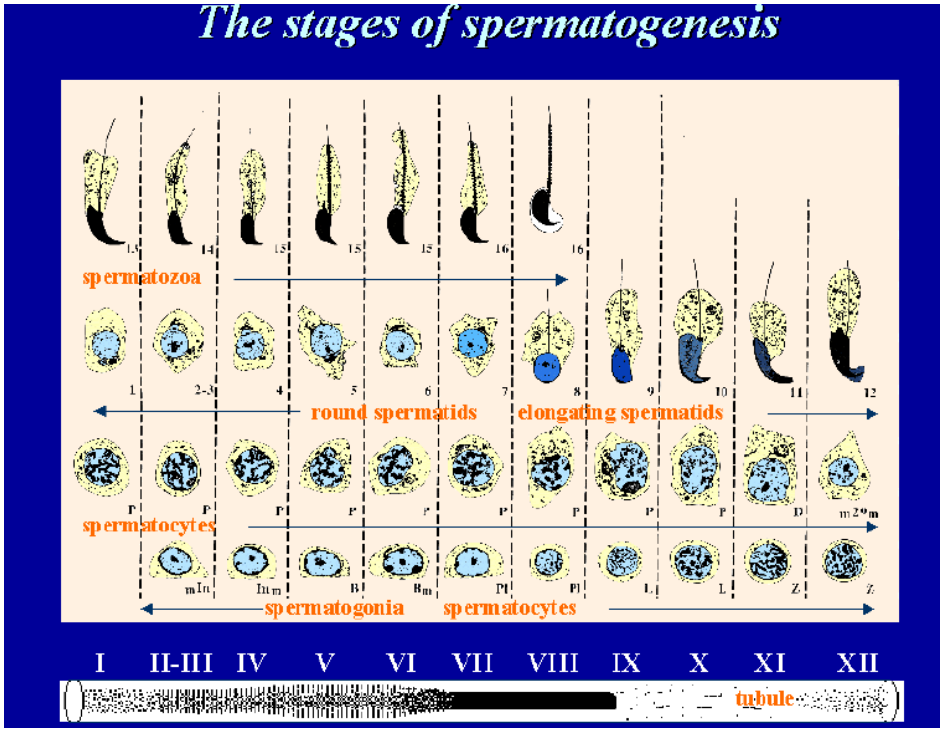

Figure 1.3: The stages of spermatogenesis. Taken from Russell et al. (1990) "Histology and Histopathological Evaluation of the Testis." Cache River Press, FL: Chapter 1.

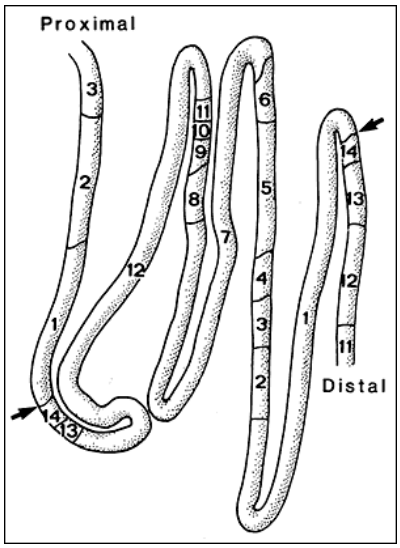

Figure 1.4: The wave of spermatogenesis. The picture shows the pattern of the stages of spermatogenesis, which occur along the tubule. Taken from de Kretser and Kerr (1994) in "The Physiology of Reproduction" E. Knobil and J. D. Neill (Eds), Lippincott Williams & Wilkins.

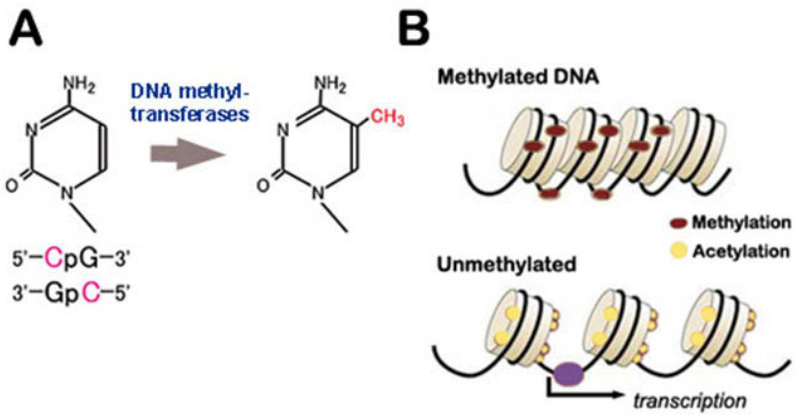

Figure 1.5: DNA methylation. (A) Methylation by DNA methyltransferases at CpG islands. (B) DNA demethylation is related to histone acetylation and transcription. Taken from Taylor (2006). "p53 and deregulation of DNA methylation in cancer". Cell science Reviews **2**: 82-93.

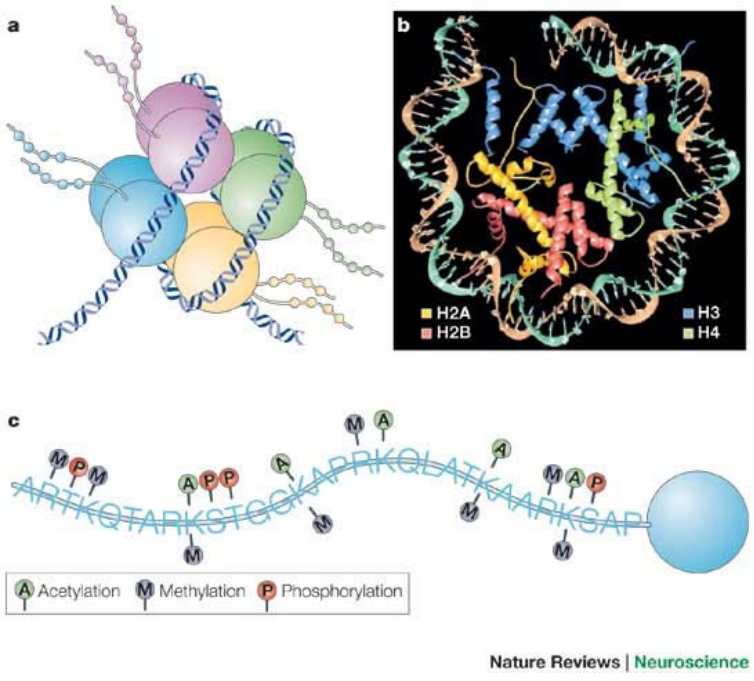

Figure 1.6: Histone modifications. (a) Schematic depiction of the nucleosome. (b) Crystal structure of the nucleosome. (c) An example of the histone tail and modifications on it. Taken from Levenson and Sweatt (2005). "Epigenetic mechanisms in memory formation". Nature Reviews Neuroscience **6**, 108-118.

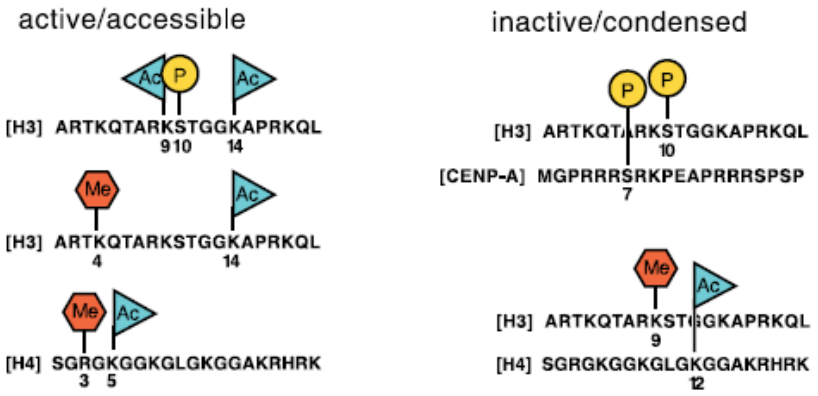

Figure 1.7: Examples of active or inactive histone modifications. Taken from Jenuwein and Allis (2001). "Translating the histone code." Science **293**(5532):1074-80.

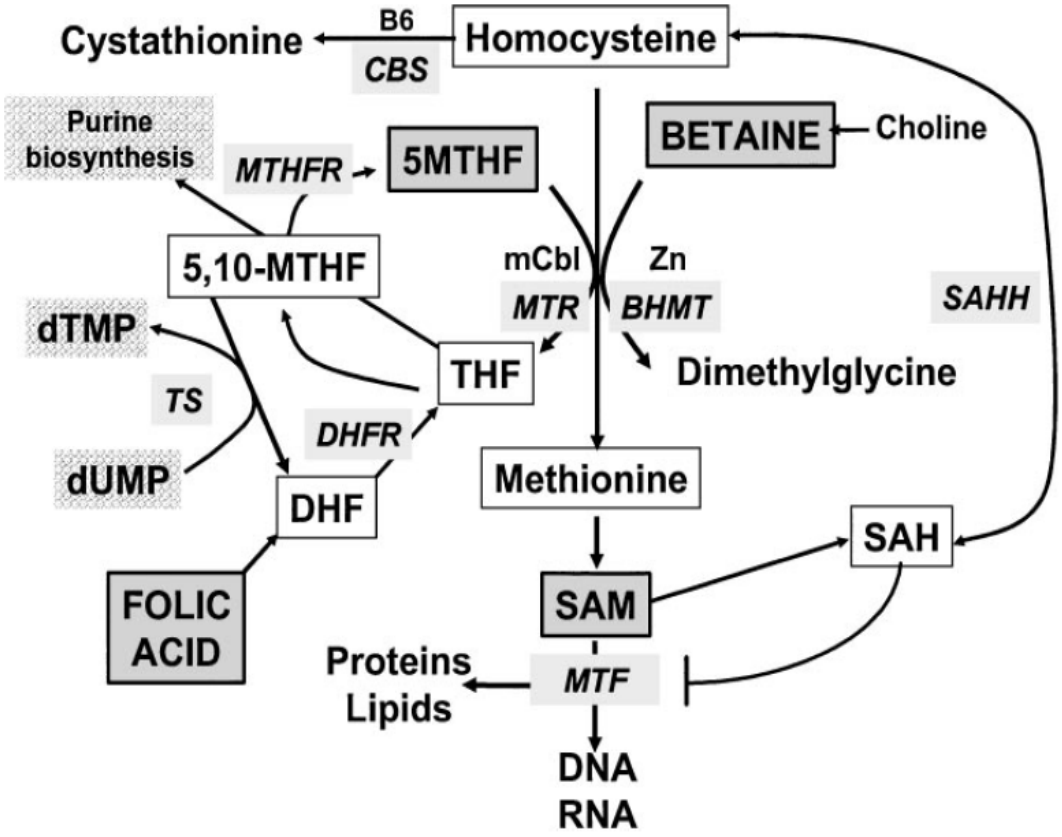

Figure 1.8: Folate metabolism. Taken from Van den Veyver, I. B. (2002). "Genetic effects of methylation diets." Annual Review of Nutrition **22**(1): 255-282.

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1.1 Spermatogenesis and somatic cells of the testis: development from the uterus to adulthood

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Chapter 3

Folate deficient male mice have an altered sperm epigenome and poor reproductive outcomes

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Introduction

Folates, also known as vitamin B_9, are a group of inter-convertible co-enzymes. A large proportion (10%-70%) of the population in developed countries is not meeting the recommended dietary folate level (Beck and Olek 2003). Folate deficiency has been associated with many diseases, such as neural tube defects, slowed growth, anemia, weight loss and colon carcinogenesis; the role of folate in male reproductive health has just begun to be investigated and is an open area in need of study. Epidemiological study showed that high intakes of meat, potatoes and whole grains are positively associated with red blood cell folate level and an increase in sperm concentration (Vujkovic et al. 2010).Several clinical trials have demonstrated the beneficial effect of folate on spermatogenesis of infertile men, of whom some gained fertility after folate supplementation (Forges et al. 2007). Experiments on animal models provided further evidence that folate is important in male reproduction. Rats fed a folate-free diet showed a reduction in testis and epididymis masses, a drastic decline in sperm counts, and higher uracil content in testis DNA (Mayr et al. 1999). In a micropigs model,

folate-depleted diets were associated to reduced testis and epididymis weights, lower sperm counts and altered sex hormone profiles. The impaired spermatogenesis is also evidenced by testicular lesions in histological analysis (Wallock-Montelius et al. 2007).

Despite the fact that folate is a determinant of male reproductive health, the underlying molecular and cellular mechanisms of folate and its role in the testis are still unknown. Moreover there are no studies relating paternal diet and offspring health. Evidence is accumulating that suggests folate is important for testis function, for example, a high-affinity folate receptor was found in human testicular tissue (Holm et al. 1999), and mice null for the reduced folate carrier(RFC1), which functions to transport 5-MTHF into cells, developed pathological changes in seminiferous tubule (Zhao et al. 2001). These results suggest that male reproductive tissues are sensitive to folate availability.

Folate availability can affect DNA integrity, gene expression, and we postulate also the developing sperm cell epigenome, which until now has not been investigated. Thus in a folate deficient setting the sperm may be impaired and contain aberrant epigenetic information and DNA damage. This compromised sperm may put the offspring's health at risk. Therefore, we undertook the following studies to assess the role of folate in male reproduction and its effect on future generations. Folates are involved in a variety of important biochemical reactions by acting as mediators for the transfer of one-carbon moieties in the nucleotide synthesis/repair pathway and in the methylation cycle. Folic acid is the synthetic form of folate and is more stable. Folic acid enters the metabolic pathway of folate via dihydrofolate (DHF) and tetrahydrofolate (THF) (Van den Veyver 2002). THF is then methylated to 5,10-methylene-tetrahydrofolate (5, 10-MethyleneTHF), which participates in DNA synthesis through conversion of dUMP in dTMP and through purine biosynthesis (Choi et al. 2009). Besides,

methylene tetrahydrofolate reductase (MTHFR) can convert 5, 10-MethyleneTHF to 5- Methyltetrahydrofolate (5-MTHF), which is the primary methyl donor in the homosysteine remethylation pathway. By gaining a methyl group from 5-MTHF, methionine is regenerated from homosysteine, so that the methyl group can be donated, via S-adenosylmethionine (SAM), to broad substrates including DNA, RNA, proteins, and phospholipids, etc. (Van den Veyver 2002). Homocysteine can be recycled from S-adenosylhomocysteine (SAH) after SAM donating the methyl group. As folate availability is a determinant to DNA synthesis and methylation, and we postulate also histone methylation, the impaired genetic and epigenetic information induced by folate deficiency in male germ line may become potential source of paternal transmitted disease.

Both *in vitro* and *in vivo* studies have correlated folate deficiency with increased DNA breaks (Blount et al. 1997; Beck and Olek, 2003; Linhart et al. 2009). The most plausible explanation is excessive uracil misincorporation into DNA when lacking of dTMP (Fenech 2001). During repair of uracil in DNA, transient single strand breaks (nicks) are formed. Two opposing nicks within 12bp can result in a less repairable and more dangerous double strand break (DSB). Of all types of DNA damage, DSB is a great threat to the genomic integrity (Ahmed, van der Vaart et al., 2007). In addition, folate deficiency attributes to accumulation of homocysteine (Selhub 1999), which is considered to be a cause of oxidative stress (Loscalzo 1996). Excessive reactive oxygen species is believed to be one of the major sources of DNA damage in spermatozoa (Agarwal et al. 2006). Damaged DNA originating in sperm has been a great concern because of its consequences of low fertilization rates, impaired preimplantation development, increased abortion and even a long effect in the offspring's health (Lewis and Aitken 2005). Several animal studies demonstrated that spermatozoa damaged by paternal exposure to cancer therapeutic agents could have adverse

effects on offspring (Trasler et al. 1986; Lewis and Aitken 2005). An example from human studies associated parenral cigarette smoking with 15% of childhood cancers (Sorahan and Lancashire 2004). Thus there is evidence suggesting that there are routes of paternal disease transmission but the potential role of the sperm epigenome and the mechanisms involved remain under-investigated.

Beyond the DNA integrity, gene expression is also at a risk of being affected when folate is deficient, since the methylation cycle regulated by folate is responsible for the fidelity of epigenetic information. Epigenetics refers to the heritable changes in gene expression that occur without changes in the DNA sequence, including DNA methylation and histone modifications (Delcuve et al. 2009). Epigenetic mechanisms manifest as the link between environment and gene expression. Environmental factors, such as nutrition, chemical, physical and even psychosocial factors, can impact the epigenome, especially during the two critical periods when a remarkable epigenetic reprogramming takes place – gametogenesis and early embryogenesis (reviewed in Kimmins and Sassone-Corci 2005; Szyf et al. 2007). Spermatogenesis is characterized by unique and extensive reorganizations to the epigenome, involving a wave of DNA demethylation, followed by *de novo* DNA methylation and chromatin remodeling, in order to give rise to a totipotent zygote (reviewed in Kimmins and Sassone-Corsi, 2005). DNA demethylation occurs as the primodial germ cells arrive in the genital ridge at E11.5 and completes by E13-14. DNA de novo methylation then takes place at E15.5 in prospermatogonia and is completed prior to the end of pachytene after birth in the first wave of spermatogenesis (Sasaki and Matsui 2008). Therefore the resetting of the sperm epigenome is on going throughout the male's lifetime. However, nothing is known about the erasure of histone modifications in this reprogramming progress. Since epigenetic mechanisms are dynamic in response to environment, failure of reprogramming may be harmful as environmental

exposure could have a life long impact on offspring health (Rhind et al. 2003; Szyf 2008). Although to date this remains a theory and has not been definitively shown.

The building block of chromatin is the fundamental repeating unit called the nucleosome, which is constituted by a protein octamer containing two molecules each of core histone (H2A, H2B, H3, and H4) and 147 bp of DNA wrapped around the octamer core (Allis et al. 2006). Histones undergo a variety of post-translational modifications, including methylation, acetylation, phosphorylation, ubiquitination, sumoylation, ADP-ribosylation, biotinylation, etc. Methylations occur on specific arginine or lysine residues predominantly on histone H3 and H4 tails, catalyzed by protein arginine methyltransferases (PRMTs) or histone lysine methyltransferases (HKMTs). These modifications are considered to be involved in altering chromatin structure, regulating gene transcription, and DNA damage repair mechanism (Bannister and Kouzarides 2005; Shailatifard 2006). Several histone methylation marks are found in heterochromatin, such as H3 lysine 9 (K9), H3K27, H3K79 and H4K20 (Bannister and Kouzarides 2005), while methylated H3K4 and H3K36 are related to gene activation by facilitating the engagement of transcription machinery (Allis et al. 2006).

Spermatogenesis is a complex and remarkable cell differentiation process that requires unique epigenetic events to accomplish, including programming of histone methylation. For example, H3K4 methylation is under a tight regulation in spermatogenesis (Godmann et al. 2007). A high level of H3K4 methylation appears in spermatogonia through to leptotene, followed by downregulation in pachytene and reappearance in elongating spermatids, suggesting its role in transcription regulation and chromatin remodeling events (Godmann et al. 2009). Disturbance of histone methylation in male germ cells may lead to failure of spermatogenesis and impaired fertility. Mice showed a complete spermatogenic

failure and infertility when Meisetz, a meiosis specific histone methyltransferase for H3K4me3, was knocked out; or when Suv39h1 and Suv39h2, which are murine H3K9 histone methyltransferases, were double knocked out (Hayashi et al. 2005; Peters et al. 2001).

During spermiogenesis, the last step of spermatogenesis, most somatic histones are sequentially replaced by transition proteins and then protamines. The haploid male genome is then compacted into a sperm head that is only 5% of the size of a somatic cell nucleus. This distinct chromatin architecture enables the sperm for safe travel in the female reproductive tract to achieve fertilization (Sassone-Corsi 2002; Jean-Pierre 2003). However, up to 15% histones are retained in human spermatozoa (Ooi and Henikoff 2007). Theoretically this amount is enough to package all coding regions in the genome (Ooi and Henikoff 2007). This histone retention may be responsible for preventing DNA methylation on imprinted genes from being erased during embryonic reprogramming. However, whether the histone retention could mediate germ line epigenetic inheritance is unknown. Recent studies provided evidence of the role of histone methylation in sperm to the embryo development (Hammound et al. 2009; Brykczynska et al. 2010). In human sperm, significant histone retention was found at loci crucial for embryo development, including embryonic transcription factors, signally pathway components, the promoters of miRNAs and imprinted genes. Remarkably, the gene active mark, H3K4me3, was retained in sperm and was localized to developmental promoters, regions in HOX clusters, noncoding RNAs, and imprinted genes, suggesting its important contribution to embryo development (Hammound et al. 2009). Moreover, the repression mark H3K27me3 was found to be enriched at genes that are repressed in early embryogenesis (Hammound et al. 2009; Brykczynska et al. 2010).

Understanding the possible causes of male subfertility and infertility are

important as these changes are not only crucial for human reproduction but may also have consequences for subsequent generations. This project is the first study to link the impact of folate on spermatogenesis, fertility, histone methylation in sperm and offspring health. The objective of this study is to determine the impact of exposure to a low folate diet, during embryonic development and into adulthood on histone methylation, DNA integrity, spermatogenesis, fertility and offspring health.

Materials and Methods

Animals and Dietary Treatments

The inbred C57⁄BL6 strain was used for all diet trials and the outbred CD-1 strain was used to assess fertility in breeding trials (Charles River Laboratories, St-Constant, Quebec, Canada). The mice were housed under controlled light/dark cycle in the Animal Facility of McGill University, Macdonald Campus. Animals were provided with food and water ad libitum. All animal procedures were approved by the Animal Care and Use Committee of McGill University, Montreal, CA. Protocol #5138 (Appendix 4).

We designed our experiments in collaboration with Dr. Rima Rozen and Jacquetta Trasler (McGill) who have been using the same folate model to study the role of folate in colon cancer (Knock et al. 2008; Li and Rozen 2006). C57⁄BL6 females were fed either the folate sufficient (2mg folic acid/kg) diet (TD.01369, Harlan Laboratories, Madison, WI) or the folate-deficient (0.3mg folic acid/kg) diet (TD.01546, Harlan Laboratories, Madison, WI) two weeks prior to breeding with C57⁄BL6 males which were fed regular mouse chow (8640 Rodent diet, Harlan Teklad, Madison, WI). The experimental diets were amino acid defined and contained 1% succinylsulfathiazole which is an antibiotic to prevent synthesis of folate by gut bacteria (Appendix 1 and 2). The effects of these diets on serum

levels of folate and homocysteine have been fully described in different mouse strains (Knock et al. 2008; Li and Rozen 2006). The same folate deficient diet used here was demonstrated to be effective in lowering folate and increasing plasma homocysteine levels, with 4.99±0.14 μmol/L homocysteine in C57⁄Bl6 males on FS diet for 12 to 14 months and 9.90±1.18 μmol/L in those on FD diet (Knock et al. 2008). To breed these C57⁄BL6 females, C57⁄BL6 males were brought to the females' cages at night and removed in the morning, in order to limit consumption of the experimental diets by the males. Each male was used to breed one female on FS diet and one on FD diet. Females were maintained on the experimental diets through pregnancy and lactation. From weaning (postnatal day 21 (PND21)), male pups were given the same experimental diets as their mothers until sacrifice. Male pups were sacrificed and testes and epididymides were collected at the following ages: PND 6, 10, 12, 14 and 18 (n=3 for all postnatal days), 6 weeks of age (FS n=7, FD n=5), corresponding to sexual maturity, 9 weeks (FS n=7, FD n=7), 15 weeks (FS n=12, FD n=8; combined from 2 trials) and 18 weeks (FS n=28, FD n=29) of age, corresponding to adulthood. The postnatal days were chosen as collection time points corresponding to the appearance of different spermatogenic cell types during the first wave of spermatogenesis. Type A spermatogonia appear on the PND6, while leptotene, zygotene, pachytene and diplotene appear at PND10, 12, 14 and 18 (Belvé et al. 1977).

Assessment of Sperm Morphology

The cauda epididymides from 15-wk old mice (FS, n=7; FD, n=3) were removed, cut and incubated at 34 ℃ for 30 minutes. The sperm suspension was spread onto a slide pre-dipped into 4% paraformaldehyde to allow for fixation of the sperm. The slides were dried and hydrolysis was performed using 0.1N HCl at 4 ℃

for 5 min. Following washing in double deionized water, sperm was stained with toludine blue for 5 minutes. Slides were than washed in double deionized water, air dried, covered with a coverslip and sealed with nail polish. Slides were viewed by light microscopy (Nikon Eclipse 80i). Pictures were captured using Nikon NIS-Elements and optimized with Photoshop. Sperms showing tail defect and/or head defect were quantified (100 sperm counted per animal).

Homogenization resistant sperm counts

A pair of caput and corpus epididymides was used for sperm counting for each animal at 15-wks (FS, n=5; FD, n=5). Tissues were thawed on ice and cut into small pieces in homogenizing solution containing 0.9% NaCl, 0.1% Thimerosal (merthiolate) and 0.5% Triton X-100. Samples were homogenized using a tissue homogenizer under the following condition: 2 x 15 sec with a 30 sec interval in between at the maximum speed. Samples were diluted in PBS and sperm heads were counted using a hemacytometer. The sperm count per gram of epididymal tissue was obtained by dividing the total count by the gram weight of the caput and corpus epididymis.

Immunohistochemistry, Immunofluorescent and Immunohistopathology Analysis

Experiments were performed as previously described (Godmann et al. 2007). Briefly, tissues were fixed in Bouins solution and embedded in paraffin and cut into 5-µm-thick sections. Sections were deparaffinized with Citrisolve (Fisher, USA) and then rehydrated in alcohol. After washing in TBS-Brij (0.61% Tris Base, 0.88% NaCl, pH 8.1, 0.03% Brij) for immunohistochemistry or PBS-Tween (0.8% NaCl, 0.02% KCl, 0.02% KH_2PO_4, 0.01% $MgCl_2{\cdot}7H_2O$, 0.22% Na_2HPO_4, 0.01% anhydrous $CaCl_2$, 0.05% Tween) for immunofluorescent, antigen retrieval was

performed by boiling in microwave oven in sodium citrate buffer (18ml of solution A (2.11% citric acid), 82ml of solution B(2.94% sodium citrate) in 1L, pH 6). When cooled down, the slides were washed in distilled water and then TBS-Brij. Endogenous peroxidase activity was blocked by incubation with 0.3% hydrogen peroxide in methanol for 30 min at room temperature. The sections were subsequently blocked in 10% BSA and 10% normal donkey/goat serum in TBS-Brij or PBS-Tween for 1 h, and then incubated with the primary antibody overnight at 4 ℃. After washing, the sections were incubated with secondary horseradish peroxidase conjugated antibody or fluorescent dye labeled antibody for 1 h at room temperature, followed by washing. For immunohistochemistry, immune complexes were revealed by diaminobenzidine (Sigma) and sections were counterstained with hematoxylin. For immunofluorecence, sections were conterstained with DAPI and then analyzed under fluorescent microscope (Nikon Eclipse 80i).

For histopathology analysis testis were fixed in Bouins overnight, followed by dehydration and embedding. Section were cut at 5 µm, deparrafinized, stained with hematoxylin and eosin and staged according to the methods described in Russell et al (Russell et al. 1990).

Antibodies

Primary antibodies used in this study were Rabbit Polyclonal SCP3 (ab15093, Abcam; 1:1000 dilution), anti-phospho Histone H2AX (Ser139) (05-636, Millipore; 1:8000 dilution), anti-mono-methyl H3 (Lys4) (ab8895, Abcam; 1:500 to 1:1000 dilution), anti-di-methyl H3 (Lys4) (ab7766, Abcam; dilution 1:1000), anti-mono-methyl H3 (Lys9) (ab8896, Abcam; dilution 1:2000), anti tri-methyl H3 (Lys9) (07-442, Millipore; 1:1000 dilution), MIS (c-20) (sc-6886, Santa Cruz), Mouse VASA homologue (MVH) (in house made by Dr. Kimmins), and monoclonal

anti-β-actin (A-1978, Sigma; 1:5000 to 1:10000 dilution). Secondary antibodies were horseradish peroxidase-conjugated donkey-anti-rabbit antibody (711-035-152, Jackson Immunoresearch Laboratories; 1:5000 dilution), horseradish peroxidase-conjugated donkey-anti-mouse antibody (715-035-150, Jackson Immunoresearch Laboratories; 1:5000 to 1:10000 dilution), Alexa Fluor 488 goat-anti- mouse (A11001, Invitrogen; 1:1000 dilution) and Alexa Fluor 594 goat-anti-rabbit (A11012, Invitrogen; 1:1000 dilution).

Spermatocyte enriched preparations for analysis of DNA double strand breaks

To determine whether reduced folate intake was associated with increased DNA damage in sperm development, DNA double strand breaks (DSBs) were assessed in enriched pachytene spermatocyte cell spreads. Cells were prepared according to the drying-down technique described by Peters et al. 1997. Briefly, tubules were removed from decapsulated testes and placed in hypotonic extraction buffer (30mM Tris-HCl, 50mM sucrose, 17mM trisodium citrate dehydrate, 5mM EDTA, 0.5mM dithiothreitol and Proteinase Inhibitor Cocktail; pH 8.2) for 45 min. Subsequently, a cloudy suspension was made by first tearing, then pipetting up and down small tubules pieces in sucrose solution (100mM; pH 8.2). The cell suspension was then dispersed on slides pre-dipped in 1% paraformaldehyde containing 0.15% Triton-X (pH 9.2). The slides were washed in 0.4% Photoflo (Kodak) solution and dried at room temperature overnight in a humid slid box. Co-immunofluorescent of synaptonemal complex protein 3 (SCP3) and the histone variant that lables DNA DSBs, γ-H2AX, was performed on spermatocyte enriched cell spreads.

Confocal Microscopy

To analyze the SCP3 and γ-H2AX counterstained spermatocyte surface-spread,

fluorescence was visualized using a Zeiss LSM 510–NLO with inverted microscope Axiovert 100M, and the objective lens was Plan-Apochromat 63x / 1.4 NA Oil immersion – DIC. Automated image analysis systems MCID Elite was used to capture images. Fluoresceins were excited by 488nm and 543nm lasers. Optimal conditions for laser scanning confocal microscopy were established: Scaling: 0.07µm X 0.07µm; Stack size: 1024X1024 pixels, 73.1µm X 73.1µm; Pinhole: 92µm for SCP3 and 94µm for γ-H2AX; Pixel time: 1.28µsec. Image stacks generated by laser scanning confocal microscopy were imported into Zeiss LSM Image Browser for analyzing.

Determining the Levels of Histone Methylation in Sperm by Western Blot

Sperm from 9-wk (FS, n=6; FD, n=6) and 18-wk (FS, n=7; FD, n=7) old mice were analyzed by western blot to determine whether altering the availability of methyl donors by diet would affect global levels of histone methylation in sperm. Sperm were collected using what is known as the swim-out method. Briefly, two to four epididymides were pooled, minced and incubated at 34 ℃ for 30 min. Sperm protein extracts were prepared in Laemelli and sonicated using the following condition: five seconds on with five seconds of rest for a total of 2 min at 25% of maximum amplitude. Samples were incubated on ice for 30 min and centrifuged at 4000g for 30 min at 4 ℃. The supernatant was transferred and stored at -80 ℃ until western blots were performed for the analysis of histone H3 lysine 4 (K4) and 9 (K9) methylation. After boiling for 5 min, equal sample (25 µl per lane) were loaded and separated by standard 12% SDS-PAGE gels and electroblotted onto PVDF membranes. Membranes were incubated in a blocking solution of 5% skimmed milk in PBS-tween (0.05%) for 45 min. Membranes were then incubated with the primary antibody overnight at 4 ℃. After washing, membranes were incubated with secondary horseradish peroxidase-conjugated antibody for 45

minutes. Immune complexes were detected by enhanced chemiluminescence (Pierce) and membranes were exposed to Kodak autoradiography BioMax film. Films were scanned and the band intensity was quantified using AlphaDigiDocTM. Each experiment was replicated 2 times.

Detection of Apoptotic Germ Cells

Germ cell apoptosis was examined in testis cross sections from 15-wk old mice using the Terminal deoxynucleotidyl transferase-mediated dUTP Nick End Labeling (TUNEL) detection protocol (Apoptag®, Chemicon International). Slides were viewed by fluorescent microscopy (Nikon Eclipse 80i) and pictures were captured by Nikon NIS-Elements. All the tubules in each testis section were counted and the ratio of tubules with a certain level of apoptotic cells (>5, >10 or >15) to total number of tubules counted was calculated. Normal levels of apoptosis have been documented to be a mean value of 1.9±0.2 apoptotic cells per tubule in testes of Bl6 mice (Kon et al. 1999).

Assessing DNA integrity in Sperm Using the COMET Assay

DNA single and double strand breaks in spermatozoa of adult male mice were evaluated by the alkaline COMET assay. The method was adapted from Delbes et al. 2007 with some modifications. Epididymal sperms were collected from 18-wk old FS and FD mice (FS, n=4; FD, n=4) by the swim-out method. Frozen sperm suspensions in PBS were thawed and 50µl was mixed with 500µl 0.7% molten low-melting-point agarose at 42 ℃. 50µl was immediately pipetted and evenly spread onto slides (Trevigen Inc). After the gel was set, cells were lysed by immersing the slides in lysis buffer (2.5 mol NaCl, 100 mmol EDTA, 10 mmol Tris-HCl, 10% DMSO, 1% Triton X-100, and 40 mmol dithiothreitol; pH 10) for 1 hr at 4 ℃. Subsequently, slides were washed in prechilled (4 ℃) distilled water for

10 min and were incubated in freshly prepared alkaline solution (1 mmol EDTA in distilled water; pH 12.3) for 45 min at 4℃. Slides were then washed twice in 1 X TAE buffer for 5 min before electrophoresis at 0.7V/cm for 10 min. Then, slides were dehydrated by prechilled 70% ethanol for 5 min and stored at room temperature until analysis. DNA was stained by 1:10 000 SYBR Green (Trevigen) in distilled water and immediately pictures were captured under microscope. For each animal, 100 cells were randomly selected and measured using the KOMET 5.0 image analysis system (Kinetic Imaging Ltd, Liverpool, UK). The software setting was the same as Delbes et al. 2007.

Determining the Effect of Diet on Male Fertility

The effect of folate deficiency on male fertility was examined by mating each male (FS, n=20; FD, n=21; 8-10 wk old) to a virgin CD-1 female over 5 days. Females were examined daily for vaginal plugs. If a plug was not detected during the first 5 days, mating was repeated with another CD-1 female in the following week. To limit the effect of folate deficiency on females, females were brought to males' cages at night and were removed to a separated cage in the next morning. Pregnancy rate was determined as the percentage of plug-positive females that became pregnant.

Examination of Fetuses and Placentas at E18.5

To determine if the offspring health was sensitive to paternal diet, CD-1 mice were mated to males fed either the FS or FD diet throughout life (FS, n=27; FD, n=29; 2-3 month old) (the procedure was described in Fertility Analysis) and pregnancy outcomes were determined at embryo day 18.5 (E18.5). For the purpose of ascertaining the stage of gestation, the day on which a vaginal plug was discovered was designated as E0.5. Also, the stage was assured by the

appearances of longer whiskers, thickened and wrinkled skin, visible eyes, and joined fingers and toes, according to the descriptions of Theiler, 1972. Females were sacrificed at noon on E18.5 and ovaries were excised and the numbers of corpora lutea (CL) were counted. The uteri were opened and the resorption sites, implantation sites, and live fetuses were determined. Preimplantation loss, representing the lack of fertilization or embryo death prior to endometrial implantation, was calculated as (CL – number of implants)/CL x 100 (Toppings et al. 2008). Postimplantation loss, representing the later impairment of embryo viability indicated by resorption, was calculated as (number of implants – viable fetuses) / number of implants x 100. The embryo weight, crown-rump length, placenta width and placenta weight were measured.

Statistical analysis

Graphical or table numbers shown represent the mean ± SEM (standard error of the mean) of at least three independent experimental units. The level of significance was set at $P < 0.05$. Statistical analysis of pregnancy rate and resorption rate was done by Fisher's exact test and chi-square test respectively. The data assessing weights, sperm counting, Sertoli cell and Leydig cell counting, meiotic tubule counting and apoptosis were analyzed for differences between the control and treated groups by Student *t*-test or Mann-Whitney *U*-test (in cases of failed normality), as indicated in the text or legends. For the fertility trial and pregnancy outcomes, all the calculation and analysis were on a per male basis. For each litter, the average of embryo weights, crown-rump lengths, and placenta widths and weights were calculated, then the collection of averages for the litters sired by FS males was compared with the collection of averages for the litters sired by FD males by Student *t*-test. When analyzing the COMET scores, the average of scores for each animal was calculated, and then the collection

averages were compared between FS and FD males by Student *t*-test. The γ-H2Ax foci counting was analyzed by subsample model. The animals are random effects nested in the fixed effects of folate level. Each cell analyzed is a subsample unit, while the animal is experimental unit. Data were analyzed with the aid of SAS 9.2 and PASW Statistics Base 18.

Results

There were no negative effects of folate deficiency on overall body condition as assessed by body weight and appearance

Generally, there was no difference in litter size, sex ratio and offspring survival between mothers fed the folate sufficient or those fed the folate deficient diet for two weeks prior to and through conception. In this study, all male offspring gained weight progressively during the trial. At only two points examined, PND6 and 6-wk of age, was there an effect on weight, where the males on the FD were significantly heavier then those on the FS diet ($p<0.01$) (Table 1). Overall, these results indicate that folate deficiency did not negatively impact body weight. By examination of the mice they appeared healthy and of normal size with good coats and normal behavior throughout the study regardless of diet.

Effect of folate deficiency on testis and epididymide weights

Testis and epididymide weights are shown as both absolute weight and relative weight (tissue weight to body weight ratio x 100) (Table 1). The testis weight of 9-wk FD mice was reduced relative to FS group ($p<0.01$), even after adjusting for the body weight ($p<0.01$). However, reduction in testis weight was not found at any other age. The epididymis weight of FD mice did not differ from FS mice at any time points examined (Table 1).

Sertoli and Leydig cell development was not affected in folate deficient mice

To determine whether somatic cells in the testis were sensitive to folate deficiency, we examined Sertoli and Leydig cell development at critical points following their proliferation and differentiation (Kluin et al. 1984; Payne and Hardy, 2007). It is believed that the total number of Sertoli cells determines the efficiency of spermatogenesis (Brehm 2005). Thus, we examined the Sertoli number at PND10 (FS, n=3; FD, n=3), since in mice Sertoli cells proliferate only in fetal/neonatal period and become almost quiescent at PND12 (Kluin et al. 1984; Vergouwen et al. 1991). Sertoli cells were identified by immunofluorescent labeling with anti-MIS and counted in 10 round tubules per animal in FS (n=3) and FD (n=3) (Figure 1A and 1B). There was no difference in Sertoli cell numbers between FS and FD mice ($p>0.05$) (Figure 1C).

Leydig cells are prominent in the interstitial space and are responsible for testosterone production (Holstein et al. 2003). In mice, the fetal Leydig cells (FLDs), which are present and observable as round- to oval-shaped with a round nucleus and are found exclusively in clusters, start to regress postnatally marked by the scattering of clusters and their decreasing number (Payne and Hardy, 2007; Haider et al. 2007; Habert et al. 2001). The histological examination of testis cross sections stained by the Leydig cell marker, 3β-HSD, revealed no detectable size or quantitative differences of FLD clusters between FS and FD males at PND6 (Figure 2A and 2B). Adult Leydig cells (ALD) first appear at PND10–13. The first stage of ALDs, from the initiation to PND25, is the progenitor Leydig cell (PLD) stage, which has a high proliferation rate. After this stage, ALDs only double once and then become quiescent (Haider et al. 2007; Habert et al. 2001). Thus, we assessed the postnatal ALD number, in order to determine whether ALD proliferation was affected by folate deficiency. In PND12 FS and FD mice testis, the number of PLD was quantified. PLD were identified as 3β-HSD positive cells

with slender-shaped cytoplasm and an elongated nucleus, located singly around seminiferous epithelium (Figure 2C-2H). The ratio of the total number of PLDs to the number of tubules counted was calculated and compared between FS and FD mice (FS, n=3; FD, n=3) (Figure 2I). There was no observable change in morphology or number of the PLDs between FD and FS testis (Figure 2).

Meiotic onset was delayed in folate deficient mice

Histological analysis revealed that meiotic onset was delayed in FD mice. Normally in the first wave of spermatogenesis, leptotene and zygotene meiotic cells first appear at PND10 and PND12 respectively (Belvé et al. 1977). Using histone H3-K4 mono-methylation as a marker of leptotene cells as we have previously described (Godmann et al. 2007), we calculated the proportion of tubules that contained at least 10 meiotic cells in PND12 FS and FD testis sections (FS, n=3; FD, n=3). As shown in Figure 3A and 3B, the FS tubules contain abundant H3K4-me positive cells while fewer tubules with meiotic cells were observed in the FD tubules. There was on average 3 times less meiotic positive tubules in FD testis in comparison to FS testis (40.77±0.97 in FS versus 12.20±6.28 in FD) ($p<0.05$) (Figure 3C).

Histopathological examination of testis cross sections from PND18 mice indicated that by this day of post-natal development there was no difference in the progression of spermatogenesis between FS and FD (data not shown). Testes were examined from ages 2-4 months and spermatogenesis appeared to be unaffected by folate deficiency. All spermatogenic stages were present, with normal cellular associations and complete progression of spermatogenesis with normal spermiation was observed (Figure 4).

Germ cell apoptosis was not increased in folate deficient mice

Given that folate deficiency has been associated to the miss-incorporation of uracil and increased DNA breaks, we hypothesized that there may be an increased incidence of apoptotic cells in testis from FD males. TUNEL immune-staining was used to detect cellular DNA fragmentation and as a marker of apoptosis. For this analysis we chose the oldest males that had been on the diet the longest as it would be most likely to find increased apoptosis in males that had been on the diet the longest (Figure 5A-D). For each 18-wk male (FS, n=7; FD, n=7), all the tubules in one testis cross section were analyzed. The tubules with different ranges of TUNEL-positive cell number (>5, >10, or >15) were quantified and the ratio to the total number of tubules in the section were calculated. The results showed no increase in the proportion of apoptotic tubules in FD group, in comparison with FS group ($p>0.05$) (Figure 5E). Also, no spermatogenic-stage specific apoptosis was observed.

Folate deficiency increased DNA damage in spermatocytes but not in sperm

To quantify the DNA damage occurring in spermatocytes, cell spreads were prepared from 9-wk old FS and FD mice (FS, n=3; FD, n=3) and co-immunostained for the synaptonemal complex protein (SCP3) which labels synapsed chromosomes and the phosphorylated histone variant, H2AX (γ-H2AX), which is involved in DNA repair activities and targeting of DNA DSBs (McManus and Hendzel 2005) (Figure 6A-D). In meiotic prophase I, γ-H2AX foci are present in response to the naturally induced DNA DSBs associated with recombination. The γ-H2AX number peaks at leptotene stage with 300 foci per nucleus and gradually decreases to 120 in early pachytene and 48 in late pachytene (Chicheportiche et al. 2007).

Consistent with previous results, the sex body, which contains the unpaired regions of X and Y chromosomes, was intensely stained by γ-H2AX. The foci

numbers observed in this study of 125.5 ±2.1 in early pachytene and 58.5 ±1.9 in late pachytene of FS mice were comparable with the previous findings (Chicheportiche et al. 2007). Remarkably, significantly increased γ-H2AX foci was observed in both early and late pachytene stage in FD group ($p<0.05$) (Figure 6E). As meiotic prophase progressed, the difference of the foci number between FS and FD cells rose from 5.9% in early pachytene to 12.5% in late pachytene. This increase in foci in spermatocytes from FD males suggests that DSBs have not been efficiently repaired in FD spermatocytes. More alarmingly, the DSBs might be retained into the matured spermatozoa.

Alkaline comet assay (pH=12.3) was carried out on 18-wk old FS (n=4) and FD (n=4) sperms to detect DNA single-strand and double-strand breaks and alkali-labile damage (Olive 1999). No folate related effect on sperm DNA fragment level represented by Tail DNA, Tail length and Tail extent moment was found ($p>0.05$) (Table 2).

Sperm Quantity and Quality

Since folate deficiency is associated with reduced cell division rate (Stempak et al. 2005), we hypothesized that the highly proliferating male germ cells may be affected as evidenced by reduced sperm counts. Homogenization-resistant sperm counting was carried out on a pair of caput and corpus epididymis for each animal at 15-wk old (FS, n=5; FD, n=5). Surprisingly, there was no significant difference in sperm number between FS and FD mice ($p>0.05$) (Figure 7A).

However, sperm morphology was changed by folate deficiency. At 15-wk, FD males produced considerably more abnormal sperm in comparison to FS males (Figure 7B-D) (FS, n=7; FD, n=3). Sperm with abnormalities including misshapen sperm tails (curled, folded or looped tail) and/or misshapen heads were identified under light microscope and counted. Folate deficiency significantly

reduced the proportion of normal morphological sperm ($p<0.01$) (Figure 7B) and significantly increased the proportion of sperm with defects in tail ($p<0.01$) (Figure 7C) and the proportion of sperm with defects in both head and tail ($p<0.01$) (Figure 7D).

Global Histone methylation was reduced in the sperm of folate deficient mice

It has been suggested that up to 15% of histones are retained in human spermatozoa (Ooi and Henikoff 2007) and in mice the content is thought to be around 1-2% (Calvin 1976; Balhorn et al. 1977; O'Brien and Bellvé 1980; Bench et al. 1986). To investigate the notion that folate availability could alter sperm epigenome by changing available methyl donors for histone methylation by histone methyltransferases, global histone H3K4 and K9 methylation levels were assessed in epididymal sperm extracts. Our results revealed that histone methylation was decreased by folate deficiency, as evidenced by significantly reduced histone methylation level at specific lysine residues. In 9-wk old, FD sperm had reduced H3K9 mono-methylation (me1), compared with FS sperm ($p<0.05$) (FS, n=6; FD, n=6) (Figure 8A); in 18-wk old, FD mice sperm had reduced H3K9 me1, compared with FS sperm ($p<0.05$) (FS, n=7; FD, n=7) (Figure 8B). These results indicate that altered SAM concentration induced by dietary folate has an impact on histone methylation level and that different histone methyltransferases may possess different sensitivity to altered methyl donor pool.

Folate deficiency compromised fertility and pregnancy outcomes

Because of the observed spermatogenic defects, we hypothesized that folate deficiency might alter fertility in adult males. To test this hypothesis, 8-10-wk old males from FS and FD (FS, n=20; FD, n=21) were mated over a period of 10 days

with 1 or 2 females, and mating success was recorded by the presence of a vaginal plug or confirmed pregnancy. No difference of mating performance was observed between the two groups (data not shown). However, fertility in FD males was compromised, as demonstrated by the reduced pregnancy rate of 52.38%, compared with 85% for FS mice ($p<0.05$) (Figure 9A), which was calculated as the percent plug-positive females that became pregnant. This data is based on two breeding trials.

Since the sperm of FD mice were determined to be carrying epigenetic defects and impaired DNA integrity in FD spermatocytes, we hypothesized that there may be consequences for embryo development and offspring health. We next investigated embryo loss and development at embryonic day 18.5 (E18.5). Data were combined from two trials and litters were sired by FS or FD males from 3 to 4 months of age (FS, n=27; FD, n=29). The litter size (Figure 9B), embryo weight and crown-rump length were not affected by folate deficiency ($p>0.05$) (Table 4), and no significant effect on growth was detected. Importantly, various developmental abnormalities were only observed in fetuses sired by FD males (Table 4; Figure 9 E-I). These malformations included: a limb hyperextension where there was straightening the hind limb, also this limb was ectrodactyly where digits were absent; another observed abnormality was an offspring with a domed head which may be due to hydrocephaly which is defined as excessive cerebrospinal fluid within the skull; in several fetuses we observed abnormalities suggesting muscle and/or skeletal defects in the region of the scapula (Table 4; Figure 6 E-I) (Hood 2006). All abnormalities were confirmed by consultation with a McGill Veterinary Pathologist, Dr. Marilene Paquet.

The level of pre- and post-implantation embryo loss was assessed (Table 3). The pre-implantation loss did not significantly differ between pregnancies sired by FS and FD males. Notably, embryonic loss after implantation was found to be 2

fold greater in pregnancies sired by FD males in comparison to those sired by FS males (Figure 9D), as detected by the presence of resorption sites (Figure 9C). However, with the number of litters examined this difference was not statistically significant.

The placenta serves essential functions for embryo development, such as exchange of gas, nutrients and waste. Development of the placenta requires accurate epigenetic information for genomic imprinting from the father (Bressan et al. 2009). Generally, placenta weight and size did not differ between the FS and FD groups. However, we observed abnormalities of some placentas in FD group (Table 4). The occurrence of fused placentas was limited to FD group, as shown in Figure 8I. The two embryos were separately connected but, interestingly, one embryo was very small in size suggesting its development was compromised by the placental abnormality.

Table 1: Effect of folate on body and tissue weights.

Table 1.A

Diet	*Age*	*Mean body weight (g) ± SEM*	*Mean testes weight (mg)± SEM*	*Relative testes weight [%] ± SEM*	*Mean epidymides weight (mg) ± SEM*	*Relative epidymides weight [%] ± SEM*
2mg/kg folic acid	PND6	1.99±0.04	1.22±0.08	0.061±0.003	1.40±0.03	0.070±0.001
0.3mg/kg folic acid	PND6	2.61±0.02**	1.23±0.22	0.047±0.008	1.33±0.11	0.051±0.004
2mg/kg folic acid	PND10	3.67±0.06	3.43±0.02	0.094±0.002	3.18±0.37	0.087±0.010
0.3mg/kg folic acid	PND10	3.88±0.13	3.17±0.54	0.081±0.011	2.95±0.70	0.075±0.016
2mg/kg folic acid	PND12	4.96±0.12	5.13±0.23	0.103±0.002	4.87±0.76	0.099±0.017
0.3mg/kg folic acid	PND12	4.56±0.08	3.77±0.28	0.083±0.006	4.23±0.32	0.093±0.007
2mg/kg folic acid	PND14	5.80±0.23	6.28±0.52	0.12±0.02	8.38±0.65	0.15±0.02
0.3mg/kg folic acid	PND14	4.16±0.83	6.02±0.21	0.16±0.04	8.72±1.01	0.23±0.05
2mg/kg folic acid	PND18	6.73±0.33	12.48±0.76	0.19±0.00	19.42±1.50	0.29±0.01
0.3mg/kg folic acid	PND18	6.20±0.32	11.30±0.53	0.18±0.00	15.78±1.97	0.25±0.02

(A) Effects of folate deficiency on postnatal testis, epididymis and body weight. Values represent absolute tissue weight or relative tissue weight which was calculated as tissue weight/ body weight X100. PND6, PND10, PND12, PND14, and PND18 mice were examined (FS, n=3; FD, n=3). Statistical differences were evaluated by t-test with adjusted probability level by Bonferroni's correction ($p< 0.05/ 5=0.01$ was considered as significant): **$p<0.01$

Table 1.B

Diet	*Age*	*Mean body weight (g) ± SEM*	*Mean testes weight (mg)± SEM*	*Relative testes weight [%] ± SEM*	*Mean epidymides weight (mg) ± SEM*	*Relative epidymides weight [%] ± SEM*
2mg/kg folic acid	6 weeks	16.83±0.44	54.75±2.14	0.32±0.01	29.38±2.21	0.18±0.02
0.3mg/kg folic acid	6 weeks	20.33±0.42**	64.62±3.65	0.32±0.02	24.16±2.60	0.12±0.01
2mg/kg folic acid	9 weeks	22.66±0.75	73.49±2.50	0.32±0.00	37.39±2.25	0.17±0.01
0.3mg/kg folic acid	9 weeks	21.59±0.77	62.99±1.61**	0.29±0.01**	39.05±2.45	0.18±0.01
2mg/kg folic acid	15 weeks	25.29±0.40	70.92±3.51	0.28±0.02	30.74±1.60	0.12±0.01
0.3mg/kg folic acid	15 weeks	24.02±0.74	65.40±2.21	0.27±0.00	26.67±2.69	0.11±0.01
2mg/kg folic acid	18 weeks	30.12±0.45	84.65±1.30	0.28±0.01	39.38±1.75	0.13±0.01
0.3mg/kg folic acid	18 weeks	30.12±0.50	84.30±1.27	0.28±0.01	40.11±1.55	0.13±0.01

(B) Effects of folate deficiency on pubertal and adult testis, epididymide and body weight. 6-wk (FS, n=7; FD, n=5), 9-wk (FS, n=7; FD, n=7), 15-wk (FS, n=12; FD, n=8), and 18-wk (FS, n=13; FD, n=14) mice were examined. Statistical differences were evaluated by t-test with adjusted probability level by Bonferroni's correction ($p < 0.05/4 = 0.0125$ was considered as significant): **$p < 0.01$.

Table 2: Assessment of sperm chromatin integrity by COMET assay

Diet	*Tail length± SEM*	*Tail DNA± SEM*	*Tail extent moment ± SEM*
2mg/kg folic acid	99.82 ±0.44	34.12 ± 0.17	63.28 ±0.40
0.3mg/kg folic acid	99.87 ±0.80	34.06 ± 0.35	63.35 ±0.84

Tail DNA (the percentage of DNA present in the tail), Tail length and Tail extent moment (the average distance migrated by the DNA multiplied by the fraction of DNA in the tail) were used for comparison of danage (Olive 1999) between folate sufficient (FS) and folate deficient (FD) sperm (FS, n=4; FD, n=4) at 18-wk old. Values are presented as Mean±SEM. Data are analyzed by t-test (2-tailed, equal variances assumed): $p>0.05$.

Table 3: Pre- and post-implantation loss was assessed at E18.5

Diet	*Number of corpora lutea (CL) ± SEM*	*Number of implantation sites ± SEM*	*Pre-implantation loss [%] ± SEM*	*post-implantation loss [%]± SEM*
2mg/kg folic acid	13.38±0.65 (n=26)	10.81±0.54 (n=27)	18.26±3.85 (n=26)	2.25±0.87 (n=27)
0.3mg/kg folic acid	13.57±0.40 (n=28)	11.83±0.54 (n=29)	13.29±3.66 (n=28)	4.47±1.20 (n=29)

Pre-implantation loss was calculated as (Number CL – Number implantations)/Number CL x 100; Post-implantation loss is calculated as (Number implantations – Number live embryos)/Number implantations x100. Values are presented as Mean±SEM. Number of CL, number of implantation sites and pre-implantation loss are analyzed using t-test ($p>0.05$). Post-implantation loss is analyzed using Mann-Whitney U test ($p>0.05$).

Table 4: Pregnancy outcomes

Diet	*Embryo weight ± SEM*	*Crown-rump length ± SEM*	*Placenta weight ± SEM*	*Placenta width ± SEM*	*Embryo malformations*	*Placenta malformations*
2mg/kg folic acid	1.39±0.02 (n=21 litters)	24.19±0.26 (n=12 litters)	0.090 ±0.006 (n=12 litters)	7.70±0.16 (n=12 litters)	0/ 285 (27 litters)	Small placenta: 7/ 126 (12 litters).
0.3mg/kg folic acid	1.42±0.02 (n=27 litters)	24.32±0.15 (n=17 litters)	0.086 ±0.004 (n=17 litters)	7.47±0.11 (n=17 litters)	4/ 328 (29 litters)	Small placenta: 9/ 199 (17 litters) Fused placenta: 2/ 199

Embryo weights were combined from two trials. Abnormalities of embryo morphology included: Tow cases of muscle-skeletal developmental problem in shoulder blade region; one case of leg hyperextention with dysgenesis of digits; one case of big head. Small placenta represents runt of placenta weight. Values are presented as Mean±SEM. For the embryo weight, crown-rump length, placenta weight and placenta width, statistical differences were analyzed using t-test (2-tailed): $p>0.05$. For the occurrence of runt of placenta weight, statistical difference was analyzed using Fisher's exact test: $p>0.05$.

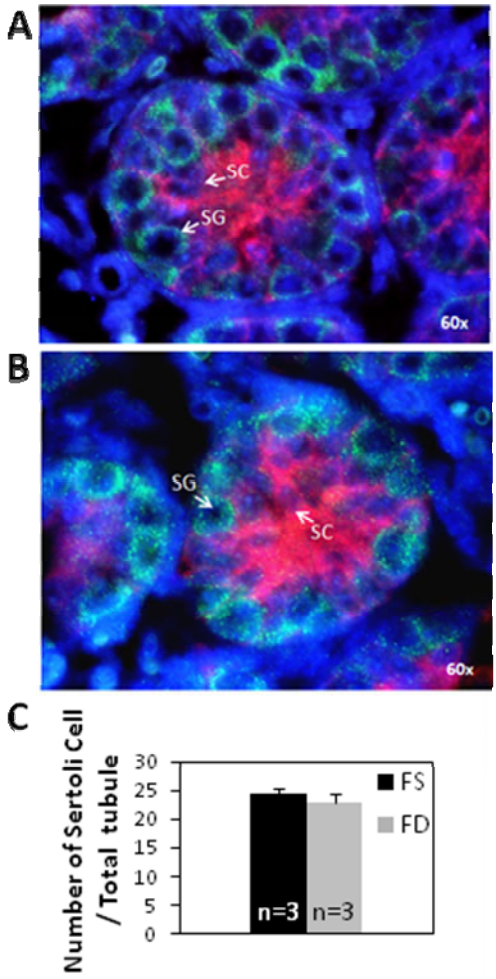

Figure 1: Folate deficiency does not alter Sertoli cell numbers as assessed at postnatal day 10 (PND10).
(A and B) Germ cells were visualized by Mouse VASA homologue (MVH) (green) and Sertoli cells were detected by Mullerian inhibiting substance (MIS) (red) on testis cross sections of PND10 folate sufficient (FS) mice (A)and folate deficient (FD) mice (B). Immunofluorescent pictures of MVH, MIS and DAPI were stacked by Photoshop. Spermatogonia (SG), Sertoli cell (SC). (C) No significant change in Sertoli cell numbers was detected in FD mice in comparison to FS mice. The graph represents Mean ± SEM. The data were analyzed by Mann-Whitney U test ($p \sim 0.05$).

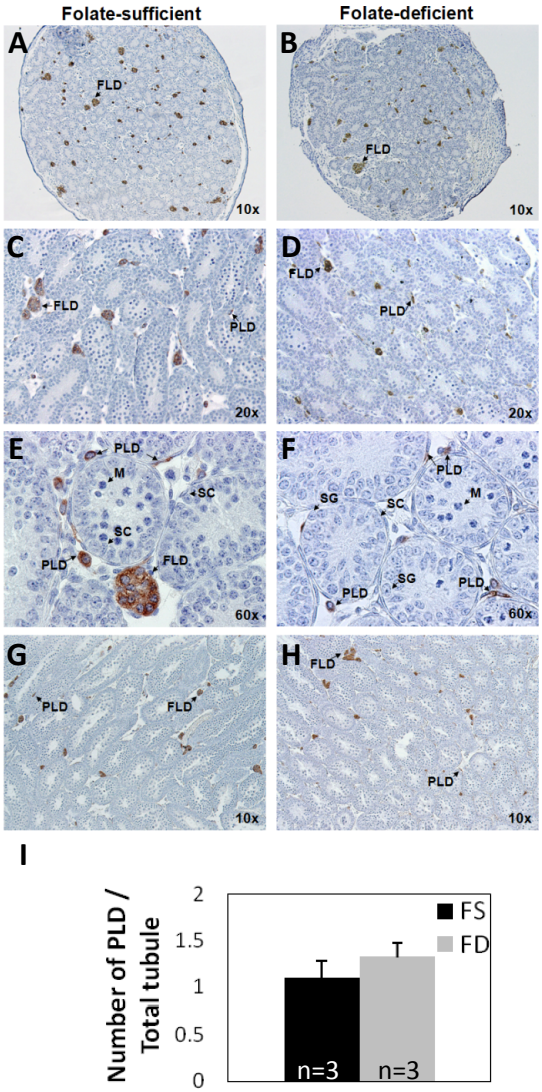

Figure 2: Folate deficiency does not alter Leydig cell proliferation in postnatal day (PND). (A-H) Testis cross sections of mice at PND6 (A-B), PND12 (C-F) and PND 18 (G-H) were stained with the Leydig cell marker 3β-HSD (brown cells indicates positive reactivity). In the interstitial space, the Fetal Leydig cells (FLDs) appear as clusters and Progenitor Leydig cells (PLDs) appear as single cells. Spermatogonia (SG), meiotic spermatocytes (m), Sertoli cell (SC), Progenitor Leydig cell(PLD), Fetal Leydig cell(FLD). (I) Quantitation of PLDs in testicular –cross sections revealed no difference in leydig cell number or appearance between folate sufficient (FS) and folate deficient (FD) mice on PND12. The graph represents Mean ± SEM. Statistical difference was evaluated by Mann-Whitney U test: $p > 0.05$.

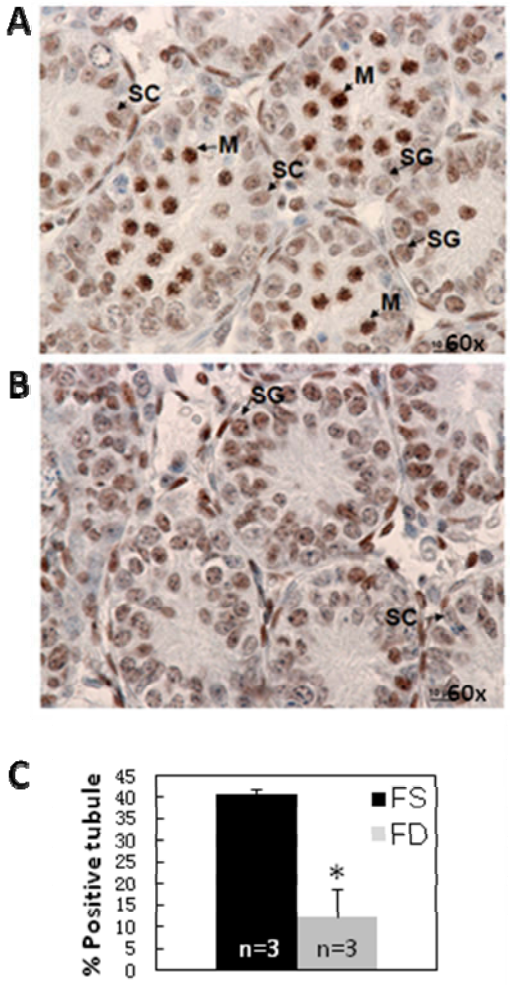

Figure 3: Meiotic onset in the first wave of spermatogenesis was delayed in Folate deficient mice. (A and B) PND12 testis cross sections of folate sufficient (FS) (A) and folate deficient (FD) mice (B) were stained using anti-histone H3 mono-methylation at lysine 4 (H3K4 me1) as a marker for early meiotic spermatocytes . Fewer meiotic cells (M) were present in FD seminiferous epithelium in comparison to FS mice. Spermatogonia (SG), Sertoli cell (SC). (C) The graph represents the ratio of meiotic tubules (~10 meiotic germ cells) to total number of tubules. Values are presented as Mean ± SEM. Statistical analysis was done by t-test (2-tailed, equal variances not assumed): $^{b}p<0.05$.

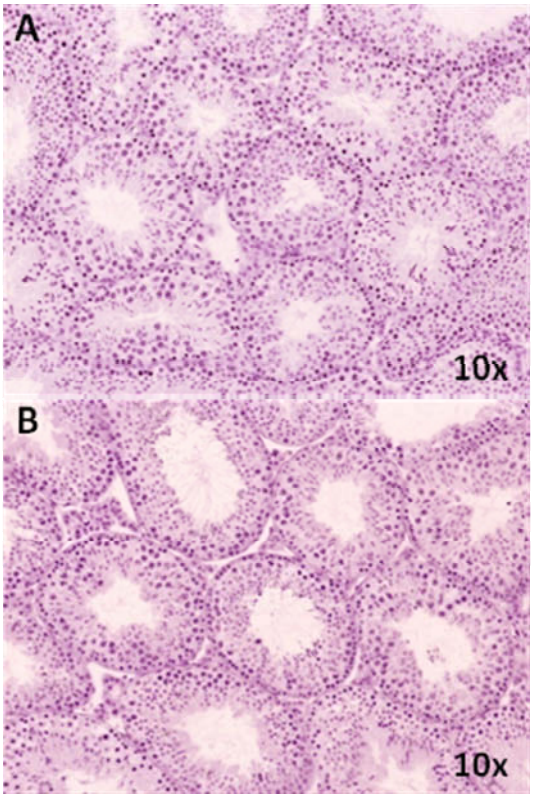

Figure 4: Histopathological analysis of spermatogenesis indicated that it was normal in folate defecient mice. Hematoxylin and eosin staining of testicular cross sections shown from 18-wk old mice reveals normal spermatogenesis in folate sufficient (A) and folate deficient (B) mice. Sections shown are representative sections of adult mice.

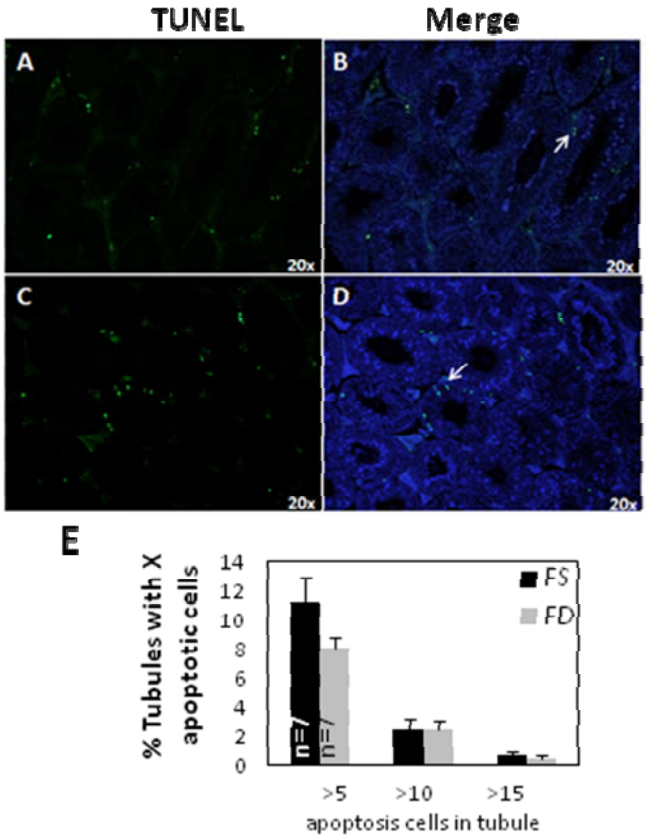

Figure 5: Effect of folate deficiency on germ cell apoptosis.
(A-D) Detection of apoptosis by TUNEL staining on cross sections of testis of 18-wk old mice in folate sufficient (FS) mice (A and B) and folate deficient (FD) mice (C and D). Arrows point to apoptotic cells. (E) Graphs represent the percentages of tubules with more than 5, 10 or 15 positive cells. Values are presented as Mean ± SEM. There was no statistical difference in the level of apoptosis between FS and FD mice as determined by the t-test (2-tailed): $p > 0.05$.

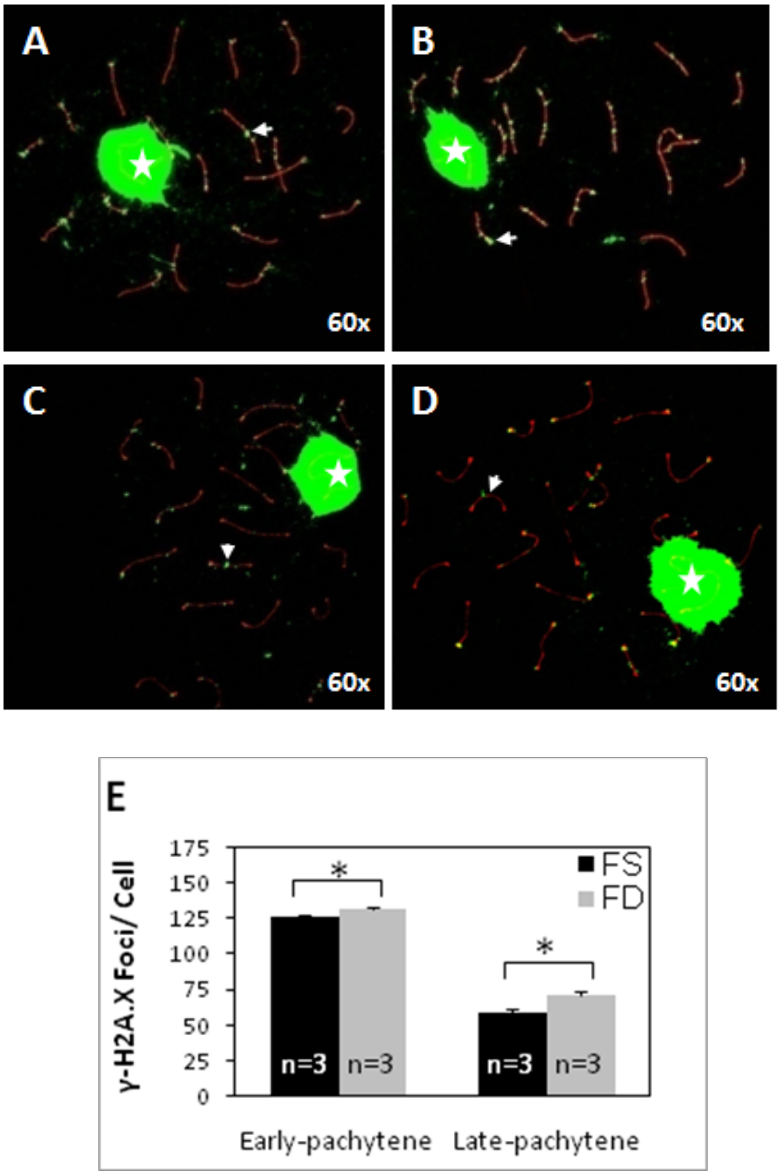

Figure 6: Folate deficiency is associated with increaced DNA double strand breaks(DSBs) in early and late pachytene spermatocytes. (A) Shown is co-immunofluorescent staining for synaptonemal complex protein 3 (red) and γ-H2AX (green). Arrows indicate DNA DSBs, and stars indicate the sex body. (B) 25 early pachytene spermatocytes and 30 late pachytene spermatocytes in each animal at 9-wk of age were analyzed. The values are shown are the mean ± SEM. Statistical analysis was done using a subsample model to test whether there was a difference between folate sufficient (FS) and folate deficient (FD) mice:*$p<0.05$.

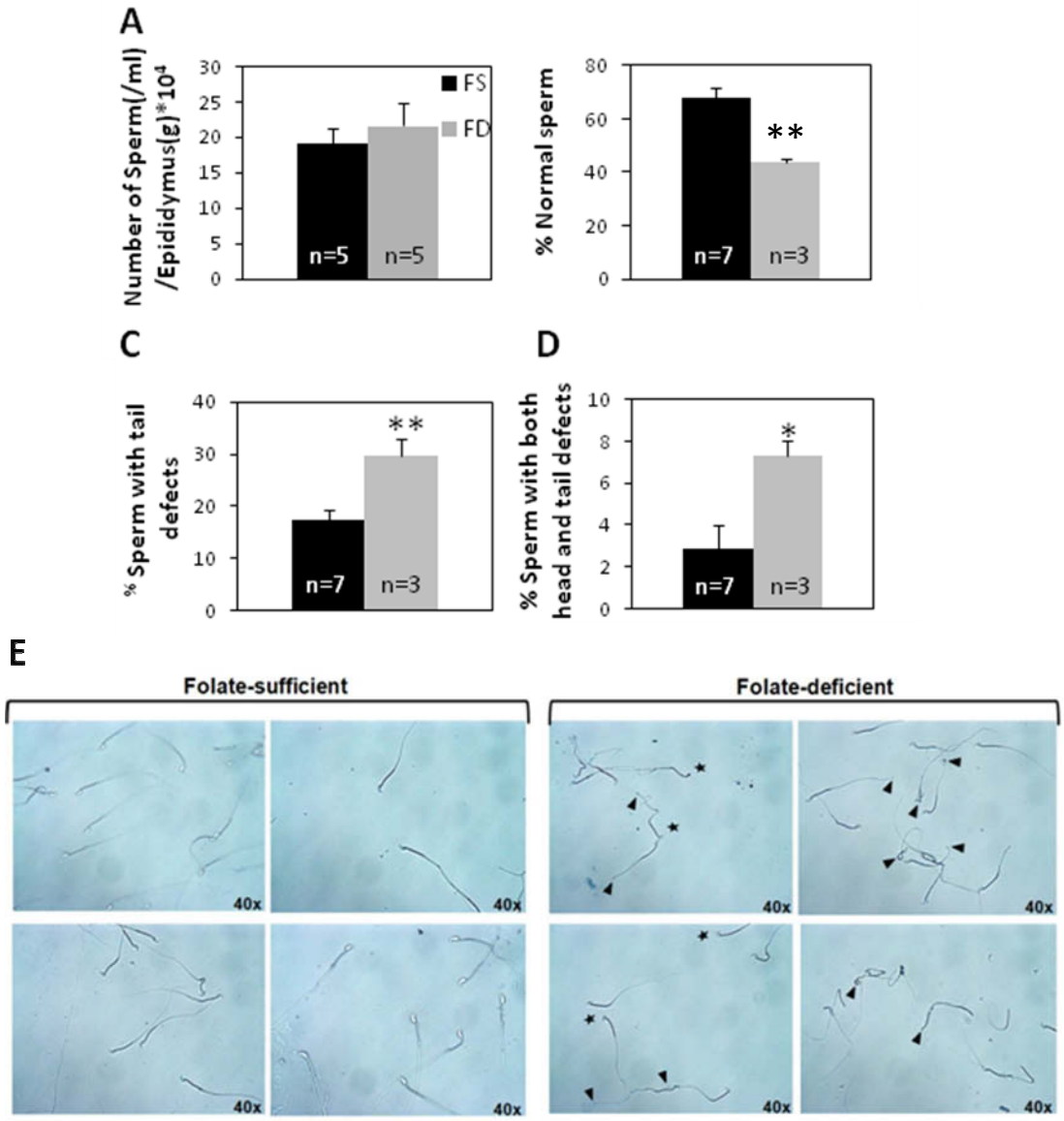

Figure 7: Sperm quantity was not altered but sperm quality was decreased in folate deficient mice. (A) Homogenization resistant sperm counts from epididymus from 15-wk mice was not changed in folate deficient (FD) mice compared with folate sufficient (FS) mice. (B-D) However, the percentage of abnormal sperm was increased significantly. Values are presented as mean ± SEM. Statistical differences between FS and FD were analyzed by t-test (2-tailed): **p< 0.01; *p< 0.05. (B) Sperm morphology of 4 month old FS and FD mice. Folate deficiency was associated with abnormal sperm morphology such as coiled tails (highlighted by arrow heads) and misshapen heads (highlighted by stars).

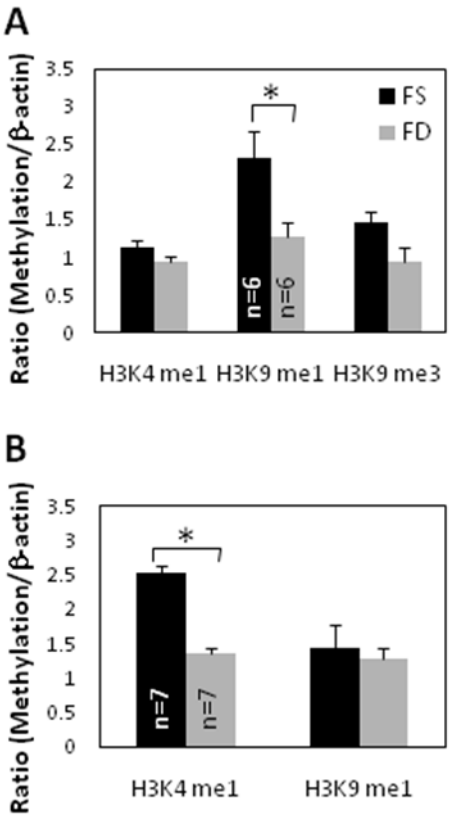

Figure 8: Effect of folate deficiency on sperm histone methylation at histone H3 lysine 4 (H3K4) and H3 lysine 9 (H3K9). Western blot was used to assess global levels of H3K4 mono-methylation (me1), H3K9 me1 and H3K9 tri-methylation (me3) in 9-wk (A) and 18-wk (B) old mice sperm . Data are presented as the ratio of methylation over β-actin. The values are Mean ± SEM. Statistical differences of methylation between folate sufficient (FS) and folate deficient (FD) mice are evaluated by t-test (2-tailed): *p<0.05.

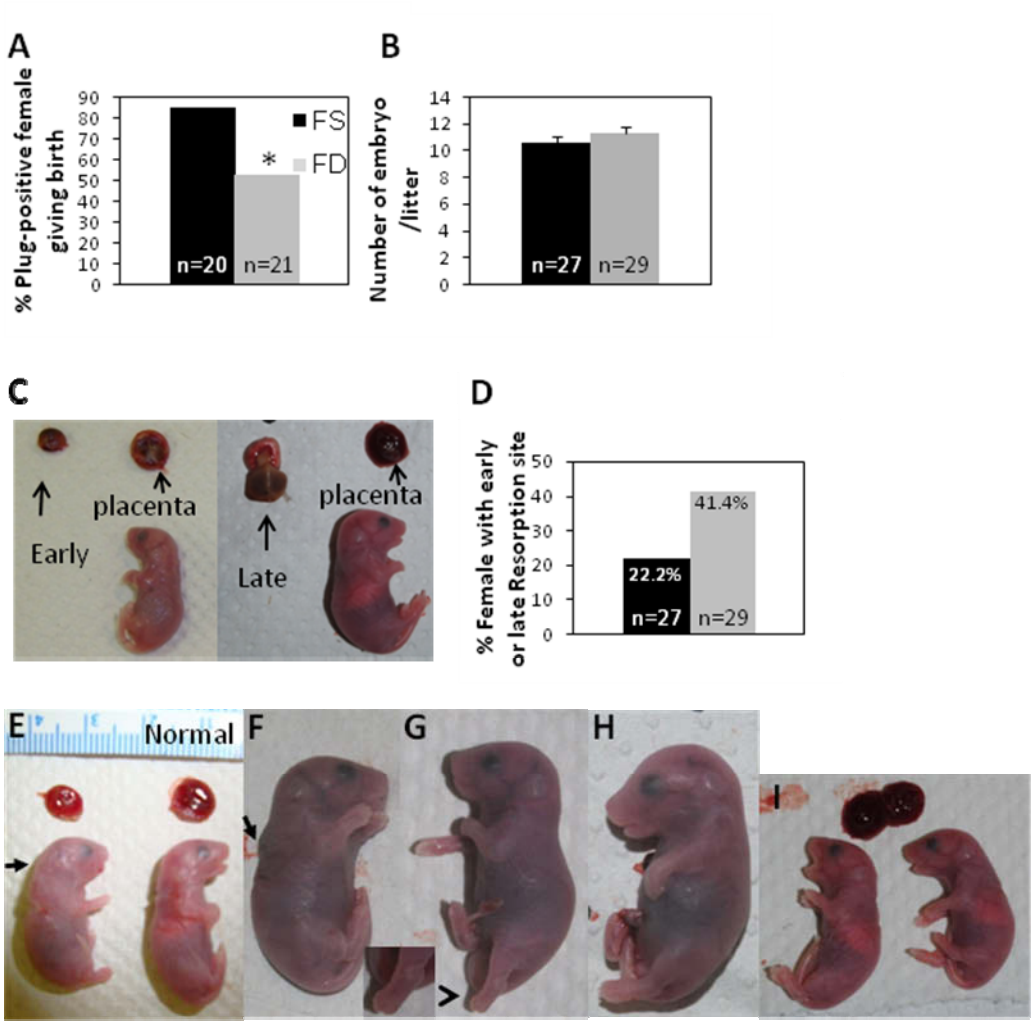

Figure 9: Folate deficiency compromised fertility and pregnancy outcomes.
3-4 month old males were bred to CD1 females and their litters were examined at E18.5 to assess pregnancy outcomes. (A) Pregnancy rate over a period of 10 days. (B) Litter size was examined at E18.5. (C) Early resorption is characterized by early-remnants of a placenta being present but no obvious signs of an embryo; Late resorption is characterized by visible remnants of an embryo present having died up to E18.5. (D) Resorption represents the frequency one or more early or late resorption site(s). All data were calculated and shown on a per male basis. Statistical differences between folate sufficient (FS) and folate deficient (FD) were evaluated: Fisher's exact test for pregnancy rate (*$p<0.05$); t-test (2-tailed) for litter size ($p>0.05$); Chi-square test for resorption rate ($p=0.125$). (E-I) Abnormal embryos and placentas sired by FD males. (E and F) Arrows suggest muscle-skeletal developmental problem in shoulder blade region. (G) The arrow head indicates abnormal leg hyperextention with dysgenesis of digits. (H) enlarged head. (I) Fused placentas linked to two embryos in which one embryo was small in size.

Discussion

This is the first study that has examined the effect of dietary folate on the sperm epigenome and offspring outcomes. Importantly our data indicate that the sperm epigenome is sensitive to folate levels in the diet. Given the most recent data on the sperm epigenome in mouse and human (Hammound et al. 2009; Brykczynska et al. 2010) which showed that histone modifications in sperm mark genes for embryo development, our results from this study are relevant in the context of male reproductive and offspring health. Here we have shown that a FD diet is associated with reduced histone H3 methylation, previously shown to mark imprinted genes in sperm (Delaval et al. 2007) and genes involved in embryo development (Hammound et al. 2009; Brykczynska et al. 2010). We found that FD increased DNA damage in developing sperm, specifically the pachytene spermatocytes, yet this damage was not sustained in mature sperm as measured by the COMET assay. We therefore suggest that the 2 fold increase in embryo resorption rate and the developmental abnormalities in the FD sired offspring were a consequence of the altered sperm epigenome. In the ongoing analysis from this study it will be key to identify the gene specific regions that contain altered epigenetic marks at the level of both histone and DNA methylation to be able to ascertain the relationship between the sperm epigenome and the offspring outcomes. As this project develops in the future we aim to be able to extend this knowledge to fertility counseling of men and to emphasize that as in females, male pre-conception health is directly tied to folate levels.

General effects of folate deficiency on reproductive organs and sperm production

Body weight of male offspring was monitored as a general gauge of health and

there was no reduction of body weigh in FD males compared with FS males. This result is consistent with the C57⁄Bl6 mice model which was on the same FD diet for 12 to 14 months (Knock et al. 2008).

In our study, the testis weights were not changed by folate deficiency at all time points examined except those at 9-wk of age. The epididymide weights of FD mice were also not changed at any age examined, which is consistent with our finding that epididymal sperm reserve was not reduced in FD mice. These results agree with those from a similar ongoing research project where BALB/c mice on folate deficient (0.3mg folic acid/kg diet) diet from weaning to 12 months have no change in reproductive organs weights, compared with those on folate sufficient (2mg folic acid/kg diet) diet (Chan et al. 2010).

Histological examination of adult testes at the light microscope level revealed no detectable morphological differences between FS and FD mice at 18-wk old. However, in FD mice, significantly increased sperms showed abnormalities in morphology, including misshapen heads and/or misshapen tails. These defects of spermatogenesis might be result from altered gene expression by disrupted epigenetic information in FD male germ cell. The morphology defects could contribute to the reduced fertility in FD mice. These results support the scenario that folate is a determinant of male fertility, as evidenced by several clinical trials which found that folate supplementation could rescue male fertility (Forges et al. 2007).

Sertoli cell and Leydig cell development were not affected by folate deficiency

Our evaluation of the Sertoli cell and Leydig cell number in postnatal day concluded that there was no folate–related effect on their proliferation. Therefore, we postulated that the development of the Sertoli cell and Leydig cell were not affected by folate deficiency. Thus, the cause of spermatogenesis

defects in FD mice should largely originate from the male germ cells themselves rather than a stress imposed by somatic cells in the testis. To further confirm the postulation that the Sertoli cell and Leydig cell function normally in FD mice, the hormone levels, such as testosterone, estradiol and LH levels, should be tested.

Meiosis onset was delayed in folate deficient mice

The first meiotic cells, the pre-leptotene appears at PND10 and is important as an onset toward fertility. Our study showed a delay of meiotic onset in FD mice. Further examination of animals in later postnatal days did not find a remained discrepancy of meiotic cell number between FS and FD males. Taking into consideration the unchanged sperm counts, we concluded that meiosis was not completely blocked in FD mice and they could catch up with FS mice in an older age. Commitment of spermatogonia to meiosis requires proper expression of numerous genes and presence of appropriate chromatin structure (Rousseaux et al. 2005). Delay of meiosis onset in FD mice could be caused by disturbed DNA synthesis or epigenetic defects, which would result in marked changes in chromatin organization and the loss of chromosome segregation. In fact, several studies with a disturbance of epigenetic information in male germ cells describe a failure of meiosis. For example, male mice that lack DNA methyltransferase Dnmt3L showed a complete failure of meiotic progression in young adult and were aspermatogonia in older mice (Bourc'his and Bestor 2004). Meisetz, a meiosis specific histone methyltransferase for H3K4me3, was demonstrated to be important in mediating meiosis progression in male germ cell (Hayashi et al. 2005). Moreover, double knockout of Suv39h1 and Suv39h2, which are murine H3K9 histone methyltransferases, the pachytene spermatocytes were almost absent due to perturbed chromosome interactions during meiosis in male mice

(Peters et al. 2001).

Folate deficiency increased DNA damage in spermatocytes but not in spermatozoa

Strikingly, our investigation on the impact of folate deficiency on the DNA integrity of the spermatocytes revealed an increased incidence of DSBs i.e. γ-H2AX foci, in early and late pachytene spermatocytes. γ-H2AX foci occur in case of formation of DSBs. A study of radiation of spermatocytes induced a peak of γ-H2AX foci, which suggests that increased γ-H2AX foci in spermatocytes might be attributed to induced DNA DSBs (Chicheportiche et al. 2007). It is unclear in our study whether the increased DSB is because of folate deficiency induced DNA damage or impaired DNA repair mechanism in spermatocytes. We propose that the underlying mechanism is that in the situation of folate deficiency, uracil is misincorporated into DNA which can then lead to miss-repair and DNA damage (Fenech 2001). Moreover, it is also possible that the DNA damage is caused by oxidative stress in the condition of a high level of homocysteine when folate is deficient (Loscalzo 1996; Agarwal et al. 2006).

Since increased sperm DNA damage is related to reduced fertility and abnormal embryo development (Chohan 2006; Hales et al. 2005), we wanted to determine whether the DNA breaks could be retained into matured spermatozoa by COMET assay. Our examination of sperm by COMET assay showed the amount of DNA breaks was similar between FD and FS males. Moreover, despite the increased DSBs in spermatocytes, male germ cell apoptosis was not increased in FD mice, as demonstrated by the TUNEL Assay. DNA fragmentation is one typical feature of apoptotic cells; however the DSBs in FD spermatocytes may not be severe enough to cause degradation of DNA and to be detected by TUNEL. Also, from these results we could conclude that DNA repair mechanism in FD male

germ cells was not impaired and managed to eliminate the DSBs efficiently after late pachytene stage.

Reproductive outcomes were compromised in folate deficient mice

The fertility was significantly reduced in FD mice. Since the E18.5 embryo analysis found that pre-implantation loss was not enhanced in females mated with FD mice, we postulated that the observed reduction in fertility as measured by pregnancy rate is mostly due to sperm defects such as poor motility, as evidenced by the increased tail defects.

Despite the reduced fertility, most FD males still sired offspring although it took longer for them to successfully impregnate. This warrants further investigation, considering that errors of genomic and epigenomic information during spermatogenesis may be transmitted to the offspring. In contrast to the absence of an effect on embryo pre-implantation loss, folate deficiency resulted in increased post-implantation loss, although this was not significant. However, the litter size was not reduced in FD males. Perhaps it is because most of the time there was only one resorption occurred in each uterus, which would not contribute to a significant change of litter size and would not be detected due to the variation in litter size. Although the embryos sired by FS and FD males have similar weights and crown-rump lengths, some embryos showed abnormalities in morphology. Because of the variable phenotypes among the abnormal embryos in FD mice, it is not possible to determine the specific cause of the malformations or the specific reason of the post-implantation loss.

It is suggested that histone retention in sperm may contribute to embryo development (Hammound et al. 2009; Brykczynska et al. 2010). In light of these recent studies our results showing that sperm from FD males had an altered sperm epigenome suggest that this could be related to embryo abnormalities

that were only observed in offspring sired by FD. In our study, since the DNA DSBs were already repaired in FD spermatozoa, we postulated that the compromised pregnancy outcomes may be due to the altered epigenetic information, in particular, histone methylation.

Also, the impact of the sperm epigenome on embryo development and pregnancy loss could be an indirect effect via the placenta. We believed that the placental development was also adversely affected, as evidenced by the observation of fused placentas in litters sired by FD males. However, the exact reason of the placenta fusion is unknown in our study. In some instances, if the embryos implant very nearby each other, the placentas can become fused (Neer 2005). We hypothesize that the developmental defects in FD placentas may be due to imprinting defects originated from the spermatogenesis. Genomic imprinting is a phenomenon that leads to the exclusive expression of paternally and maternally inherited alleles of a subset of genes (Trasler 2006; Allegrucci et al. 2005). Paternally imprinted genes are transcriptionally silenced by DNA methylation and histone modifications on the paternal-origin allele and are expressed from the maternal-origin allele (Allegrucci et al. 2005). Imprints are erased and reestablished during the epigenetic reprogramming that occurs in germ cells. However, the genome-wide demethylation during the pre-implantation stage spares the imprints (Bressan et al. 2009). This indicates that the period of germ cell development is quite essential for the establishment of correct imprinting information and the normal development of the embryo and placenta. The placenta is an important organ of imprinted gene action (Bressan et al. 2009). Interestingly the imprinted genes found in placenta are all repressed on the paternal-origin allele and expressed from the maternal-origin allele (Wagschal and Feil 2006). A resent study found that maintenance of imprinting in placenta depend more on repressive histone methylation than on

DNA methylation (Wagschal et al. 2008; Bressan et al. 2009). Therefore the altered histone methylation in FD mice may account for the observed defects in placenta development.

Folate deficiency disrupted Histone methylation in sperm

With accumulated evidence suggesting that epigenetic errors in sperm may be a route for paternal transmitted disease, it is urgently needed to investigate the impact of environmental factors like diet on the epigenetic programming in the developing male germ cells and sperm. The folate-derived methyl group is a main methyl donor for homocysteine remethylation, which is subsequently converted to methionine and then SAM, the ubiquitous methyl donor with a wide variety of substrates, including DNA, RNA, and perhaps histones (Choi et al. 2009). Until now it was not known whether dietary folate levels can influence histone methylation. For the first time, our study linked dietary folate deficiency with disrupted histone methylation in sperm in a mouse model. The underlying mechanism is postulated to be that in the condition of folate deficiency, the SAM regulated methylation process of histone is not able to keep pace with the rapid cell division of the male germ cells.

Gametogenesis is one critical time window in the life time that epigenetic changes are sensitive to the environmental exposure (Szyf et al. 2007; Dolinoy et al. 2007). Extensive epigenetic reprogramming takes place during the development of male germ cell in order to reset imprints, erase epimunations and give rise to totipotent zygotes (Allegrucci et al. 2005). The DNA methylation pattern undergoes a genome-wide erasure in primodial germ cells from E11.5 to E14, followed by the *de novo* methylation that starts from E15.5 and complete prior to the end of pachytene after birth in the first wave of spermatogenesis (Sasaki and Matsui 2008). Not only the DNA methylation, but also the histone

modification in the male germ cell is found to be dynamic throughout life time. During spermatogenesis, H3K4 methylation is under a tight regulation (Godmann et al. 2007). A high level of H3K4 methylation appears in spermatogonia through to leptotene, followed by down-regulation in pachytene and reappearance in elongating spermatids, suggesting its role in transcription regulation and chromatin remodeling events (Godmann et al. 2007). Importantly in this dramatic epigenetic programming process, the fidelity of the epigenome must be ensured for male reproductive health and prevention of paternal disease transmission (Godmann et al. 2009).

How histone methylation in sperm contributes to epigenetic inheritance and embryo development is largely unknown. However this issue has stared to be focused in recent years and several studies have provided evidence suggesting histones retained in sperm are not random but has specific function in both human and mice, such as marking imprinted genes and genes for embryo development (Gatewood et al. 1987; Gardiner-Garden et al. 1998; Delaval et al. 2007; Hammound et al. 2009; Brykczynska et al. 2010). It is found that in mice the active allele of imprinted genes is associated with H3K4 dimethylation and H3 acetylation, while the silent allele is marked by H3K9 trimethylation (Delaval et al. 2007). Epigenetic inheritance by histone retention might be manifested as preventing DNA methylation on imprinted genes from being erased during embryonic reprogramming (Ooi and Henikoff 2007). Most strikingly, recent studies suggested that significant histones are associated to many loci important for embryo development in both mouse and human sperm (Hammound et al. 2009; Brykczynska et al. 2010). The gene active mark, H3K4me3, is retained in sperm and is localized to developmental promoters, regions in HOX clusters, noncoding RNAs, and imprinted genes, supporting a role in embryo development (Hammound et al. 2009).

Several *in vivo* and *in vitro* studies showed that folate status can alter DNA methylation (Stempak et al. 2005; Kim 2005). In human trials, leukocyte DNA is hypomethylated in response to inadequate folate intake in postmenopausal women (Jacob et al, 1998; Rampersaud et al. 2000). Interestingly, a study using restriction landmark genomic scanning (Chan et al. 2010) showed that in BALB/c mice, FD diet (0.3mg folic acid/kg diet) from weaning to 12 months of age results in DNA hypermethylation compared with those on FS diet (2mg folic acid/kg diet). To explain the observations that FD diet resulted in decreased H3K4 methylation but DNA hypermethylation in mice sperm, we must take into consideration that histone modification and DNA methylation are mutually regulated (Li 2002). Now accumulating studies revealed that histone methylation is required for DNA methylation. In *Neurospora crassa*, the disruption of H3K9 methyltransferase results in a complete loss of DNA methylation (Bannister and Kouzarides 2005; Li 2002). In mouse embryonic stem cells, the double mutation of *Suv39h* (H3K9 methyltransferases) is associated to DNA hypomethylation in major satellite DNA in pericentric regions (Freitag and Selker 2005). While the gene repressive mark H3K9 methylation is assumed to trigger DNA methylation, the active mark H3K4 methylation is suggested to repulse DNA methylation (Ooi et al. 2007; Hu et al. 2009). DNMT3L triggers the de novo methylation mediated by DNMT3a2 upon contact with nucleosomes that contain unmethylated H3K4 (Ooi et al. 2007; Hu et al. 2009). Therefore, the possible scenario could be that in the circumstances of unmethylated H3K4, the DNMT3a2 is falsely stimulated and DNA de novo methylation is carried out in some chromatin region.

Another possibility could be that DNA methylation was affected by the folate deficiency and further led to the alteration of histone methylation. DNA methylation is crucial in initiating chromatin remodeling and gene expression regulation. For example, DNMT1 recruits HDACs and an H3K9 trimethylase

Suv39h1. (Turek-Plewa and Jagodzinski 2005). Also, the Methy1-CpG-binding protein MeCP2 recruits H3K9 methylation to reinforce gene silencing (Fuks et al. 2003). Besides, DNA demethylation could contribute to marked changes in chromatin organization and the loss of chromosome segregation (Lamprecht and Lipkin 2003). Therefore, in ongoing studies from these trials the DNA methylation level in the sperm is being measured.

In conclusion, the folate deficient mice in this study were determined to produce sperm bearing disturbed epigenetic information yet were still fertile. This is an appropriate animal model for investigating the improvement of male pre-conceptional health and epigenetic errors as a route for paternal transmitted disease, compared with other animal models carrying mutations in chromatin modifiers which result in infertility and are not representative of real-life situation in humans (reviewed in Godmann et al. 2009). This study is the first to extensively assess the impact of dietary folate deficiency on male reproductive health. Our results strongly support that adequate folate intake is required for maintaining male reproductive health, in particular the correct epigenetic programming during spermatogenesis.

Summary

While it is becoming increasingly clear that paternal exposures contribute significantly to infertility and adverse progeny outcomes, the underlying mechanisms are not well understood. The purpose of this dissertation is to advance our current knowledge of the mechanisms by which preconceptional paternal exposure to folate deficient diet alters male germ cell quality and have consequences for fertility and early embryo development. The results of our studies provide novel information that deficient dietary folate alters the epigenome of male germ cell in a manner that adversely impacts the next

generation and has implications for improving male reproductive health. The future studies will be focused on looking through and verifying the ChIP-sequence data on sperm histone H3K4 trimethylation and histone H3K9 trimethylation, in order to specify genes that have an altered histone methylation level, and to further relate comfirmed disturbed histone methylation with abnormalities observed in offspring development. In the ongoing studies using this model it will be important to assess the global DNA methylation. In addition, it would be of interest to investigate more about folate metabolism in male reproductive system, including locating folate receptors and evaluating folate carriers in the testis.

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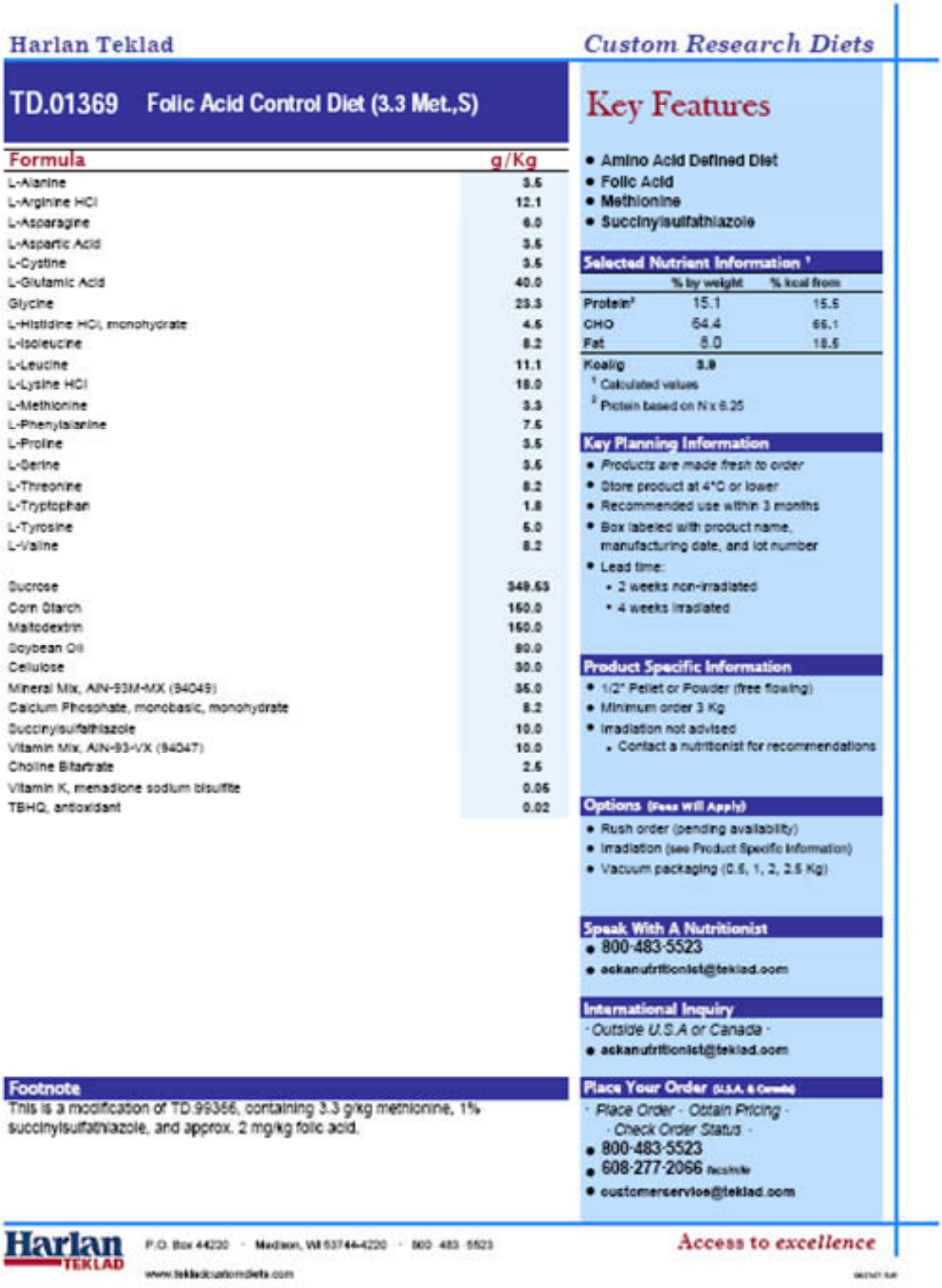
APPENDIX

Appendix 1: Harlan Laboratories Folate-sufficient diet TD.01369



Harlan Teklad — Custom Research Diets

TD.01369 Folic Acid Control Diet (3.3 Met.,S)

Formula	g/Kg
L-Alanine	3.5
L-Arginine HCl	12.1
L-Asparagine	6.0
L-Aspartic Acid	3.5
L-Cystine	3.5
L-Glutamic Acid	40.0
Glycine	23.3
L-Histidine HCl, monohydrate	4.5
L-Isoleucine	8.2
L-Leucine	11.1
L-Lysine HCl	18.0
L-Methionine	3.3
L-Phenylalanine	7.5
L-Proline	3.5
L-Serine	3.5
L-Threonine	8.2
L-Tryptophan	1.8
L-Tyrosine	5.0
L-Valine	8.2
Sucrose	349.55
Corn Starch	150.0
Maltodextrin	150.0
Soybean Oil	90.0
Cellulose	30.0
Mineral Mix, AIN-93M-MX (94049)	35.0
Calcium Phosphate, monobasic, monohydrate	8.2
Succinylsulfathiazole	10.0
Vitamin Mix, AIN-93-VX (94047)	10.0
Choline Bitartrate	2.5
Vitamin K, menadione sodium bisulfite	0.05
TBHQ, antioxidant	0.02

Footnote
This is a modification of TD.99366, containing 3.3 g/kg methionine, 1% succinylsulfathiazole, and approx. 2 mg/kg folic acid.

Key Features

- Amino Acid Defined Diet
- Folic Acid
- Methionine
- Succinylsulfathiazole

Selected Nutrient Information [1]

	% by weight	% kcal from
Protein[2]	15.1	15.5
CHO	64.4	66.1
Fat	6.0	18.5
Kcal/g	3.9	

[1] Calculated values
[2] Protein based on N x 6.25

Key Planning Information

- *Products are made fresh to order*
- Store product at 4°C or lower
- Recommended use within 3 months
- Box labeled with product name, manufacturing date, and lot number
- Lead time:
  - 2 weeks non-irradiated
  - 4 weeks irradiated

Product Specific Information

- 1/2" Pellet or Powder (free flowing)
- Minimum order 3 Kg
- Irradiation not advised
  - Contact a nutritionist for recommendations

Options (Fees Will Apply)

- Rush order (pending availability)
- Irradiation (see Product Specific Information)
- Vacuum packaging (0.5, 1, 2, 2.5 Kg)

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Appendix 2: Harlan Laboratories Folate-deficient diet TD.01546

Harlan Teklad — Custom Research Diets

TD.01546 Folic Acid Defic. Diet (3.3 Met., S)

Formula	g/Kg
L-Alanine	3.6
L-Arginine HCl	12.1
L-Asparagine	6.0
L-Aspartic Acid	3.6
L-Cystine	3.6
L-Glutamic Acid	40.0
Glycine	23.3
L-Histidine HCl, monohydrate	4.6
L-Isoleucine	8.2
L-Leucine	11.1
L-Lysine HCl	18.0
L-Methionine	3.3
L-Phenylalanine	7.6
L-Proline	3.6
L-Serine	3.6
L-Threonine	8.2
L-Tryptophan	1.8
L-Tyrosine	6.0
L-Valine	8.2
Sucrose	368.1787
Corn Starch	160.0
Maltodextrin	160.0
Soybean Oil	80.0
Cellulose	30.0
Mineral Mix, AIN-93M-MX (94049)	35.0
Calcium Phosphate, monobasic, monohydrate	8.2
Succinylsulfathiazole	10.0
Choline Bitartrate	2.6
Vitamin K, menadione sodium bisulfite	0.06
TBHQ, antioxidant	0.02
Green Food Color	0.1
Niacin	0.03
Calcium Pantothenate	0.016
Pyridoxine HCl	0.007
Thiamin HCl	0.006
Riboflavin	0.006
Folic Acid	0.0003
Biotin	0.0002
Vitamin B_{12} (0.1% in mannitol)	0.026
Vitamin E, DL-alpha tocopheryl acetate (500 IU/g)	0.16
Vitamin A Palmitate (500,000 IU/g)	0.008
Vitamin D_3, cholecalciferol (500,000 IU/g)	0.002
Vitamin K_1, phylloquinone	0.0008

Footnote

This is a modification of TD.01370. Formula includes succinylsulfathiazole at 1%.

Key Features

- Amino Acid Defined Diet
- Folic Acid
- Methionine
- Color Coded

Selected Nutrient Information [1]

	% by weight	% kcal from
Protein[2]	15.1	15.5
CHO	64.4	66.1
Fat	8.0	18.5
Kcal/g	3.9	

[1] Calculated values
[2] Protein based on N x 6.25

Key Planning Information

- *Products are made fresh to order*
- Store product at 4°C or lower
- Recommended use within 3 months
- Box labeled with product name, manufacturing date, and lot number
- Lead time:
  - 2 weeks non-irradiated
  - 4 weeks irradiated

Product Specific Information

- 1/2" Pellet or Powder (free flowing)
- Minimum order 3 Kg
- Irradiation not advised
  - Contact a nutritionist for recommendations

Options (Fees will Apply)

- Rush order (pending availability)
- Irradiation (see Product Specific Information)
- Vacuum packaging (0.5, 1, 2, 2.5 Kg)

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