PHARMACOLOGICAL AND GENETIC APPROACHES TO ALTERING DNA METHYLATION IN THE MOUSE MALE GERM LINE: EFFECTS ON SPERMATOGENESIS AND EMBRYOGENESIS

by

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This thesis is dedicated with great love to my parents who always stood by me and encouraged me along the way when I lost hope, and to my grandfather who was always so proud *(and is right now, somewhere wearing a tux..)*.

"Earthman, the planet you lived on was commissioned, paid for, and run by MICE...These creatures you call mice, you see, they are not quite as they appear. They are merely the protrusion into our dimension of vast hyper-intelligent pan-dimensional beings. The whole business with the cheese and the squeaking is just a front....they've been experimenting on you, I'm afraid."

- Douglas Adams, The Hitchhiker's Guide to the Galaxy

...and to my mice, I couldn't have done it without you.

ABSTRACT

DNA methylation is an epigenetic phenomenon catalysed by a family of DNA methyltransferases (DNMTs) and is tightly regulated throughout spermatogenesis. In these studies, I employed pharmacological and genetic means to disrupt DNA methylation in the male germ line. First, to lower levels of DNMT activity, I treated adult wild-type (*Dnmt1*$^{+/+}$) and DNMT-deficient (*Dnmt1*$^{c/+}$) male mice with the anticancer agent 5-aza-2'-deoxycytidine (5-azaCdR), which incorporates into DNA and inhibits methylation. *Dnmt1*$^{+/+}$ males treated with clinical doses of 5-azaCdR for 7 weeks, to expose germ cells throughout their development, exhibit abnormal testicular histology, as well as dose-dependent reductions in testis weight, sperm count, fertility, and sperm motility. In contrast, *Dnmt1*$^{c/+}$ males are evidently partially protected from the deleterious effects of 5-azaCdR, likely because of lowered levels of DNMT1. Although 5-azaCdR-treated *Dnmt1*$^{c/+}$ males display reduced testis weight, they do not have altered sperm number or testicular histology; these males also have greater changes in sperm DNA methylation relative to treated *Dnmt1*$^{+/+}$ males. Interestingly, elevated preimplantation loss is observed in females mated with treated males of either genotype. This preimplantation loss appears to result from a reduction in oocyte fertilisation, perhaps because of altered sperm motility. Moreover, higher levels of blastocyst loss were observed in embryos derived from matings with treated *Dnmt1*$^{+/+}$ males. I suggest that the testicular effects and reduced fertility of treated *Dnmt1*$^{+/+}$ males are the result of both the cytotoxic and hypomethylating effects of 5-azaCdR, whereas the changes observed in 5-azaCdR-treated *Dnmt1*$^{c/+}$ males may be due more to hypomethylation and its consequences.

Our second genetic model targetted the methyl supply necessary for DNA methylation. Methylenetetrahydrofolate reductase (MTHFR) is integral to the folate

pathway and is necessary for methionine and S-adenosylmethionine formation; levels of MTHFR are highest in the testis, which suggests a role for this enzyme in spermatogenesis. Indeed, severe MTHFR deficiency (*Mthfr*$^{-/-}$) results in severely abnormal spermatogenesis and infertility. Within my studies, I showed that maternal administration of betaine, an alternative methyl donor, throughout gestation and nursing, results in reduced germ cell apoptosis in male pups soon after birth. When betaine supplementation is maintained post-weaning, spermatogenesis is partially restored and fertility increases significantly. Here, I provide evidence that perturbation of the components of the DNA methylation pathway detrimentally affects male germ cell development and fertility.

RÉSUMÉ

La méthylation de l'ADN est une modification épigénique qui est strictement contrôlée lors de la spermatogénèse et est catalysée par l'enzyme ADN-methyltransférase (DNMT). Dans cette thèse j'ai employé des moyens pharmacologiques et génétiques pour parvenir à modifier la méthylation dans les céllules souches mâles afin de déterminer si ceci aurait un effet dans la fécondation et la fertilité. Pour réduire le niveau d'activité de l'enzyme DNMT j'ai traité les souris wild type (*Dnmt1*$^{+/+}$) et des souris déficientes en DNMT (*Dnmt1*$^{c/+}$) avec la drogue anti-cancer 5-aza-2'-deoxycytidine (5-azaCdR), qui s'incorpore dans l'ADN et inhibe la méthylation. Les mâles, *Dnmt1*$^{+/+}$ traités avec une dose clinique pendant 7 semaines démontrent des réductions en matières de masse testiculaire, niveaux de fertilité et la motilité du spermatozoïde, ainsi qu'une histologie anormale. J'ai constaté par contre que les souris, *Dnmt1*$^{c/+}$ sont partiellement protégées des effets de cette drogue. Par contre, des niveaux élevés de perte à la pré-implantation ont étées observés à la suite de couplements avec les mâles traités dans les deux groupes de souris, qui pourrait être attribués à une réduction dans la fertilité des oocytes, à la suite de la réduction dans la motilité spermatozoïde. Des niveaux plus élevés ont été signalés au niveau de la perte des blastocytaire, chez les mâles *Dnmt1*$^{+/+}$. Je suggère que ceci est dû à une réduction de fertilité des mâles lié à la cytotoxicité et hypométhylation tandis que les changements dans les souris traitées *Dnmt1*$^{c/+}$ seraient dûs plutôt à la méthylation et ses conséquences.

Notre deuxième modèle génétique visait cibler le fournissement en groupes méthyles nécéssaires pour la methylation de l'ADN. L'enzyme, methylenetetrahydrofolate reductase (MTHFR) est intégral pour la production de acide folique, est nécessaire à la formation de la methionine et S-adenosylmethionine et c'est au testicules que s'exprime le plus haut niveau de MTHFR, suggérant une fonction de celui-ci dans la spermatogénèse.

Une pénurie sévère de MTHFR (*Mthfr*$^{-/-}$) chez la souris a comme effet d'induire de sévères abnormalités au niveau de la spermatogénèse et de la fertilité. Lors de ma recherche, j'ai démontré que l'administration de la bétaine aux méres porteuses et allaitantes, réduisait l'apoptose des cellules germinales chez les mâles peu après leur naissance. Si la diète de bétaine est maintenue pendant la période post-allaitement, la spermatogénése est partiellement restaurée et la fertilité augmente de façon importante. Alors, ma thèse met en évidence que des pertubations dans la méthylation de l'ADN induisent des effets néfastes sur la fertilité et le développement des céllules germinales mâles.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

For the purposes of clarity, treatment lengths are referred to as numerals rather than words throughout the text (e.g., 7 weeks as opposed to seven weeks).

5-aza	5-azacytidine
5-azaCdR	5-aza-2'-deoxycytidine
ALH	amplitude of lateral head displacement
BCF	beat cross frequency
BHMT	betaine homocysteine methyltransferase
C57BL/6	my mice!
CASA	computer assisted sperm analysis
CD1	my girl mice!
DNA	deoxyribonucleic acid
DMR	differentially methylated region
DNMT	DNA methyltransferase
dpc	days post-coitum
dpp	days post-partum
ES cells	embryonic stem cells
FSH	follicle stimulating hormone
GCNA	germ-cell nuclear antigen
GD	gestational day
H3K9	histone H3, lysine 9
hCG	human chorionic gonadotropin
HbF	fetal hemoglobin
HDAC	histone deacetylase
IAP	intracisternal A particle
ICF	immunodeficiency, centromeric region instability and facial anomalies syndrome
ICM	inner cell mass
ICSI	intracytoplasmic sperm injection
i.p.	intra-peritoneal injection
i.v.	intravenous injection
IVF	in vitro fertilisation
LH	luteinising hormone
LIN	linearity
LINE	long interspersed nuclear elements
m5C	(5-)methylcytosine
MeCP	methyl CpG binding protein
MTHFR	methylenetetrahydrofolate reductase
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PGC	primordial germ cell
Pgk-2	phosphoglycerate kinase 2
PMSG	pregnant mare's serum gonadotropin
RLGS	restriction landmark genome scanning
RNA	ribonucleic acid

RT-PCR	reverse transcriptase polymerase chain reaction
SAM	S-adenosylmethionine
SAH	S-adenosylhomocysteine
SCE	sister chromatid exchange
SEM	standard error of the mean
siRNA	short/small interfering RNA
STR	straightness
TLC	thin layer chromatography
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling
VAP	average path velocity
VCL	curvilinear velocity
VSL	progressive velocity

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As I unwrapped my last syringe, labelled it with "Magic" tape, and calculated my last dose, I thought back on my Ph.D., and realised so many people helped me, often in countless little ways, over the course of these six years. These words, in no way, do them justice.

Firstly, I must thank my supervisor, **Dr. Jacquetta Trasler**, for taking me on as a student despite my lack of "small" lab skills. I am eternally grateful for your encouragement, your faith in my abilities, and for your understanding. Your enthusiasm and balanced approach to life continues to inspire your students. P.S. I'm sorry for torturing you with pink, orange and small fonts....

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To my **Mom** and **Dad**, who watched me enter kindergarten and wondered so many years later just when exactly I would finish school....the perpetual student is finished! It is because of your encouragement and unflagging support that I have completed this project. Your unfailing belief in me has meant the world. And to my **Grandfather**, who always believed in me.

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To my friends: To **Suzana Anjos**, if you're reading this – we're finished!! Thanks for making me laugh since I met you in Ecotox and for sharing the joy of writing. I know there's a big bottle of port waiting for us. To my best and oldest friend **Stephanie Boyd**, who even from Peru has always encouraged me to think of the "bigger picture" and has made me see that the application of science is endless. I am indebted to **Tonia Doerksen** for her encouragement at the sidelines, even after leaving the lab and her friendship (good meals, wine and providing me with "family" time) and for her critical reading of this thesis. Even if this project failed I would have gained your friendship. I am ever thankful to **Cerrie Rogers**, for hours of laughter over misheard quotes, the drawing of the chemical structures within this thesis, endless patience and the editing of this thesis. You have shown me that a Ph.D. can lead to something fun and absolutely rewarding.

The members of my lab were invaluable to my thesis experience. They all, at some point or another, contributed great ideas. I would like to thank: **Diana Lucifero** (for our fishbowl chats about everything from boys to eggs), **Sophie La Salle** (for Grand Canyon experiences!), **Chris Oakes** (how to get great quality DNA & diamonds), **Oana Neaga** (for saving me from the mice), **Marc Toppings** (for chats on everything from the environment to politics), and **Josée Martel** (for keeping us sooo organised!).

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And again, to my **mice** – I'm sorry, but I couldn't have done it without you.

It is a mistake to think you can solve any major problems just with potatoes.

- Douglas Adams

PREFACE

Format of the Thesis

This thesis comprises five chapters and two appendices, three chapters of which are data chapters in the form in which they were submitted or will be submitted for publication. Chapter I, is an introduction that includes background material relevant to this thesis. Some of this material has been published in *Clinical Genetics* (65: 247-260, 2004). Chapter II has been published in the *Journal of Andrology* (24: 822-830, 2003). Also included are two appendices to Chapter II, detailing the preliminary work regarding choice of drug and dosage and reversibility of drug effects. Chapter III was published in *Biology of Reproduction* and was available online November 17, 2004, and in complete journal format in March, 2005 (72: 667-677, 2005). Chapter IV will be submitted to *Reproduction*. Connecting texts are provided in accordance with Section C of the Guidelines for Submitting a Doctoral or Master's Thesis as a manuscript based thesis.

CONTRIBUTIONS OF AUTHORS

The candidate performed all of the experiments described in Chapter II. Our colonies of mice deficient in DNA methyltransferase 1 (*Dnmt1*$^{c/+}$), used in the experiments of Chapter II (and Appendices I and II, Chapter IV), were derived from mice which were developed by, and a gift of, Dr. En Li; for this reason Dr. Li's name appears as a co-author for this paper. All male mice used, both *Dnmt1*$^{+/+}$ males and *Dnmt1*$^{c/+}$ males, were raised in our own facilities by the candidate. All aspects of Appendix I were performed by the candidate. Xinying He did histological sectioning of testis samples in Appendix II, in preparation for TUNEL staining conducted by the candidate; the candidate performed all other work included in Appendix II. In Chapter III, all of the experiments were carried out by the candidate with the exception of genotyping, GCNA staining, and germ cell counts, which were completed by Oana R. Neaga. The methylenetetrahydrofolate-deficient (MTHFR) mice used in Chapter III were from the lab of Dr. Rima Rozen of the Departments of Pediatrics, Biology, and Human Genetics, McGill University. From these mice, the author and Oana Neaga derived and maintained our own colony of MTHFR mice. Both Dr. Rozen and Dr. Bernd Schwahn provided supervision and discussion for these experiments; Dr. Schwahn also provided the postnatal day 6 testes. The candidate, Oana R. Neaga and Xinying He, performed sectioning of the testis samples used in Chapter III. Chapter IV is a collaborative effort between the candidate and Christopher C. Oakes. Christopher C. Oakes completed the RLGS assay and Southern blot analysis; the candidate did all other experiments, including animal breeding, treatments, matings and tissue collection. The candidate participated actively in the planning of all experiments and prepared the manuscripts, and all figures therein, included in this thesis. Included in Chapter V, is a discussion of all results presented within this thesis, as well as potential future studies and a list of Original Contributions.

CHAPTER I

INTRODUCTION

1.0 INTRODUCTION

The male germ cell, or spermatozoon, is possibly the most recognisable cell of the body. It is a highly differentiated, morphologically unique cell designed especially to convey genetic information from the father to the embryo upon fertilisation. Despite this highly differentiated nature, the sperm must be able to undergo a transformation upon fertilisation, which renders the resulting embryo totipotent, enabling it to have unlimited developmental potential. In this way, the embryo can develop the diverse cell populations required for normal development.

Normal differentiation and maturation of male germ cells relies on highly specific changes in gene expression. Regulation of such developmentally specific expression can be at the level of transcription, translation and post-translation. Transcriptional regulation can occur via cell-specific protein-DNA interactions (*e.g.*, transcription factors) and the accessibility of DNA to these factors by virtue of changes in chromatin structure. Epigenetics is one such specialised level of transcriptional gene regulation and can act to silence gene expression and also to modulate changes in chromatin structure. It is the nature of this epigenetic programming in relation to male germ cell differentiation with which this thesis is concerned.

1.1 Statement of Investigation

Epigenetics refers to covalent modifications of DNA and core histones that regulate gene activity without altering DNA sequence. Over the last five to ten years there has been an exponential increase in our knowledge of the enzymes that epigenetically modify the genome and the diseases associated with anomalies in DNA modification or chromatin modifying enzymes, such as the immunodeficiency, centromeric region instability and facial anomalies syndrome (ICF Syndrome) and Rett Syndrome. Other human disorders associated with DNA methylation abnormalities

include rare imprinting diseases, and cancer.

DNA methylation does not act alone, but rather as part of a host of other chromatin and histone modifications that work together to coordinate chromatin structure and gene expression (reviewed in Goll & Bestor, 2002; Li, 2002). For example, the binding of methyl CpG binding protein 2 (MeCP2) to methylated DNA and the subsequent recruitment of histone deacetylases aid in silencing of transcription (Jones *et al.*, 1998; Nan *et al.*, 1998; Cameron *et al.*, 1999). However, for the purposes of brevity, only the components of the DNA methylation system are discussed in this thesis.

To date, the best-characterised DNA modification associated with the modulation of gene activity is methylation of cytosine residues within CpG dinucleotides. Methylation of DNA within the mammalian genome is catalysed by a family of DNA (cytosine-5)-methyltransferase (DNMT) enzymes and occurs primarily within CpG dinucleotides, at approximately 30 million sites throughout the genome (reviewed in Bestor & Tycko, 1996). DNA methylation is involved in a number of processes, including gene regulation, cancer and chromatin stability. CpG methylation, particularly within the promoter region of genes, is associated with transcriptional repression and provides a means to control gene expression (Bird, 2002). Furthermore, DNA methylation is implicated in a number of specialised biochemical functions, including allele-specific gene expression (genomic imprinting), transcriptional silencing of parasitic sequence elements, and X-chromosome inactivation (reviewed in Bestor, 2000). The periods of germ cell and early embryo development are critical times when patterns of DNA methylation are initiated and maintained. During gametogenesis, genomic methylation patterns are laid down in a sex- and sequence-specific manner, with sex-specific differences in methylation patterns most evident at imprinted gene loci and repetitive sequences (Bestor, 2000). Early studies indicated that the DNA of mature sperm is hypermethylated relative to that of mature oocytes (Monk *et al.*, 1987; Sanford

et al., 1987) and may be linked to the extremely condensed nuclear state required of sperm.

Because of the close association between methylation and spermatogenesis, I hypothesised that perturbation of methylation within the male germ line would have detrimental effects on male germ cell development and fertility. In this thesis, I examine genetic and pharmacological methods with which to alter, but not completely abrogate, methylation of male germ cells in order to explore the effects of perturbed methylation on spermatogenesis. Here, we treated male mice with the hypomethylating agent 5-aza-2'-deoxycytidine in order to induce changes in male germ cell methylation. As well, two genetic mouse models, each with altered enzymes important to the methylation pathway, were employed to target DNA methylation.

This introduction consists of background relevant to this project, including: a brief overview of the male reproductive system; methylation dynamics within the male germ line; a summary of the enzymes responsible for the methylation of DNA; and several approaches to altering DNA methylation within the male germ line, outlining the rationale for our methodological choices. As the focus of this thesis is on the male germ line, I will begin by briefly describing the mechanics of spermatogenesis.

1.2 The Male Reproductive System

The processes of the male reproductive system are intricate and there are a number of reviews that describe the regulation, kinetics and dynamics of spermatogenesis (Clermont, 1972; Ewing *et al.*, 1980; Hecht, 1998; Hess, 1998; de Rooij & Russell, 2000; Grootegoed *et al.*, 2000; Cooke & Saunders, 2002; Dadoune *et al.*, 2004). For the purposes of this thesis I will present only a brief review of the male reproductive system necessary to understand the rationale and design of the studies presented here.

The male reproductive system comprises the testes, epididymides, the excurrent

ducts and the seminal vesicles. Two gonadotropins, both produced by the anterior pituitary, luteinising hormone (LH) and follicle-stimulating hormone (FSH), help to control the functions of the testes. In turn, the testes function as endocrine glands, producing several steroids. In response to these hormonal signals the testes yield male germ cells; this tissue is extremely proliferative and in the human it is estimated that 100 million cells are produced daily (Amann & Howards, 1980).

1.2.1 Testis Structure

The testis is enclosed by the tunica, a trilayered capsule; within the tunica, the testicular parenchyma is comprised of the seminiferous tubules and the interstitial tissue (Fig. 1.1). The seminiferous tubules are a series of convoluted tubes in which spermatogenesis takes place. The seminiferous tubule is encircled by the basal lamina, which leads into the seminiferous epithelium. The epithelium is a stratified structure and in the adult is made up of the non-proliferating Sertoli cells and the proliferating germinal cells (Fig. 1.1). When viewing a cross-section of a tubule, concentric circles of successive germ cell generations are visible. Closest to the basal lamina are the most immature spermatogonia; the more mature germ cells are found as one moves from the periphery towards the lumen, encountering successively the spermatogonia, spermatocytes, elongating spermatids and immature spermatozoa.

The Sertoli cell is the only somatic cell within the seminiferous tubule and these cells are distributed randomly along the basal epithelium, forming a non-dividing stable population of cells in the mature testis. In addition to nourishing the surrounding germ cells, Sertoli cells are responsible for the compartmentalisation within the seminiferous epithelium, dividing the tubule into the basal and adluminal regions (Fig. 1.1), thus forming the "blood-testis" barrier (reviewed in Hutson, 1997). Each Sertoli cell is in contact with 30 to 50 germ cells, all at various stages of development; this contact is essential for germ cell maturation (reviewed in Mruk & Yan Cheng, 2000; Siu & Yan

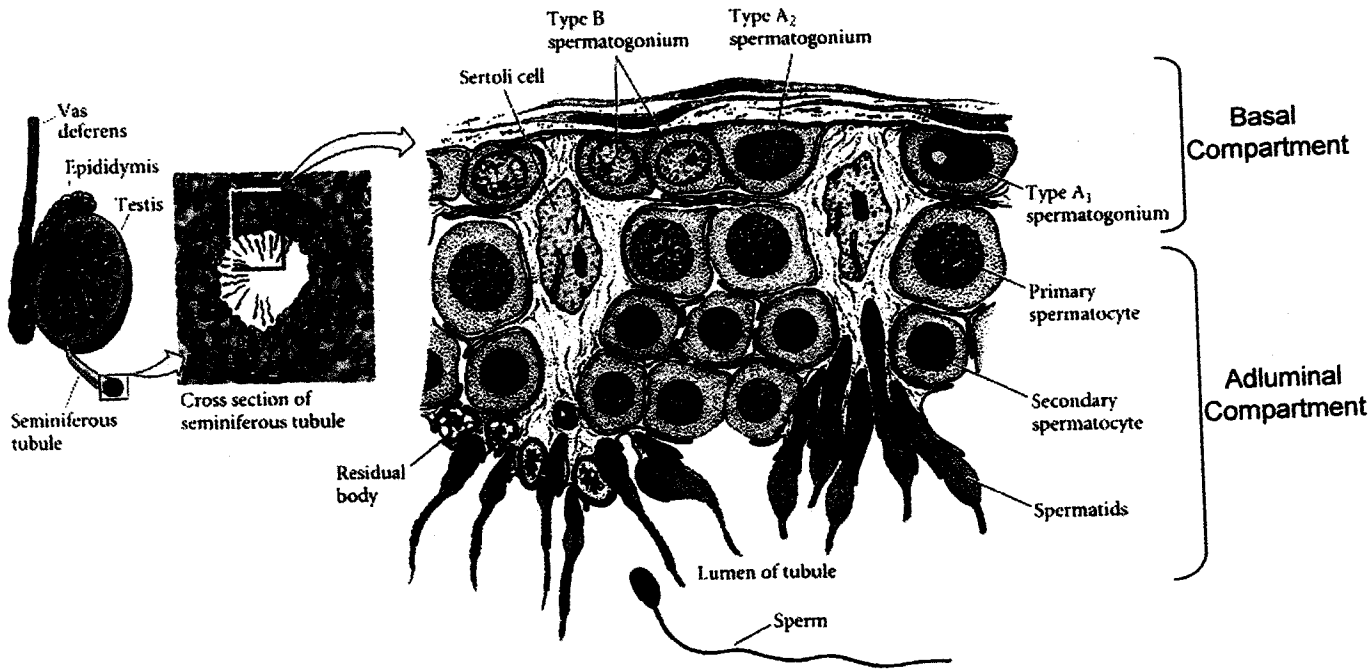
Cheng, 2004). Other functions of Sertoli cells include: secretion of fluid to form the tubular lumen, phagocytosis of residual bodies and damaged germ cells, secretion of proteins, regulation of the spermatogenic cycle and mediation of hormone effects (reviewed in Sharpe, 1994; Griswold, 1998; Mruk & Yan Cheng, 2000).

The testosterone-producing Leydig cells, nerves, blood vessels, lymphatic channels and macrophages are all found within the connective interstitial tissue. Leydig cells are the most frequent cell type (~12%) within this interstitial environment and contribute to the high levels of testosterone found within the testis (reviewed in Hutson, 1997). In addition to testosterone production, Leydig cells are believed to secrete oxytocin, which is involved in the peristaltic movement of the seminiferous tubules (reviewed in Hutson, 1997). Without proper interaction between the germ cells and somatic cells of the testis, spermatogenesis cannot function normally (reviewed in Syed & Hecht, 2002; Saunders, 2003).

1.2.2 Testicular Development

In the developing embryo, the primordial germ cells (PGCs) migrate into the genital ridge from the endodermal yolk sac epithelium, localising within the embryonic testicular cords (precursors to the seminiferous tubules). During this period of prespermatogenesis, the germ cells – referred to as gonocytes at this stage – are situated centrally within the seminiferous cords, in the space where the lumen later develops. In the mouse the gonocytes actively divide until 14.5 to 16.5 days post-coitum (dpc) at which point they arrest in the G_1 phase; the timing of mitotic cessation is strain-specific (Vergouwen *et al.*, 1991; Nagano *et al.*, 2000). Throughout this time, Sertoli cells continue to divide, but stop dividing within the week after birth (Vergouwen *et al.*, 1991; Nagano *et al.*, 2000). About 1.5 to 2 days after birth gonocytes begin to divide again and start to relocate to the basement membrane. It is unclear whether reproliferation after birth is related to gonocyte migration from the lumen to the periphery (McGuiness &

Figure 1.1. Schematic of the seminiferous tubules within the testis. All populations of germ cells are shown, with immature spermatogonia lying along the basal lamina and spermatids near the lumen. The compartmentalisation of the seminiferous tubule is also shown (basal and adluminal compartments). Adapted from Gilbert, 2000 and reproduced with permission of Sinauer Associates, Inc.


Vas deferens
Epididymis
Testis
Seminiferous tubule
Cross section of seminiferous tubule
Type B spermatogonium
Type A2 spermatogonium
Sertoli cell
Type A1 spermatogonium
Primary spermatocyte
Secondary spermatocyte
Spermatids
Residual body
Lumen of tubule
Sperm
Basal Compartment
Adluminal Compartment

Orth, 1992a, 1992b; Nagano *et al.*, 2000). Increases in testicular weight after birth are coincident with the onset of spermatogenesis and hence increased germ cell number and tubular length and diameter. Interestingly, this rapid period of germ cell proliferation and testicular growth all occurs before the appearance of fully functional adult Leydig cells (reviewed in Mendis-Handagama & Ariyaratne, 2001).

1.2.3 Spermatogenesis

The seminiferous epithelium is a complex yet highly regulated amalgam of germ cells at different stages of development. However, spermatogenesis can be broken down into three distinct phases: proliferation, meiosis and spermiogenesis (Fig. 1.2A) (reviewed in Hess, 1998). All germ cells pass through these phases as they mature and move inward from the basal lamina to the lumen of the tubule. Because of incomplete cytokinesis following mitotic and meiotic divisions, all spermatids derived from the same spermatogonium are linked via intercellular bridges and remain so until the end of differentiation. At the base of the epithelium sit the rapidly mitotically dividing spermatogonia (Fig. 1.1), which are categorised mainly according to the amount of chromatin lying along the nuclear envelope. Aside from Sertoli cells, spermatogonia are the only cells within the tubules in contact with the basal lamina.

The exact nature of spermatogonial renewal and differentiation is under debate and currently two hypotheses, one (the reserve stem cell theory) by Clermont and Bustos-Obregon (1968) and the other (the single stem cell theory) proposed by Huckins (1971) and Oakberg (1971) predominate and are reviewed in de Rooij and Russell (2000). In both schemes, a subset of stem cells exists which is less vulnerable to insult due to its ability to remain dormant for extended periods. In this way, the testis can possibly evade acute periods of injury, and sustain a viable population of spermatogonia (reviewed in Clermont, 1972; de Rooij & Russell, 2000; de Rooij, 2001).

The mature spermatogonia differentiate and divide to form the primary

spermatocytes, which then undergo the two reductive divisions of meiosis, from which the haploid spermatids arise (Fig. 1.2A). In more detail, type B spermatogonia divide to form primary spermatocytes, which then undergo the lengthy process of meiosis I. DNA of leptotene spermatocytes is present as condensed and unpaired chromatin. During zygotene, chromosomal pairing occurs and the synaptonemal complex begins to form and is complete by pachytene. Crossing over occurs during pachytene, the longest stage of meiosis I, and in the brief diplotene phase, the nucleus achieves its maximum size and the chromosomes remain connected only by chiasmata. The secondary spermatocytes are very short-lived and divide rapidly to form the haploid round spermatids.

The round spermatids complete spermiogenesis, a series of cytological changes during which they differentiate into the characteristic morphologically unique spermatozoa (Figs. 1.2A-B). The number of steps in spermiogenesis is species-specific; in the mouse, there are 16 steps in the spermiogenic process and the timing of these steps is tightly regulated. It is during this postmeiotic maturation that the nucleus elongates and compacts and both the flagellum and the acrosome form (reviewed in Oko & Clermont, 1998). In the early steps of spermiogenesis an increase in transcription occurs that halts once nuclear elongation begins; translation of many of these sperm-specific proteins occurs later in spermiogenesis (reviewed in Hecht, 1998; Oko & Clermont, 1998). Extensive chromatin remodelling occurs during spermiogenesis in order to achieve the tightly packed inert chromatin configuration characteristic of spermatozoa (reviewed in Dadoune *et al.*, 2004). DNA packaging occurs via replacement of histones by transition proteins and protamines (reviewed in Sassone-Corsi, 2002; Meistrich *et al.*, 2003). In the final steps of spermiogenesis, excess cytoplasm is "pinched off" in the form of the residual body which is then phagocytosed by the Sertoli cells (reviewed in Clermont, 1972; Russell *et al.*, 1990), effectively breaking the intercellular bridges that have maintained communication between all the cells of one germ cell population, within

a stage, since they were spermatogonia. Sperm are then released into the fluid-filled lumen, yet it is not until the spermatozoa pass through the epididymis that they reach final maturation and are able to fertilise ova. Sperm pass from the epididymis into the vas deferens where they are combined with the secretions of accessory sex tissues to form the semen.

1.2.4 The Stages of Spermatogenesis

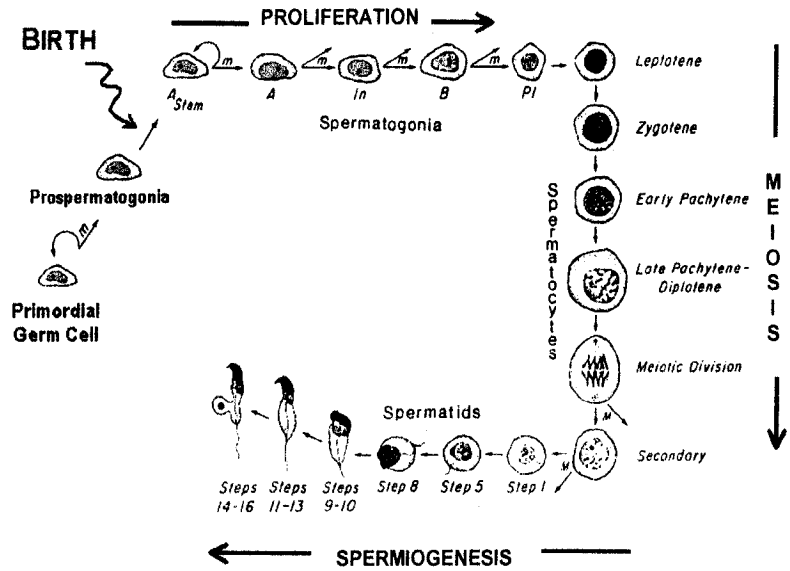
Spermatogenesis is a highly regulated process, whereby different generations of germ cells are found together, in specific cellular associations, within the seminiferous epithelium. There are twelve cellular associations in the seminiferous epithelium of the mouse, each one representing a specific stage (Fig. 1.2B) (reviewed in Russell *et al.*, 1990; Hess, 1998). Stages, by definition, remain in one part of the tubule and have relatively synchronous development because of the intracellular bridges that link the germ cells. Each germ cell thus passes through all 12 stages approximately 4.5 times (Fig. 1.2B). The duration of spermatogenesis is species-specific and in the mouse is approximately 35 days (Oakberg, 1956). Thus, the spermatogenic process is very precise and requires the time-appropriate expression of the many genes associated with sperm development. DNA methylation is one such way to control gene expression and its role in spermatogenesis is discussed in Section 1.3.

1.2.5 Hormones and Spermatogenesis

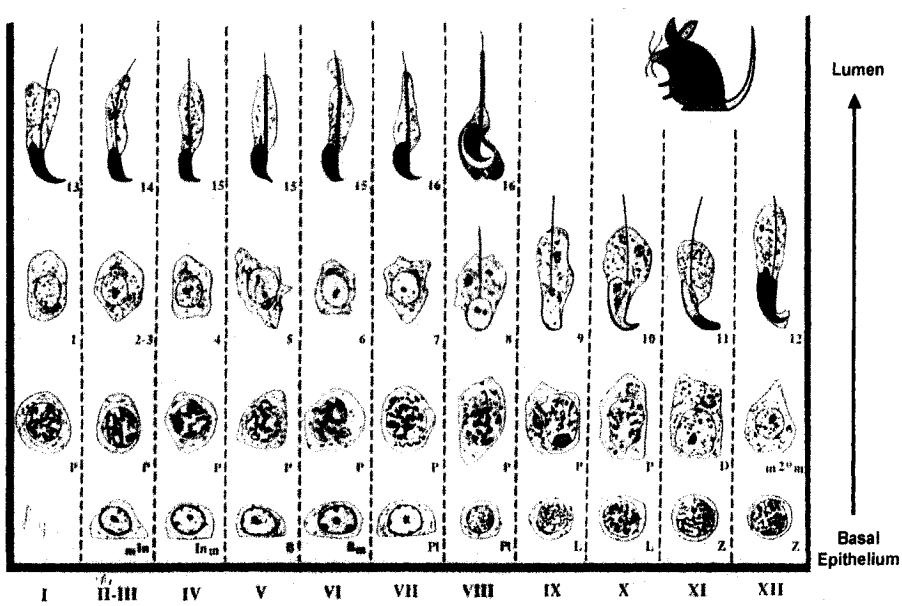
Normal testicular development and spermatogenesis are highly dependent on hormones such as FSH and testosterone (reviewed in Cooke & Saunders, 2002). Interspecific differences in the spermatogenic effects of FSH make it difficult to draw general conclusions for the role of this hormone in spermatogenesis (reviewed in Zirkin, 1998; de Kretser *et al.*, 2000; McLachlan, 2000; Heckert & Griswold, 2002; Dohle *et al.*, 2003). Whereas FSH is necessary for normal sperm production and quality in man and primates (reviewed in McLachlan, 2000), FSH-deficient male mice are fertile despite

Figure 1.2. **(A)** The three phases of spermatogenesis in the mouse: proliferation, meiosis and spermiogenesis. *M*, meiotic division; *m*, mitotic division; *A*, type A spermatogonia; *In*, intermediate spermatogonia; *B*, type B spermatogonia; *Pl*, preleptotene. Adapted from Meistrich, 1977 and reproduced with permission of Elsevier. **(B)** The distinct cellular associations found in each of the 12 stages of the cycle of the mouse seminiferous epithelium. Each stage is determined by the step(s) of the spermatids present. Roman numerals indicate stage of spermatogenesis; numbers indicate steps of spermiogenesis. m, mitotic division; In, intermediate spermatogonia; B, type-B spermatogonia, Pl, pre-leptotene; L, leptotene; Z, zygotene; P, pachytene; D, diplotene. From Russell *et al.*, 1990 Reprinted with permission of Cache River Science, an imprint of Quick Publishing, LC, 888-PUBLISH, fax 314-993-4485, email:cacheriverpress@sbcglobal.net.

A



B



STAGES OF THE CYCLE OF THE SEMINIFEROUS EPITHELIUM

reduced testis size (Kumar *et al.*, 1997). In neonates FSH administration stimulates Sertoli cell proliferation, resulting in increased spermatogenic production (Singh & Handelsman, 1996) but as rodents age, spermatogenesis becomes increasingly reliant on testosterone (reviewed in Zirkin, 1998; McLachlan, 2000).

The role of testosterone in male germ cell development is more clearly defined than that of FSH. Testosterone is produced by the interstitial Leydig cells in response to LH, and within the testis is found in high local concentrations. This androgen is necessary for spermatogenesis and consequently fertility; testosterone inhibition alters spermatid adhesion to Sertoli cells and thus prohibits further maturation of these germ cells (Cameron & Muffly, 1991; Sharpe, 1994; Perryman *et al.*, 1996). Were a drug to affect levels of testosterone, particularly in the adult, spermatogenesis could thusly be compromised. To estimate whether or not a drug has affected testosterone levels, male toxicology studies routinely measure seminal vesicle weights, as these accessory sex organs are vulnerable to even small changes in androgen status.

1.3 DNA Methylation in the Male Germ Line

Around the time PGCs enter the gonad during embryonic development, DNA methylation patterns begin to be erased; following erasure, sex-specific epigenetic patterns are set down in the male and female germ cells, with further modifications occurring in the early embryo (Fig. 1.3) (reviewed in Reik *et al.*, 2001; Surani, 2001). Recently, new techniques, including germ cell-specific fluorescent markers and bisulphite sequencing, have permitted a more precise assessment of DNA methylation patterns and their developmental timing in the germ line and embryo. Because this thesis is primarily concerned with methylation in the male germ line, methylation of oocyte DNA will be touched on only where necessary.

The most striking modulations in methylation during gametogenesis and

embryogenesis occur for imprinted genes and repetitive sequence elements. To date there are over 70 known imprinted genes (Beechey *et al.*, 2005), those genes that are expressed exclusively from the maternal or the paternal genome. DNA methylation is the most well studied epigenetic mark known to distinguish the maternal and paternal alleles of imprinted genes, and most imprinted genes have been shown to contain sequences that are differentially methylated in the gametes (Tycko & Morison, 2002; Spahn & Barlow, 2003). Typically, the two parental alleles have different levels of DNA methylation, and that methylation is often located in one area, called a differentially methylated region (DMR), within or close to the imprinted gene. As a heritable, reversible, epigenetic mark, DNA methylation of imprinted genes can be stably propagated after DNA replication and can maintain monoallelic gene expression throughout life.

The majority of CpGs in the mammalian genome are contained within repetitive DNA sequence elements (Yoder *et al.*, 1997), such as endogenous retroviruses (*e.g.* intracisternal A particle, *IAP*), long interspersed nuclear elements (*LINE*s) and satellite sequences. It has been postulated that DNA methylation of retrotransposons may be necessary to prevent their reverse transcription and transposition (Smit, 1996; Yoder *et al.*, 1997) events that could lead to deleterious mutations and cancer. Satellite sequences are localised to the centromeres and are thought to play a role in centromere function and kinetochore assembly; the methylation of satellite sequences has been implicated in chromosomal stability (Viegas-Pequignot & Dutrillaux, 1976; O'Neill *et al.*, 1998; Xu *et al.*, 1999).

1.3.1 Methylation of Testis-Specific Genes

DNA methylation within the 5' promoter region of tissue-specific genes is often associated with transcriptional repression and provides an epigenetic means by which to control gene expression (reviewed in Bird, 2002; *e.g.*, Bruniquel & Schwartz, 2003). During the course of spermatogenesis, some testis-specific genes exhibit changes in

methylation, including transition protein 1, protamines 1 and 2, and phosphoglycerate kinase 2 (*Pgk2*) (Trasler *et al.*, 1990; Ariel *et al.*, 1991, 1994). For example, Ariel and colleagues (1991, 1994) demonstrated that the 5' end of *Pgk2* is hypomethylated in germ cell types where it is actively transcribed (pachytene spermatocytes and round spermatids), but is hypermethylated in non-expressing germ cells. Hypomethylation preceded formation of a decondensed chromatin structure (Kramer *et al.*, 1998) and increased DNase I hypersensitivity (Kumari *et al.*, 1996), and may facilitate access to the DNA by transcription factors (McCarrey, 1998). Recently, more detailed studies of *Pgk2* methylation using bisulphite sequencing revealed the gradual formation of a demethylated region that occurs in the absence of DNA methylation; methylation is regained upon transit through the epididymis (Geyer *et al.*, 2004).

1.3.2 Erasure of Methylation Patterns in the Male Mouse Germ Line

Experimental evidence suggests that DNA methylation is largely erased in PGCs with each reproductive cycle. Methylation is then re-established at distinctly different developmental times, according to the sex of the organism, in the immature male and female gametes of the embryonic and postnatal gonads (Fig. 1.3). More recently, studies of imprinted gene and repetitive sequence methylation, via bisulphite sequencing, have begun to better define the timing of gene-specific early epigenetic events in PGCs. In the mouse, erasure of DNA methylation within PGCs takes place around the time these cells enter the gonad, at 10.5 to 12.5 dpc (Figs. 1.3 & 1.4) (Monk *et al.*, 1987, Chaillet *et al.*, 1991; Kafri *et al.*, 1992; Brandeis *et al.*, 1993; Szabo & Mann, 1995; Kato *et al.*, 1999; Hajkova *et al.*, 2002; Lee *et al.*, 2002; Szabo *et al.*, 2002).

Hajkova *et al.* (2002) demonstrated that a number of imprinted genes, including *Peg3*, *Lit1*, *Snrpn* and *H19*, as well as non-imprinted genes such as *α-actin*, become demethylated between 10.5 and 13.5 dpc. In fact, demethylation may occur even earlier than 10.5 dpc, as more recent studies suggest (Sato *et al.*, 2003; Yamazaki *et al.*, 2003).

Studies of imprinted gene expression in PGCs are consistent with the DNA methylation data (Szabo *et al.*, 2002), suggesting that erasure of epigenetic patterns begins before PGCs reach the genital ridge.

Whereas most imprinted and single copy sequences examined to date become demethylated in PGCs, at least some repetitive sequences do not follow this pattern. For example, while *LINE-1* sequences lose much of their methylation between 11.5 to 13.5 dpc, demethylation is less extensive for *IAP* sequences, in which substantial CpG methylation is maintained (Hajkova *et al.*, 2002; Lane *et al.*, 2003). The authors postulate this resistance of *IAP* to demethylation may prevent retrotransposition events of these elements that could lead to deleterious mutations.

The phenomenon of PGC demethylation is cell-intrinsic and is independent of gonadal environment (Sato *et al.*, 2003). Similarly, differentiation of PGCs is also a cell-intrinsic programme, but appears to be reliant on the extent of demethylation (Maatouk & Resnick, 2003). As of yet, it is unknown whether differentiation is induced because of the demethylation of genes involved in germ cell differentiation or because a threshold level of global demethylation has been reached. Regardless, improper erasure of methylation marks has the potential to interfere with the cellular differentiation programme of PGCs and/or could interfere with the establishment of sex-specific methylation imprints later in germ cell development (Davis *et al.*, 1999, 2000); it is only during germ line passage that methylation of imprinted genes may be acquired (Tucker *et al.*, 1996).

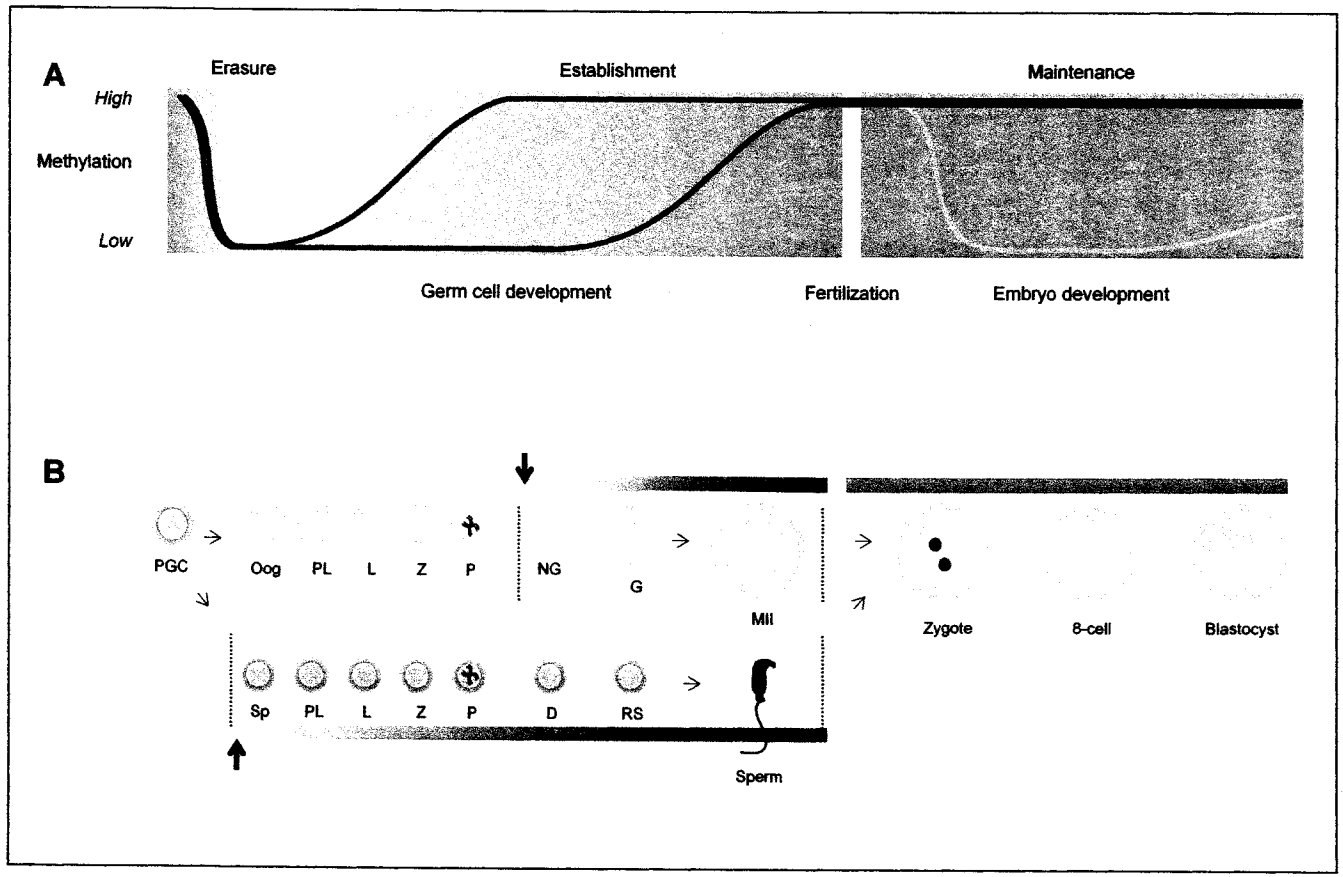
1.3.3 Methylation Pattern Acquisition in Male Mouse Germ Cells

Following the nearly genome-wide demethylation in PGCs, sex- and sequence-specific genomic methylation patterns are re-established in the male and female gametes (Fig. 1.3) (reviewed in Reik *et al.*, 2001). Although there is a second phase of genomic demethylation that occurs in preimplantation embryos, some sequences, notably

imprinted genes and some repeat sequences, retain their gamete-acquired methylation marking while the rest of the genome is demethylated. The gamete methylation that is retained during the wave of preimplantation demethylation is likely important for subsequent postimplantation embryo development.

In the male, a number of techniques have shown that the acquisition of DNA methylation patterns begins before birth in prospermatogonia and is completed, for many sequences, after birth, prior to the end of the pachytene phase of meiosis (Figs. 1.3 & 1.4) (Kafri *et al.*, 1992; Walsh *et al.*, 1998; Davis *et al.*, 1999; Davis *et al.*, 2000; Ueda *et al.*, 2000; Lees-Murdock *et al.*, 2003). In the male mouse this initial acquisition of methylation occurs between 15.5 and 18.5 dpc; corroborating these results, during this same time window, germs cells begin to stain positively with an antibody directed to methylated cytosine (m^5C), indicating an increase in genomic methylation (Coffigny *et al.*, 1999). Some of the most detailed male germ cell methylation studies have been done on the maternally expressed *H19*, a region of which becomes methylated in the male germ line but remains unmethylated in oocytes. A 2 kilobase (kb) region approximately 2 to 4 kb from the transcription start site of *H19* begins to acquire its methylation in day 15.5 to 18.5 prenatal male germ cells. Methylation is completed postnatally, by the pachytene stage of meiosis, and persists in sperm (Davis *et al.*, 1999, Davis *et al.*, 2000; Ueda *et al.*, 2000). The paternal alleles of *H19* became methylated prior to (*i.e.*, in earlier germ cell types) the maternal alleles, suggesting that paternal alleles may retain some 'memory', whether it be via chromatin structure or residual methylation, of their origin as methylated alleles (Davis *et al.*, 1999; Davis *et al.*, 2000). The paternally methylated imprinted genes, *Gtl2* (Takada *et al.*, 2000, 2002) and *Rasgrf1* (Yoon *et al.*, 2002) acquire their methylation at the same time as *H19*, with substantial, but not complete, methylation by 17.5 dpc (Li *et al.*, 2004). Repetitive sequences, including *LINE-1*, *IAP*, and centromeric minor satellite sequences, undergo a more rapid

Figure 1.3. Changes in imprinted gene methylation during male and female germ cell development, and in the preimplantation embryo. **(A)** Methylation patterns of both maternally (red line) and paternally (blue line) imprinted genes. During gametogenesis, the methylation of non-imprinted genes is similar to that of imprinted genes. Post-fertilisation, the methylation dynamics of non-imprinted genes are different than those of imprinted genes. The paler red and blue lines represent non-imprinted gene methylation. **(B)** Methylation imprint acquisition during gametogenesis in the female and male (degree of methylation is indicated by the intensity of red and blue shading above and below the female and male germ cells respectively). Purple shading above the preimplantation embryos illustrates the maintenance of genomic imprints. PGC, primordial germ cells; Oog, oogonia; Sp, spermatogonia; PL, preleptotene; L, leptotene; Z, zygotene; P, pachytene; D, diplotene; NG, non-growing oocytes; G, growing oocytes; RS, round spermatids; MII, metaphase II oocytes. From Lucifero *et al.* (2004) and reproduced with permission of Oxford University Press/Human Reproduction.


A
Erasure
Establishment
Maintenance
High
Methylation
Low
Germ cell development
Fertilization
Embryo development
B
PGC
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Z
P
NG
G
MII
Zygote
8-cell
Blastocyst
Sp
PL
L
Z
P
D
RS
Sperm

remethylation than imprinted genes with complete methylation established by 17.5 dpc (Walsh *et al.*, 1998; Lees-Murdock *et al.*, 2003). Studies using intracytoplasmic round spermatid and sperm injections support the observation that paternal imprints are complete prior to the haploid phase of spermatogenesis (Shamanski *et al.*, 1999).

In rodent germ cells, the level of methylation is associated with the degree of chromosome compaction. Methylation of heterochromatic pericentromeric sequences in male germ cells increases progressively after birth, while euchromatic regions become successively demethylated with each cell division (Coffigny *et al.*, 1999; Bernardino *et al.*, 2000; Marchal *et al.*, 2004). Regardless of the germ cell-type, strong chromatin compaction is associated with high levels of DNA methylation and weak compaction with lower DNA methylation levels (Marchal *et al.*, 2004). Methylation and compaction may also be related to chromosome cohesion, providing a structural role for DNA methylation in gametogenesis; progressive methylation of centromeric regions of germ cells is correlated with increased chromosome cohesion (Coffigny *et al.*, 1999; Bernardino *et al.*, 2000; Bernardino-Sgherri *et al.*, 2002; Marchal *et al.*, 2004), suggesting that DNA methylation may play a role in chromosome pairing in the male germ line.

1.3.4 DNA Methylation Patterns in Early Mouse Embryos

Dynamic alterations in DNA methylation take place during preimplantation development in the mouse. Within approximately four hours of fertilisation, shortly after protamine removal, the male pronucleus undergoes a rapid, presumably active, process of demethylation (Mayer *et al.*, 2000; Oswald *et al.*, 2000; Santos *et al.*, 2002). In contrast, the maternal genome becomes demethylated more slowly, consistent with a passive process whereby maintenance methylation does not occur following cell division (Howlett & Reik, 1991; Rougier *et al.*, 1998; Mayer *et al.*, 2000). Demethylation of the paternal genome is coincident with pronuclear formation (Santos *et al.*, 2002) and is regulated by both sperm-specific and oocyte-specific factors (Beaujean *et al.*, 2004). If

expression of these sperm-specific factors is perturbed, paternal demethylation may not occur to the extent necessary for early embryo development to proceed normally.

Genomic methylation is lowest at the morula stage of preimplantation development, at which time little methylation is detected using an antibody against m^5C. This is closely followed by genome-wide *de novo* methylation of the inner cell mass (ICM), but not the trophectoderm, of the blastocyst (Santos *et al.*, 2002). However, imprinted genes maintain their gamete-derived methylation during the preimplantation wave of demethylation, thus allowing for appropriate allele-specific, monoallelelic expression at later in embryo development (Olek & Walter, 1997; Tremblay *et al.*, 1997; Hanel & Wevrick, 2001; Reik & Walter, 2001). Interestingly, some repetitive sequence elements, such as *LINE* sequences, become demethylated during preimplantation development while others, in particular *IAP* elements, are treated like imprinted genes and retain their methylation (Lane *et al.*, 2003) perhaps to prevent deleterious retrotransposition events. In support of this suggestion, transcriptional activation of *IAP*s is seen in *Dnmt1* homozygous mutant mice that have only 5% of the normal level of genomic methylation (Walsh *et al.*, 1998) and in the germ cells of male mice deficient in DNMT3l (Bourc'his & Bestor, 2004).

1.4 DNA Methylating Enzymes - DNA (Cytosine-5)-Methyltransferases

In mammals, the DNA (cytosine-5)-methyltransferases catalyse methylation of cytosine residues, whereby a methyl group donated by the cofactor, S-adenosylmethionine (SAM) is transferred to the 5' carbon of cytosine's pyrimidine ring (Fig. 1.5A-B). Currently, three families of DNMT enzymes have been described and are classified according to similarities in their C-terminal catalytic domains (reviewed in Bestor, 2000). DNMT1 is the predominant mammalian DNMT, although several other enzymes including DNMT2 (Yoder & Bestor, 1998), DNMT3a, DNMT3b (Okano *et al.*,

1998) and more recently, DNMT3l have been characterised (Aapola *et al.*, 2000; Bourc'his *et al.*, 2001). To date, DNMT1, DNMT3a and DNMT3b are the only known catalytically active DNMTs.

Three types of enzyme activity are required to erase, establish and perpetuate methylation patterns in the genome: demethylation, *de novo* methylation, and maintenance methylation. Demethylation can come about actively, although the enzymes involved *in vivo* have not been identified, or passively, if methylation is not maintained at the time of replication. In early germ cells and peri-implantation stage embryos, DNMTs with specific *de novo* methylation activity are necessary to establish new patterns of DNA methylation; *de novo* DNMT activity is reduced in differentiated cells (Lei *et al.*, 1996). Because the methylation of cytosine residues occurs post-replicatively, maintenance DNMTs, such as DNMT1, ensure accurate propagation of DNA methylation at the time of cell division. Recent studies indicate that the various DNMTs may interact; each enzyme may be able to methylate specific sequences individually, but methylation of some elements may require cooperation between DNMT1, DNMT3a, DNMT3b and DNMT3l (Hata *et al.*, 2002; Liang *et al.*, 2002; Margot *et al.*, 2003; La Salle *et al.*, 2004; Suetake *et al.*, 2004). In the next section, I will review what is known about the DNMTs from mouse studies, including their expression in germ cells and early embryos and the reproductive consequences of their disruption using gene-targetting approaches (Table 1.1).

1.4.1 DNMT1

The first of the mammalian DNMT enzymes to be characterised, DNMT1 (Bestor *et al.*, 1988), is perhaps the best described. The mouse *Dnmt1* gene is regulated through alternative splicing and produces two major protein products, a somatic isoform and an oocyte-specific isoform; a pachytene spermatocyte-specific transcript is expressed at high levels in the testis but is not translated (Mertineit *et al.*, 1998). The oocyte-specific

isoform, DNMT1o, lacks 118 amino acids of the N-terminus of the somatic form of DNMT1; this is the only form of DNMT1 present in the early embryo prior to zygotic gene activation (Carlson *et al.*, 1992; Mertineit *et al.*, 1998). DNMT1 shows a preference for hemimethylated DNA and is essential for the maintenance of methylation patterns during DNA replication (Bestor, 2000). Levels of methylation are severely lowered (to ~5% of normal levels) in embryonic stem (ES) cells and embryos deficient in this enzyme (Li *et al.*, 1992; Lei *et al.*, 1996). However, recent data suggests that an unknown factor assists DNMT1 in maintaining methylation patterns post-replicatively. Human colon cancer cells deficient in DNMT1 experience only a 20% decrease in genomic methylation, primarily at juxtacentromeric satellite sequences (Rhee *et al.*, 2000; Bird, 2002), but this result may reflect cell-specific or species-specific differences. Recent studies with DNMT1-deficient ES cells indicate that DNMT1 may be primarily responsible for methylation of CpG-poor sequences (Liang *et al.*, 2002); it is within these regions that the majority of m^5C is found (Bird *et al.*, 1985).

Levels of *Dnmt1* mRNA are higher in the testis and ovary than in other adult tissues and there are germ line-specific differences in DNMT1 expression pre- and peri-natally (Trasler *et al.*, 1992; La Salle *et al.*, 2004). Throughout spermatogenesis in the rat and the mouse, DNMT1 expression is tightly regulated (Trasler *et al.*, 1992; Benoit & Trasler, 1994; Jue *et al.*, 1995; Mertineit *et al.*, 1998; Sakai *et al.*, 2001; La Salle *et al.*, 2004). High levels of DNMT1 exist in the nuclei of most PGCs from 10.5 to 13.5 dpc, suggesting that the DNA may be shielded from this enzyme during the wave of demethylation that takes place at this time (Hajkova *et al.*, 2002; La Salle *et al.*, 2004). DNMT1 expression is also high in mitotically dividing male germ cells at 13.5 dpc, but gradually decreases, and by 18.5 dpc it is no longer detected (Sakai *et al.*, 2001; La Salle *et al.*, 2004). During the time of methylation acquisition in prenatal male gonocytes, DNMT1 expression is conspicuously absent (Sakai *et al.*, 2001; La Salle *et al.*, 2004),

indicating that another DNMT must be responsible for *de novo* methylation at this time. DNMT1 expression peaks soon after birth, when differentiation to spermatogonia occurs, and mitotic division of spermatogonia resumes (Jue *et al.*, 1995; Sakai *et al.*, 2001; La Salle *et al.*, 2004). In early meiotic germ cells (preleptotene, leptotene and zygotene spermatocytes), significant levels of DNMT1 protein are found; protein levels are down-regulated by pachytene (Trasler *et al.*, 1992; Benoit & Trasler, 1994; Jue *et al.*, 1995), when an untranslated 6.0kb transcript is observed. This transcript is the result of an alternative first exon (exon 1p) of *Dnmt1* and appears to be unique to the testis (Trasler *et al.*, 1992; La Salle *et al.*, 2004). DNMT1 is predicted to play a role in maintenance DNA methylation in the germline; whether it plays functional roles, such as in repair mechanisms at other times such as in early meiotic cells, is currently unknown.

Gene-targetting of *Dnmt1* clearly demonstrated that DNA methylation is essential for embryonic development. Mice homozygous for targetted partial ($Dnmt1^{n/n}$ and $Dnmt1^{s/s}$) and complete ($Dnmt1^{c/c}$) loss-of-function mutations in *Dnmt1* displayed growth retardation, mid-gestational lethality, and, for the $Dnmt1^{c/c}$ mice, only 5% of wild-type methylation levels (Table 1.1) (Li *et al.*, 1992; Lei *et al.*, 1996). In addition, these embryos had biallelic expression of imprinted genes (Li *et al.*, 1993), ectopic X-chromosome inactivation (Panning & Jaenisch, 1996), hypomethylation and transcription of normally silent IAP sequences (Walsh *et al.*, 1998), and increased levels of apoptosis (Li *et al.*, 1992). Due to embryonic lethality of mice homozygous for targetted deletions in *Dnmt1*, determining a role for DNMT1 in spermatogenesis will require germ cell-specific knockout or knockdown approaches; based on the high levels of DNMT1 expression and evidence of tight regulation during male germ cell development from PGCs to mature sperm, it is predicted that such experiments would be very informative.

1.4.2 DNMT3a and DNMT3b

Members of the DNMT3 family are more closely related to multispecies DNMTs

than to DNMT1 or DNMT2 (Bestor, 2000). DNMT3a and DNMT3b are postulated to function predominantly as *de novo* methyltransferases. While different essential genes encode *Dnmt3a* and *Dnmt3b*, both are highly expressed in undifferentiated ES cells and this expression is downregulated upon differentiation (Okano *et al.*, 1998, 1999). DNMT3B is currently the only DNMT known to be mutated in a human disease. The human autosomal recessive genetic disorder ICF (immunodeficiency, centromeric region instability and facial anomalies syndrome) is caused by mutations in the *DNMT3B* gene (Xu *et al.*, 1999) and results in cytogenetic abnormalities that predominantly affect the pericentric regions of chromosomes 1, 9 and 16; these regions contain a type of satellite DNA that is methylated in normal individuals but is almost completely unmethylated in the DNA of ICF patients (Jeanpierre *et al.*, 1993). The demethylation of inactive X-chromosome loci and minor satellite sequences in ICF patients points to a role for DNMT3B in methylation of specific repeat sequences and CpG islands on the inactive X chromosome. No patients homozygous for null alleles of DNMT3B have been reported, suggesting that complete loss of DNMT3B function may be lethal, as it is in mice (Okano *et al.*, 1999).

DNMT3a and DNMT3b exhibit different developmental expression profiles, but similar to DNMT1, are uniquely regulated in both germ lines (Fig. 1.4) (La Salle *et al.*, 2004). DNMT3a expression is highest during the prenatal and early postnatal stages of testicular development, whereas DNMT3b has a pattern reciprocal to that of DNMT3a and is expressed at low levels prior to birth, with higher levels seen in the early postnatal period. La Salle *et al.* (2004) suggested these data support a role for DNMT3a in the *de novo* acquisition of DNA methylation patterns during the prenatal germ cell development, and a role for DNMT3b in maintenance and/or continued *de novo* methylation in the early mitotic germ cells. Expression pattern analyses by Sakai and colleagues (2004) support these findings. Interestingly, Watanabe *et al.* (2004) found that DNMT3b was highly

expressed in adult type A spermatogonia and was localised to both euchromatin and heterochromatin, whereas significant levels of DNMT3a were localised to the euchromatin of type-B spermatogonia and preleptotene spermatocytes.

Evidence from gene-targetting experiments suggests that DNMT3a and DNMT3b have overlapping functions, but are each so essential that one cannot compensate for the loss of the other (Table 1.1) (Okano *et al.*, 1999). Cells deficient in both DNMT3a and DNMT3b are unable to methylate newly integrated retroviral DNA, although mutants lacking one or the other enzyme retain this ability (Okano *et al.*, 1999). DNMT3a-deficient mice are underdeveloped, die 3 to 4 weeks after birth, and exhibit spermatogenic defects, while DNMT3b deficiency results in a more severe mid-gestation embryo lethal phenotype. Deficiency of both DNMT3a and DNMT3b acts synergistically; these embryos fail to develop past gastrulation and at 9.5 dpc have DNA methylation levels similar to the low levels observed during preimplantation development (Okano *et al.*, 1999), thus providing more evidence for their role in *de novo* methylation.

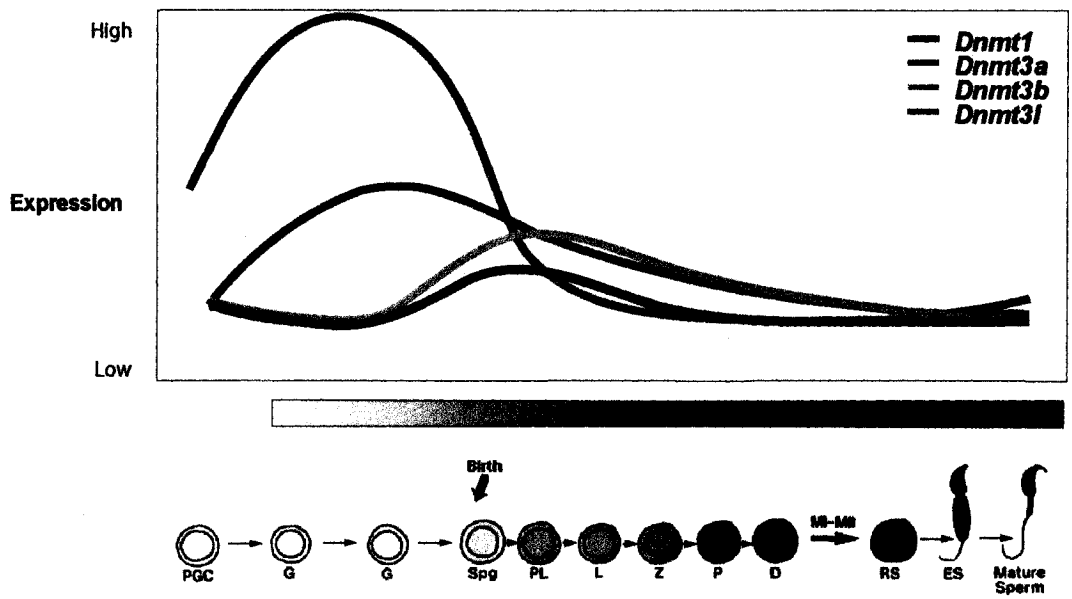
Recently, germ cell-specific conditional mutants of DNMT3a and DNMT3b were created through TNAP-Cre-mediated deletion of DNMT3a or DNMT3b in germ cells by 14.5 dpc (Table 1.1) (Kaneda *et al.*, 2004). In male germ cells, Cre-mediated deletion of *Dnmt3a*, but not *Dnmt3b*, resulted in a dramatic reduction in testis size and abnormal spermatogenesis. Unlike females, male DNMT3a mutants do not produce any mature germ cells; despite almost normal germ cell populations at 11 days post-partum (dpp), by 11 weeks of age few spermatogonia and no spermatocytes, spermatids or spermatozoa are evident. Bisulphite sequencing of early germ cells revealed hypomethylation of *H19* and *Gtl2*, but not *Rasgrf1*, suggesting that DNMT3a is indispensable for establishing paternal imprints (Kaneda *et al.*, 2004).

Table 1.1: Phenotypes associated with gene-targetting of DNA (cytosine-5) methyltransferases (DNMTs) (adapted from Kelly & Trasler, 2004 with permission of Blackwell Publishing).

Gene	Function	Mouse Mutant Phenotype	Reference
Dnmt1$^{-/-}$	Maintenance MTase	Embryo lethal at 8.5dpc, loss of imprints, ectopic X-linked gene expression; hypomethylation of various repeat sequences; increased *IAP* transcription	Li et al., 1992; Li et al., 1993 Lei et al., 1996; Panning & Jaenisch, 1996; Walsh et al., 1998
Dnmt1o$^{-/-}$	Oocyte-specific Maintains imprints at 8-cell stage	*Dnmt1o*$^{+/-}$ progeny of *Dnmt1o*$^{-/-}$ dams embryo lethal; loss of maternal imprints	Howell et al., 2001
Dnmt2$^{-/-}$	Unknown	Normal	Okano et al., 1998
Dnmt3a$^{-/-}$	*De novo* MTase	Die at ~4 weeks; spermatogenic defects	Okano et al., 1999
Germ cell -specific ***Dnmt3a***$^{-/-}$		**Females:** *Dnmt3a*$^{+/-}$ progeny of *Dnmt3a*$^{-/-}$ dams embryo lethal; loss of maternal imprints **Males:** hypogonadism; spermatogenic defects (infertility); disrupted paternal germ cell imprints	Kaneda et al., 2004
Dnmt3b$^{-/-}$	*De novo* MTase	Embryo lethal E14.5-18.5; hypomethylation of minor satellite DNA	Okano et al., 1999
Germ cell-specific ***Dnmt3b***$^{-/-}$		Normal	Kaneda et al.,2004
Dnmt3a$^{-/-}$***, 3b***$^{-/-}$	*De novo* MTase	Embryo lethal E8.5; absence of *de novo* methylation after implantation	Okano et al., 1999
Dnmt3l$^{-/-}$	No catalytic activity Regulation of methylation	**Females:** *Dnmt3l*$^{+/-}$ progeny of *Dnmt3l*$^{-/-}$ dams embryo lethal; loss of maternal imprints **Males:** hypogonadism; spermatogenic defects (infertility); disrupted paternal imprints	Bour'chis et al., 2001; Hata et al., 2002; Bourc'his & Bestor, 2004; Kaneda et al., 2004

MTase, DNA methyltransferase

Figure 1.4. Dynamics of *Dnmt1, Dnmt3a, Dnmt3b* and *Dnmt3l* expression (relative levels) during male germ cell development (*Dnmt3l* levels are not to scale). Methylation of imprinted and non-imprinted genes during male germ cell development is indicated by intensity of blue shading. Adapted from La Salle *et al.* (2004) and reproduced with permission of Elsevier.


High
Expression
Low
Dnmt1
Dnmt3a
Dnmt3b
Dnmt3l
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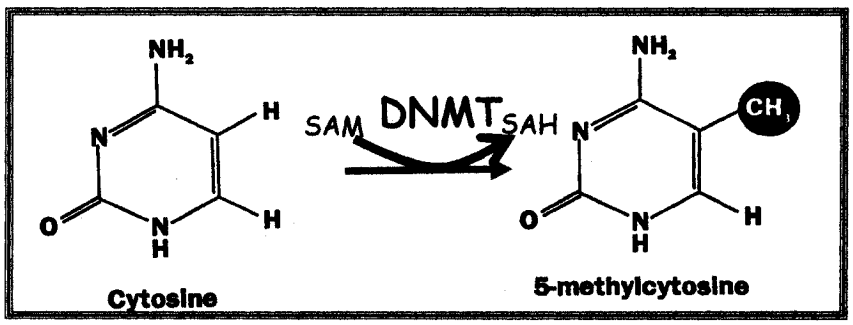
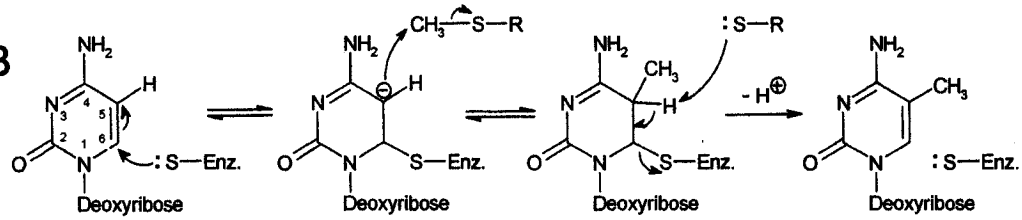
RS
ES
Mature Sperm

Figure 1.5. **(A)** Methylation of cytosine residues by DNA methyltransferase (DNMT) and the cofactor S-adenosylmethionine (SAM), whereby a methyl group is transferred to the 5' position of cytosine's pyrimidine ring. SAH, S-adenosylhomocysteine. **(B)** Mechanism of cytosine methylation. In order for methylation to occur, the cytosine base must first be flipped completely out of the DNA helix (not shown). Through nucleophilic attack on C6 by the cysteine residue in the catalytic centre of DNMT, a covalent bond forms to C6. The resonance-stabilised carbanion then attacks the methyl group of S-adenosylmethionine (SAM), and the methyl group is transferred to C5. β-elimination of the DNMT results in disassembly of this intermediate. However, **(C)** when nitrogen is present at the fifth position of the ring, as is the case for 5-aza and 5-azaCdR, the intermediate formed following nucleophilic attack is stable at physiological conditions and the DNMT remains covalently bound.

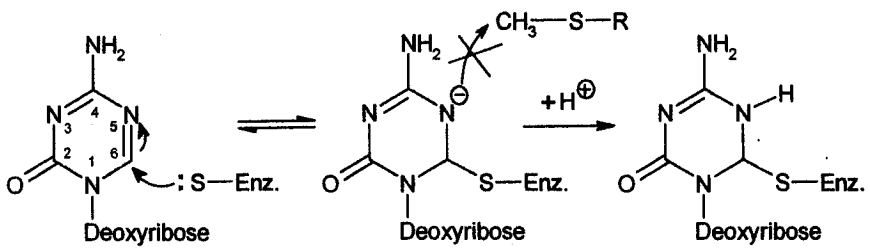
A


NH2
H
N
O
N
H
H
SAM
DNMT
SAH
NH2
CH3
N
O
N
H
H
Cytosine
5-methylcytosine

B


NH2
H
: S—Enz.
Deoxyribose
CH3—S—R
S—Enz.
: S—R
CH3
- H⊕
CH3

C


NH2
: S—Enz.
Deoxyribose
CH3—S—R
+H⊕
S—Enz.
H

1.4.3 DNMT3l

The most recently characterised member of the DNMT family, DNMT3l (Aapola *et al.*, 2000, 2001; Bourc'his *et al.*, 2001; Hata *et al.*, 2002) does not appear to have any methyltransferase activity. However, deficiencies in this enzyme demonstrate its importance to the methylation process (Bourc'his *et al.*, 2001; Hata *et al.*, 2002; Bourc'his & Bestor, 2004; Kaneda *et al.*, 2004).

Testicular levels of *Dnmt3l* mRNA are highest before birth, during the window when imprint acquisition begins in the male (15.5 – 18.5 dpc), and then decrease dramatically following birth when prospermatogonia differentiate into dividing spermatogonial stem cells (La Salle *et al.*, 2004). This profile is in agreement with evidence of DNMT3l in prospermatogonia of the fetal testis, but being downregulated in postnatal and adult testes (Bourc'his *et al.*, 2001; Bourc'his & Bestor, 2004). Co-expression of *Dnmt3l* and *Dnmt3a* in male gonocytes from 14.5 to 18.5 dpc suggests that these enzymes are responsible for the *de novo* methylation of the male genome during this period (La Salle *et al.*, 2004; Sakai *et al.*, 2004).

Gene-targetting of *Dnmt3l* showed that this enzyme is crucial for normal reproduction in both sexes (Table 1.1), but the reproductive impact of DNMT3l deficiency appears to be more severe in males as they suffer from hypogonadism and are azoospermic. The testicular phenotype of DNMT3l deficiency is indistinguishable from that associated with germ cell-specific DNMT3a deficiency (Bourch'his *et al.*, 2001; Hata *et al.*, 2002; Bourc'his & Bestor, 2004; Kaneda *et al.*, 2004). Although seminiferous cords and spermatogonia number appear normal at 1-week of age, few differentiated spermatocytes (only spermatogonia, leptotene and zygotene spermatocytes) are evident in the testes of 4-week old *Dnmt3l*$^{-/-}$ mice (Hata *et al.*, 2002). Detailed examination revealed abnormal synapsis (including non-synapsis) in all spermatocytes, and none progressed to the pachytene stage (Bourc'his & Bestor, 2004). Bourc'his and Bestor (2004) suggested

that the lack of post-zygotene spermatocytes was due to apoptosis because of non-synapsed chromosomal regions (Odorisio *et al.*, 1998). Additionally, global methylation was significantly decreased in DNMT3l-deficient male germ cells, as was the methylation of *LINE-1*, *IAP* and the imprinted gene *H19* (but not *Rasfgr1*) (Bourc'his & Bestor, 2004; Kaneda *et al.*, 2004). Consistent with this methylation profile, substantial expression of *LINE-1* and *IAP* elements was observed in spermatogonia at 2 dpp; expression persisted in the adult in spermatogonia and all spermatocytes (Bourc'his & Bestor, 2004), providing evidence for suppression of retrotransposons by methylation. Silencing may involve DNMT3l recruiting DNMT3a to *de novo* methylate specific sequences (Chédin *et al.*, 2002). Bourc'his and Bestor (2004) suggested that the perturbation of spermatogenesis is due to a failure to establish methylation on dispersed repeat sequences in prospermatogonia, resulting in ectopic gene expression and/or strand breaks from replicative retrotransposition.

Because it lacks any methyltransferase activity, DNMT3l is speculated to act as a regulator/cofactor of *de novo* DNA methylation, perhaps with one of the other DNMTs. In support of this hypothesis, DNMT3l has been shown to stimulate *de novo* methylation through DNMT3a, but not DNMT3b, at some imprinted loci *in vitro* (Chédin *et al.*, 2002). This ability to enhance methylation by the catalytically active DNMT3 enzymes likely occurs via binding of DNMT3l to these enzymes (Suetake *et al.*, 2004).

1.4.4 Demethylating Enzymes

DNA methylation is remarkably dynamic and is reversible, particularly during early development. Demethylation of the genome or specific loci can occur through two mechanisms: passive demethylation and active demethylation. Passive demethylation occurs when several cycles of replication take place in the absence of maintenance methylation. This situation could arise if methylation was blocked by protein binding to hemimethylated DNA, or through an active repression of the methyltransferase

machinery (Hsieh, 1999). On the other hand, active demethylation can occur within the timeframe of one replication cycle. Several lines of evidence suggest that active DNA demethylation does exist. For example, in the absence of replication the promoter of the testis-specific *Pgk2* becomes gradually demethylated in prospermatogonia (Geyer *et al.*, 2004). Similarly, demethylation of transition protein, *TP1,* occurs during meiotic prophase, after completion of DNA replication (Trasler *et al.*, 1990). These results, in addition to observations of PGC demethylation in the absence of replication (*e.g.*, Hajkova *et al.*, 2002; see section 1.3.2) and the rapid demethylation of the paternal pronucleus immediately after fertilisation (Mayer *et al.*, 2000; Oswald *et al.*, 2000; Santos *et al.*, 2002), point to the presence of an active demethylation event and argue for the existence of a demethylating enzyme. However, the enzymes or complex responsible has not yet been identified, although several candidates have been put forward.

Razin and colleagues (1986) hypothesised that demethylation could occur by replacement of methylated cytosines with unmethylated cytosines through the action of a m^5C-DNA glycosylase. Indeed, Vairapandi and colleagues (2000) demonstrated that a m^5C-DNA glycosylase, found in human HeLa cells, acts specifically to remove methylated cytosines from fully methylated CpG sites, producing abasic sites. This glyosylase was shown to associate with unidentified RNA factors as well as proliferating cell nuclear antigen (PCNA), which plays a role in nucleotide excision repair and mismatch repair (Pan *et al.*, 1995; Umar *et al.*, 1996). Jost *et al.* (1995) also reported a chicken m^5C-DNA glycosylase that preferentially removes m^5C from hemimethylated DNA. Differentiation is often associated with loss of methylation (reviewed in Haaf, 1995); in mouse myoblasts, differentiation was preceded by demethylation of hemimethylated DNA by a m^5C-DNA glycosylase (Jost *et al.*, 2001).

Demethylation could also occur through removal of the methyl group from the 5' position of cytosine's ring. In 1999, such an enzyme was reported, putatively identified

as methyl binding domain protein 2b (MBD2b). This enzyme was able to demethylate a methylated plasmid, yielding demethylated cytosines and methanol (Bhattacharya *et al.*, 1999; Ramchandani *et al.*, 1999). The existence of such a demethylase is disputed for several reasons, however, particularly because efforts to reproduce these findings have failed (Ng *et al.*, 1999; Wade *et al.*, 1999; Wolffe *et al.*, 1999).

The germ line provides an opportune tissue in which to search for a demethylating enzyme. Active demethylation occurs in both PGCs and, shortly after fertilisation, in the paternal genome. Moreover, recent work suggests that the major factor in post-fertilisation paternal demethylation resides in the oocyte, but is facilitated by a sperm-specific factor (Beaujean *et al.*, 2004). Perhaps a detailed examination of oocyte-specific factors will help to reveal the elusive demethylase.

1.5 Methods of Altering Male Germ Cell DNA Methylation

Several ways to alter DNA methylation exist, but only a few are applicable to studies involving the male germ line. One of the most common methods with which to manipulate DNA methylation is through the use of the hypomethylating cytosine analogues 5-azacytidine (5-aza) and 5-aza-2'-deoxycytidine (5-azaCdR) (Fig. 1.6). In the studies described in this thesis, we employed the drug 5-azaCdR, which inhibits methylation once it is incorporated into DNA. Use of these cytosine analogues offers an advantage as these agents likely inhibit methylation by both known (Weisenberger *et al.*, 2004) and unknown methyltransferases. In addition, several genetic mouse models lend themselves to studies of DNA methylation. In our studies, we utilised two mouse models: one haploinsufficient for the primary methyltransferase, DNMT1; and the other deficient in methylenetetrahydrofolate reductase (MTHFR), a gene integral to the folate pathway, the primary source of methyl groups. Moreover, combination of the 5-azaCdR drug treatment with the DNMT1-deficient males provided an attractive method

with which to challenge further the DNA methylation system. Our approaches to manipulating DNA methylation in the male germ line will now be discussed in more detail.

1.5.1 5-Azacytidine and 5-Aza-2'-Deoxycytidine

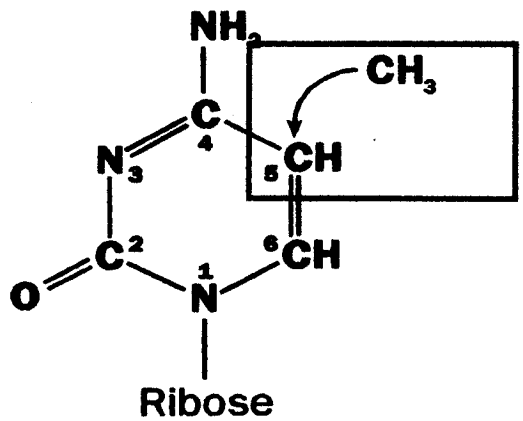
In our studies, we chose to take a pharmacological approach to disrupt DNA methylation within the male germ line. Several drugs, including 5-aza and 5-azaCdR (Fig. 1.6) (Jones & Taylor, 1980) are known to inhibit DNA methylation. Developed in 1964 by Sorm and colleagues (Sorm *et al.*, 1964; Sorm & Vesely, 1964), 5-aza and 5-azaCdR were originally slated for use as anticancer drugs. They are now among the best characterised and most studied of the known methylation inhibitors and are commonly used to manipulate DNA methylation.

1.5.1.1 Metabolism and Pharmacokinetics

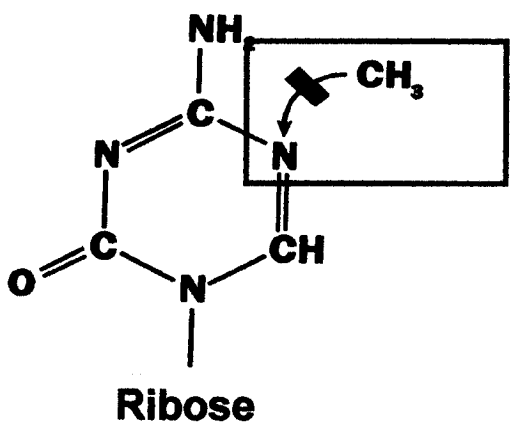
5-Aza and 5-azaCdR are transported into the cell via the facilitated uridine/cytidine nucleoside transport system, and after phosphorylation by their respective kinases, base-pair normally, as cytosine does, with guanine (Jones *et al.*, 1983a; reviewed in Haaf, 1995). The plasma half-life of 5-aza is 3.8 hours in the mouse after intravenous injection (i.v.) (Von Hoff *et al.*, 1976), whereas that of 5-azaCdR is considerably shorter, at approximately 25 to 31 minutes (Chabot & Momparler, 1984; Covey & Zaharko, 1985). Oral administration of 5-azaCdR has limited bioavailability compared to i.v. administration (Chabot & Momparler, 1984) thus i.v. and intra-peritoneal injection (i.p.) are the preferred routes of administration. 5-Aza and 5-azaCdR can be inactivated by cytidine deaminase to 5-azauridine and 5-aza-2'-deoxyuridine respectively (Pinto & Zagonel, 1993; reviewed by Christman, 2002). Both the drugs and their metabolites are rapidly excreted via the urine; 50% of 5-azaCdR is excreted in the urine within eight hours of administration (reviewed in Momparler, 1985).

Figure 1.6. Structures of cytosine and the cytosine analogues. **(A)** Cytidine, **(B)** 5-azacytidine (5-aza), and **(C)** 5-aza-2'-deoxycytidine (5-azaCdR). The presence of the nitrogen at the 5' position of the pyrimidine ring is indicated with a red box.

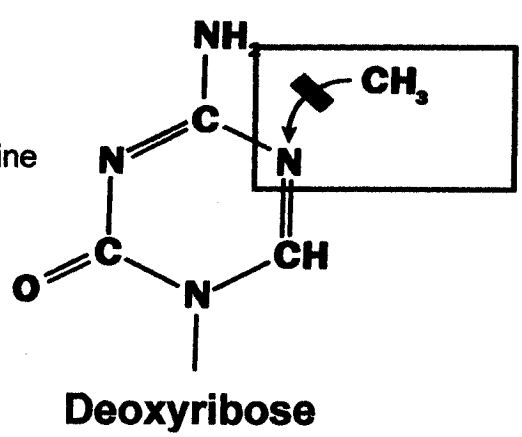
A. Cytidine



B. 5-Azacytidine
(5-aza)



C. 5-Aza-2'-deoxycytidine
(5-azaCdR)



1.5.1.2 Mechanism of Action

In order to exert their biological effects on DNA methylation, 5-aza and 5-azaCdR must be phosphorylated and incorporated into replicating DNA (Creusot *et al.*, 1982). The effects of 5-aza and 5-azaCdR are not random; it is only after incorporation into a site that would normally be methylated that 5-aza and 5-azaCdR directly inhibit methylation of that site (Friedman, 1986; reviewed in Christman, 2002). The presence of the nitrogen moiety at the fifth position of the pyrimidine ring of these analogues (Figs. 1.6) impedes the complete transfer of a methyl group from SAM, and as a result DNMT and SAM remain irreversibly bound as a covalent adduct at the sixth position of the pyrimidine ring (Fig. 1.5C) (Taylor & Jones, 1982; Santi *et al.*, 1984; Gabbara & Bhagwat, 1995). If left unrepaired, these adducts are toxic and potentially mutagenic (Jüttermann *et al.*, 1994; Jackson-Grusby *et al.*, 1997).

Using HPLC, Jones and Taylor (1980, 1981) discovered that the extent of hypomethylation in drug-substituted DNA was greater than the actual drug incorporation rate suggested. In fact, in addition to directly preventing methylation at drug-substituted sites, 5-aza and 5-azaCdR cause indirect hypomethylation and rapid loss of DNMT activity through covalent trapping of the DNMTs (Fig. 1.5C) (Creusot *et al.*, 1982; reviewed in Christman, 2002). In support of this, Weisenberger *et al.* (2004) recently showed that treatment of cell cultures with 5-azaCdR results in severely decreased DNMT (DNMT1, DNMT3a, DNMT3b) protein levels while mRNA levels remained unaffected. However, a recent study suggests that 5-azaCdR may also promote demethylation of DNA through a more active, but currently unknown, mechanism (Gius *et al.*, 2004). The mechanism of action of these cytosine analogues is complex and may involve many events, the initial effect of which may be reduction of DNA methylation. Notably, decreases in methylation can occur at non-cytotoxic concentrations of 5-aza and 5-azaCdR that do not significantly impair DNA synthesis (Mondal & Heidlberger, 1980;

Glazer & Knode, 1984; reviewed in Haaf, 1995).

1.5.1.3 Cytotoxicity

Both 5-aza and 5-azaCdR effectively deplete cells of functional methylating activity and cause a profound degree of hypomethylation after several rounds of DNA replication. However, the two drugs differ in their substrate specificity and primary mechanisms of cytoxicity (Glazer & Knode, 1984; reviewed in Haaf, 1995). As 5-aza is a ribonucleoside, it incorporates primarily into RNA and to a lesser extent into DNA after reduction to the deoxyribose form (Li *et al.*, 1970; Cihák, 1974). Integration of 5-aza into RNA has several deleterious effects including: disassembly of polyribosomes, defective protein methylation, alterations in transfer RNA, and inhibition of RNA processing and protein synthesis (reviewed in Haaf, 1995; Christman, 2002). Due to its incorporation into RNA, 5-aza has a greater effect on growth inhibition than does 5-azaCdR. Because of these non-DNA specific effects, 5-aza is not the ideal analogue for studies of DNA methylation as it is difficult to distinguish those effects due to DNA hypomethylation and those resulting from altered protein or RNA synthesis. In contrast, 5-azaCdR incorporates solely into DNA and thus does not directly impair protein synthesis (Haaf, 1995). At equivalent doses, it is a 10-fold more potent inhibitor of DNMT than is 5-aza (Jones & Taylor, 1980; Haaf, 1995).

At high doses, formation of drug-DNMT adducts can block DNA synthesis, resulting in cellular toxicity (reviewed in Haaf, 1995). In 1994, Jüttermann and colleagues proposed that the primary toxicity associated with 5-azaCdR exposure was not because of extensive hypomethylation, but rather due to covalent adduct formation between the methyltransferase and incorporated drug (Fig. 1.5C). Formation of these adducts is, in turn, dependent on the amount of methyltransferase present in the cell.

As mentioned earlier, demethylation of DNA by 5-aza or 5-azaCdR can be achieved at doses far lower than those causing cytotoxicity. In fact, 5-azaCdR appears to

have two mechanisms, depending on the dose. At high doses, exposure to the drug results in profound cytotoxicity, and the ability of 5-azaCdR to induce differentiation is lost; at low doses, the cellular effects are due to hypomethylation, and are distinct from immediate cytotoxicity (reviewed in Issa, 2003). In addition, 5-aza and 5-azaCdR may cause cells to undergo apoptosis (Yamada *et al.*, 1996; Ferguson *et al.*, 1997; Wang *et al.*, 1998), perhaps due to induction of apoptosis genes via promoter demethylation. Similar results have been observed in DNMT1-deficient mice, in which levels of apoptosis are increased (Li *et al.*, 1992). When cells exposed to 5-aza were treated with protein inhibitors, apoptosis was inhibited (Kajikawa *et al.*, 1998) indicating 5-aza-induced altered expression of pro-apoptotic genes.

1.5.1.4 Alterations in Gene Expression

Cultured cells treated with 5-aza analogues exhibit a number of changes including activation of normally silent genes, particularly imprinted genes (Jones *et al.*, 1982; Jones, 1985; Eversole-Cire *et al.*, 1993; Bender *et al.*, 1998). Hypomethylation of CpGs is correlated with gene activity and differentiation (reviewed in Bird, 2002; Haaf, 1995), and thus, reactivation of genes may involve hypomethylation followed by the formation of a more open chromatin conformation as shown by increased sensitivity to nucleases in 5-aza treated cultures (Litt *et al.*, 1997). Re-expression of hypoxanthine-guanine phosphoribosyltransferase (HPRT) increased 10-fold when 5-aza-hypomethylated cells were allowed to continue dividing; this suggests that formation of an active (open) chromatin structure may be a driving force behind induction of gene expression by 5-aza and 5-azaCdR (Homman *et al.*, 1987). Gene expression changes could also be induced by changes in DNMT interaction with other proteins (Christman *et al.*, 1985; reviewed in Christman, 2002). However, using antisense (siRNA) technology, Robert *et al.* (2003) demonstrated that the primary mechanism for reactivation of genes after exposure to the cytosine analogues is trapping of DNMT. Given that trapping of DNMT underlies

increased gene expression in cells exposed to 5-aza or 5-azaCdR, it is logical that, at equivalent doses, 5-azaCdR is a more potent inducer of gene and phenotypic expression than 5-aza (reviewed in Haaf, 1995). However, it is unclear whether a gene is demethylated and then becomes active, or if changes in chromatin structure precede gene activation, followed by demethylation (Bird, 2002).

In vitro, both 5-aza and 5-azaCdR are able to restore the normal methylation state of hypermethylated tumour suppressor genes, such as *p16* (*e.g.*, Cote & Momparler, 1997; Bender *et al.*, 1998; Gonzalgo *et al.*, 1998; Takita *et al.*, 1998; Guo *et al.*, 2002; Kim *et al.*, 2003; Qui *et al.*, 2004). *In vivo* experiments have corroborated these results (Daskalakis *et al.*, 2002). This ability of 5-azaCdR to restore normal methylation of various tumour suppressor genes holds great therapeutic potential.

1.5.1.4.1 Changes in Cellular Differentiation

Cellular differentiation involves changes in gene expression that bestow characteristics specific to certain cell types (*e.g.*, muscle cells); these changes are elicited through epigenetic modifications, such as DNA methylation (Ohgane *et al.*, 2002; Shiota *et al.*, 2002; Shiota & Yanagimachi, 2002). Early studies with 5-aza and 5-azaCdR demonstrated the ability of these drugs to alter or induce cellular differentiation (Constantinides *et al.*, 1977, 1978; Taylor & Jones, 1979; Jones & Taylor, 1980; Creusot *et al.*, 1982; Jones *et al.*, 1983b). These changes in the differentiated state of exposed cells occurred at drug concentrations that decreased DNA methylation, but did not inhibit DNA, protein, RNA or DNA synthesis; this suggests that hypomethylation, and thus activation of specific genes, was the basis for the phenotypic changes observed. In support of this, Jones and Taylor (1980) demonstrated that while 5-azaCdR could induce differentiation, cytosine analogues that do not inhibit methylation (*e.g.*, 6-azacytidine) could not.

In 1995, Raman & Narayan found exposure of neonatal mice to 5-azaCdR

blocked differentiation of spermatogonia to spermatocytes, perhaps through ectopic gene expression. This suggests that gene expression of male gametes can be modified through 5-azaCdR exposure. A recent study showed that culture of embryoid bodies for 6 hours, followed by a recovery period, resulted in increased expression of ES cell-specific markers (*e.g., SSEA-1,* alkaline phosphatase, *Oct4*), thus reversing the differentiation state possibly through demethylation of these ES cell-specific genes (Tsuji-Takayama *et al.*, 2004).

1.5.1.5 Alterations in Chromatin Structure

Treatment of cells with 5-aza or 5-azaCdR can alter chromatin structure significantly. Cells exposed to either drug exhibit both chromatin decondensation and micronuclei formation (Schmid *et al.*, 1984; Davidson *et al.*, 1992; Stopper *et al.*, 1995), with the extent of chromatin decondensation dependent on the concentration and duration of treatment. Some chromosome structures are more vulnerable than others to the effects of these drugs. In fact, inhibition of chromatin condensation appears to be largely restricted to inactive G-band chromatin and constitutively repressed chromatin; hypomethylation by 5-aza analogues results in significant inhibition of condensation of m^5C-rich heterochromatic regions in human and mouse chromosomes (Viegas-Péquignot & Dutrillaux, 1976; Schmid *et al.*, 1984; Rattner & Lin, 1987; Joseph *et al.*, 1989; reviewed in Haaf, 1995). In contrast, chromatin in which the majority of the housekeeping genes reside does not appear to be affected by exposure to 5-aza. Pulse treatment with 5-aza during S phase induces undercondensation specifically in the constitutive heterochromatin of chromosomes 1, 9, 15, 16 and/or Y (Schmid *et al.*, 1984) and can be seen in some cells at even very low doses. These same undercondensed areas often exhibit pairings with each other (Schmid *et al.*, 1983; Haaf *et al.*, 1986), resulting in somatic recombination and consequently chromosomal rearrangements in the following cell generations (Kokalj-Vokac *et al.*, 1993). Interestingly, cells exposed to 5-aza and

then allowed to recover for various amounts of time maintained their decreased methylation and undercondensed state for many cell generations (Parrow *et al.*, 1989). This continued undercondensation of chromatin in exposed cells may be related to decreased levels of linker histone synthesis; these histones tend to localise with G bands (Breneman *et al.*, 1993).

Exposure to 5-aza and 5-azaCdR may also result in chromatin instability and induce chromosomal damage such as rearrangements and micronuclei formation, in addition to generating single- and double-strand breaks in DNA (Karon & Benedict, 1972; Benedict *et al.*, 1977; Snyder & Lachmann, 1989; Stopper *et al.*, 1992). Micronuclei formation is observed after the first cell cycle of 5-aza incorporation (Stopper *et al.*, 1992); these micronuclei generally favour incorporation of chromosomes particularly vulnerable to the effects of 5-aza (Guttenbach & Schmid, 1994). 5-aza analogues can also induce fragile sites, regions that break in a non-random manner, in specific chromosome bands and can increase the frequency of sister chromatid exchange (SCE), once DNA is completely demethylated (Banerjee & Benedict, 1979; Hori, 1983; Albanesi *et al.*, 1999). The majority of these SCEs are located at fragile sites and may persist for some time, even after methylation levels return to normal, creating a heritable predisposition to chromosome recombination and rearrangement (Glover & Stein, 1987; Schmid *et al.*, 1987; Wenger *et al.*, 1987; Feichtinger & Schmid, 1989). It remains unclear whether effects of 5-azaCdR on chromatin stability are mediated through hypomethylation or DNMT adduct formation.

1.5.1.6 Clinical Use of 5-Aza and 5-AzaCdR

Recently, there has been renewed interest, with encouraging results, in the clinical use of 5-aza and 5-azaCdR. These drugs are currently proposed as therapies for sickle cell anemia, ß-thalassemia and some forms of cancer because of their ability to reactivate genes silenced by DNA methylation (reviewed in Egger *et al.*, 2004).

DNA methylation is postulated to play a role in carcinogenesis, though its exact function in this process is the subject of considerable debate. Methylated CpG dinucleotides are considered "mutagenic hotspots"; deamination of m^5C residues has been linked to tumour formation (reviewed in Egger *et al.*, 2004). As well, abnormal DNA methylation patterns, including loss of imprinting, are commonly observed in tumours although whether the aberrant patterns are the cause, or the result of, neoplasmic transformation has not yet been elucidated. Generally, global methylation levels in tumour tissues are below normal. Paradoxically, many cancers also display increased DNMT expression and regional hypermethylation at genes involved in tumour suppression, mismatch repair and imprinting (Liang *et al.*, 1998; Schmutte & Jones, 1998; Jones & Laird, 1999; Malumbres *et al.*, 1999). Thus, normal DNA methylation patterns and appropriate levels of DNMTs may be critical in the prevention of certain cancers.

Both 5-aza (Vidaza) and 5-azaCdR (Decitabine) have been used in numerous Phase I, II and III trials, with the best results in advanced myelodysplastic syndrome (reviewed in Christman, 2002; Dowell & Minna, 2004; Egger *et al.*, 2004). The anticancer effect of 5-azaCdR is, however, poorly understood; it is unclear whether response to this drug is due to cytotoxicity or induction of hypomethylation (reviewed in Issa, 2003). Because *in vivo* response to 5-azaCdR is slow and occurs at doses lower than the maximally tolerated dose (*e.g.* 15 mg/kg, 3 times/day), Issa (2003) suggests that the mechanism of 5-azaCdR is likely noncytotoxic. Gene re-activation due to hypomethylation may play a major role in the anticancer effects of 5-azaCdR. It has been suggested that activation of the p53 DNA damage response pathway or upregulation of tumour antigens may underlie the anticancer success of 5-azaCdR (Karpf *et al.*, 2001; Chan *et al.*, 2004).

Both sickle cell anemia and ß-thalassemia are marked by lowered expression of fetal hemoglobin (HbF) (Atweh *et al.*, 2003; Steinberg, 2003; Lal & Vichinsky, 2004).

Promoter methylation of the γ-globin gene corresponds to its expression (van der Ploeg & Flavell, 1980; Mavilio *et al.*, 1983), and because 5-azaCdR is a hypomethylating agent it has been considered to hold promise as a therapeutic drug for these diseases (Saunthararajah *et al.*, 2003). Indeed, chronic daily dosing with 5-azaCdR (~0.2 mg/kg/day) resulted in γ-globin promoter demethylation and a four-fold increase in HbF of sickle cell patients (Koshy *et al.*, 2000; Saunthararajah *et al.*, 2003). For cancer, sickle cell anemia and β-thalassemia, there is the potential for a greater therapeutic response when methylation inhibitors such as 5-azaCdR are used in combination with histone deacetylase (HDAC) inhibitors; and may allow for lower, less frequent dosings with 5-azaCdR (Leone *et al.*, 2003; Egger *et al.*, 2004; Hurtubise & Momparler, 2004; Lemaire *et al.*, 2004; Saunthararajah & DeSimone, 2004).

1.5.1.7 Teratogenicity

Many studies with rodents have shown that both 5-aza and 5-azaCdR are teratogenic when administered *in utero*. In the mouse and the rat, 5-azaCdR-induced defects are related to the day of *in utero* exposure (*e.g.*, Schmahl *et al.*, 1984; Rosen *et al.*, 1990; Cummings, 1994; Rogers *et al.*, 1994; Londei *et al.*, 1995; Branch *et al.*, 1996, 1998, 1999; Rosen & Chernoff, 2002). For example, administration of 5-azaCdR to pregnant mice on gestational day (GD) 8 to 9 affects skeletal growth while exposure on GD 11 specifically causes digital defects (Branch *et al.*, 1996); these malformations have been linked to reduced cell proliferation and increased cell death in affected areas (Rosen & Chernoff, 2002), and ectopic gene expression (Branch *et al.*, 1998). Males exposed on GD 10 display altered mating behaviour despite normal testicular and epididymal development, perhaps due to effects on the central nervous system (Cisneros & Branch, 2004); growth retardation of these males correlated with reduced levels of IGF-1, a regulator of body size (Cisneros *et al.*, 2003). Induction of *p53*, which plays a role in cell growth arrest and apoptosis (May & May, 1999), occurs when pregnant rats are

treated on GD 13 (Ueno *et al.*, 2002). Despite malformations in embryos exposed at later gestational stages, early embryos (*i.e.*, 1 – 3 dpc) appear to be protected from the teratogenic effects of 5-aza (Cummings, 1994; Vlahovic *et al.*, 1999).

1.5.1.8 Chronic Studies

Despite its use as a human chemotherapeutic, few studies of chronic exposure to 5-aza and 5-azaCdR have been done. Ironically, those that exist have looked at the ability of these drugs themselves to induce cancer. Treatment of BALB/c mice once a week for 50 weeks with 2 mg/kg of 5-aza induced a variety of cancers, such as lung tumours, lymphomas and skin tumours in exposed mice (Cavaliere *et al.*, 1987). Likewise, male Fisher rats treated for 9 months with 5-aza doses similar to those used in our studies (2.5 mg/kg & 5 mg/kg, Appendix I) developed tumours, most commonly testicular tumours. Similar results were obtained when rats were treated with considerably lower doses (0.025 mg/kg, 0.25 mg/kg and 2.5 mg/kg) but for a longer period of time (12 months) (Carr *et al.*, 1984, 1988). One study, in chickens, found that young chickens exposed to doses of 0.5 mg/kg or 1 mg/kg for six weeks had increased risk of developing autoimmune disorders (Schauenstein *et al.*, 1991).

1.5.1.9 5-Aza and the Male Rat

Previous studies in our lab demonstrated that disruption of DNA methylation patterns in the male germ cells of Sprague-Dawley rats has severe consequences for both germ cells and progeny outcome (Doerksen & Trasler, 1996; Doerksen *et al.*, 2000). Chronic administration of 5-aza, exposing both mitotic and/or meiotic populations of developing male germ cells, was associated with a significant increase in preimplantation loss as well as decreased sperm DNA methylation. Additionally, dose-dependent decreases in sperm counts were observed and as 50% to 70% of the testis is comprised of germ cells, this was reflected in relative reductions in testis weight. Because exposure to the non-hypomethylating agent 6-azacytidine resulted in no differences from saline

controls, this suggested that the observed effects of 5-aza were mediated by changes in DNA methylation (Doerksen & Trasler, 1996). These experiments provide further evidence that proper DNA methylation patterns are essential for normal male germ cell development and subsequent embryo development.

1.5.2 Genetic Approaches to Altering DNA Methylation

Although studies in the rat were informative on a basic level, the rat model is limited by both molecular and genetic techniques. In the mouse, numerous gene sequences (including imprinted genes) and genetic models are available, including those that allow perturbation of the methylation pathway from several vantage points. These include mice with reduced DNMT levels and mice deficient in the methyl groups necessary for methylation of DNA.

1.5.2.1 DNMT1 Deficiency

While nullizygous mouse models of the various DNA methyltransferases do exist, these models are inappropriate for the study of male germ cell development, because most mice die prior to mid-gestation, before the onset of full spermatogenesis (Li *et al.*, 1992; Lei *et al.*, 1996; Okano *et al.*, 1999; Bourc'his *et al.*, 2001). Furthermore, conditional knockouts of the DNMTs within the male germ line result in a severe spermatogenic phenotype, in which no mature sperm are recovered. It was our aim to create a system in which we could examine the effects of lowered methylation on male germ cell development, but also to generate germ cells that had reduced methylation levels yet were still viable. In this way, we could assay the effects of a moderately hypomethylated paternal genome on fertilisation ability and subsequent embryo outcome.

Of potential use in these studies are male mice heterozygous for a null mutation located in the highly conserved catalytic domain of DNMT1, the primary methyltransferase. These *Dnmt1*$^{c/+}$ mice are indistinguishable from their wild-type littermates and maintain normal methylation levels, despite DNMT1 enzyme levels that

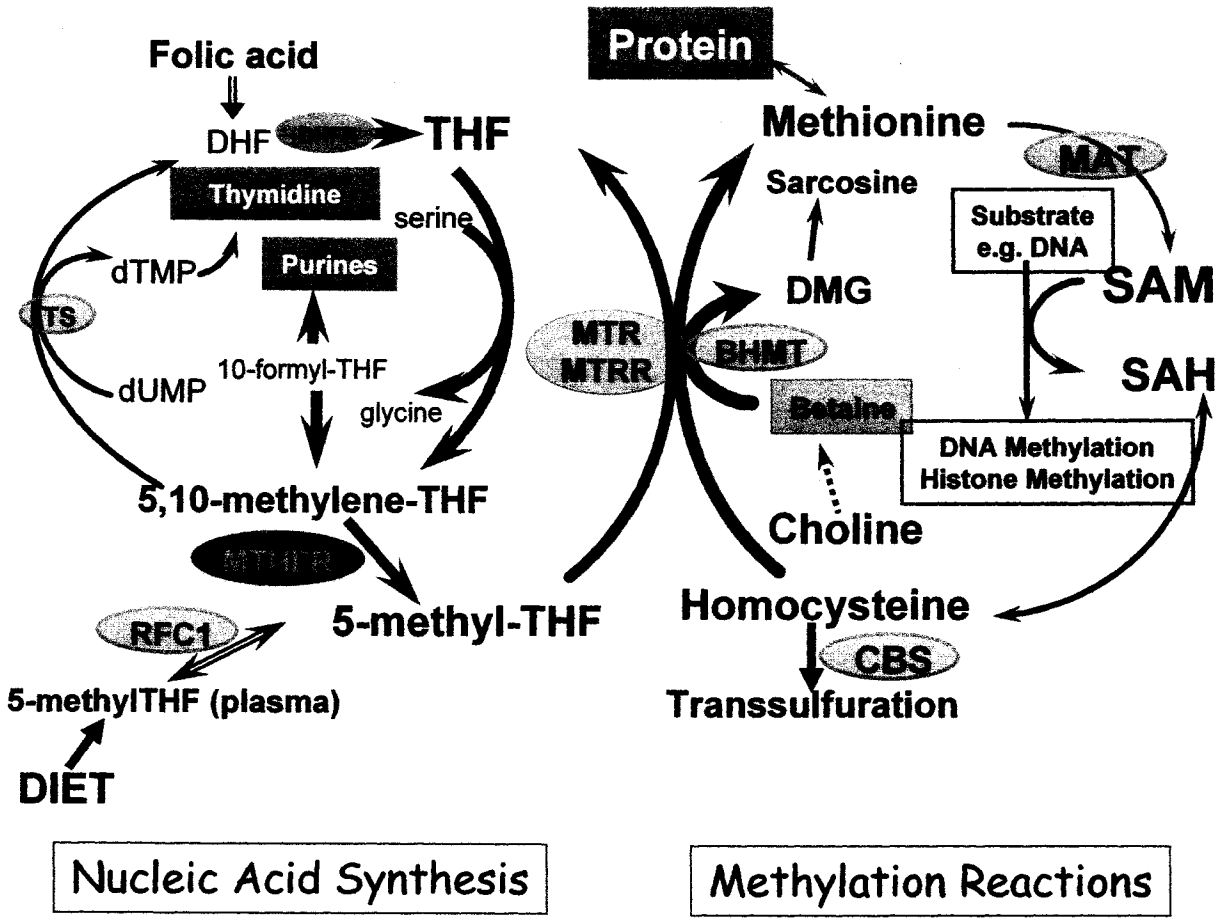
are 50% of normal (Li *et al.*, 1992, 1993). Male reproduction in these animals has never been thoroughly investigated.

1.5.2.2 Methylenetetrahydrofolate Reductase (MTHFR) Deficiency

Another genetic model of interest to us approaches the DNA methylation pathway via the availability of the methyl groups necessary for DNA methylation. MTHFR is a key enzyme in the folate pathway, which is involved in a number of biochemical processes, including methionine production (Fig. 1.7). Essentially, two pathways are linked by the reduction of 5-methyltetrahydrofolate to tetrahydrofolate via methionine synthase and the subsequent remethylation of homocysteine to methionine. Conversion of homocysteine to methionine provides the methyl groups necessary to form SAM, a ubiquitous methyl donor in numerous cellular reactions. When MTHFR is inhibited, decreases in methionine, SAM and consequently methylation of DNA follow (Chen *et al.*, 2001). Thus, MTHFR is integral to the production of the methyl groups necessary for methylation of a number of substrates including DNA.

In humans, the common 677C→T polymorphism results in a thermolabile enzyme with reduced activity that results in mild hyperhomocysteinemia when folate status is low (Frosst *et al.*, 1995; Jacques *et al.*, 1996; Christensen *et al.*, 1997). This polymorphism is thought to confer a reduced risk for colon cancer and acute leukemia, although the evidence for protection against colon cancer is conflicting (Little *et al.*, 2003; Sharp & Little, 2004). At the same time, the 677C→T polymorphism is associated with increased risk for neural tube defects and cardiovascular disease (reviewed in Schwahn & Rozen, 2001). More recently, and of relevance to this work, the 677C→T polymorphism has been linked to male infertility. In one study, the frequency of 677C→T homozygosity in infertile men was double that of the general population, suggesting that MTHFR activity may have a role in the pathogenesis of some types of male infertility (Bezold *et al.*, 2001). In support of a role for MTHFR in spermatogenesis, activity of MTHFR is highest

Figure 1.7. The folate pathway depicting the relationship between DNA methylation and remethylation of homocysteine to methionine via methylenetetrahydrofolate reductase (MTHFR). BHMT, betaine homocysteine methyltransferase; CBS, cystathione β-synthase; DHF, dihydrofolate; DHFR, dihydrofolate reductase; DMG, dimethylglycine; MAT, methionine adenosyltransferase; MTR, methionine synthase; MTRR, methionine synthase reductase; RFC1, reduced folate carrier 1; SAH, s-adenosylhomocysteine; SAM, s-adenosylmethionine; THF, tetrahydrofolate; TS, thymidylate synthase; Adapted from Kelly & Trasler, 2004 with permission of Blackwell Publishing.


Folic acid
DHF
THF
Thymidine
serine
dTMP
Purines
TS
dUMP
10-formyl-THF
glycine
5,10-methylene-THF
MTHFR
5-methyl-THF
RFC1
5-methylTHF (plasma)
DIET
Protein
Methionine
MAT
Sarcosine
Substrate
e.g. DNA
DMG
SAM
MTR
MTRR
BHMT
SAH
Betaine
DNA Methylation
Histone Methylation
Choline
Homocysteine
CBS
Transsulfuration
Nucleic Acid Synthesis
Methylation Reactions

in the adult mouse testis (Chen *et al.*, 2001), perhaps indicating a function for MTHFR in sperm development. The effects of MTHFR deficiency on male germ cell development, however, have not been examined before the work presented in this thesis.

1.6 Formulation of Project

It is estimated that approximately 15% of couples are infertile (Cooke & Saunders, 2002) and roughly 30 to 50% of this infertility can be partly or wholly attributed to male factor infertility, two-thirds of which remains unexplained. Some male factor infertility is known to result from defects in chromatin integrity, leading to chromosomal aberrations, such as deletions, translocations and aneuploidies (reviewed in Agarwal & Said, 2003; Feng, 2003). Other cases of male infertility may be attributed to genetic defects that impair the maturation, motility or egg binding ability of the sperm (reviewed in Agarwal & Said, 2003; Chemes & Rawe, 2003; Egozcue *et al.*, 2003). Just as genetic causes may underlie some male infertility, so might defects in epigenetic modifications of DNA, such as DNA methylation or modification of histone tails. DNA methylation is the most well-studied of the epigenetic phenomena and gametogenesis is the period when the sex-specific patterns of DNA methylation are established. As mentioned previously, during male germ cell development, the dynamics of DNA methylation are tightly regulated as is the expression of the corresponding DNMTs. Recent evidence in humans suggests that abnormal methylation of sperm may be correlated with poor sperm production and inability of sperm to fertilise (Benchaib *et al.*, 2003; Marques *et al.*, 2004). As well, subtle perturbation of germ cell methylation may not impair fertilisation ability, but may have more insidious consequences for the embryo or progeny. Alterations in sperm DNA methylation, particularly at repeat sequences or imprinted loci, could result in embryo failure, imprinting diseases, increased cancer risk, or behavioural abnormalities in progeny derived from abnormally methylated sperm (reviewed in Egger *et al.*, 2004; Lucifero *et al.*, 2004).

In the past year, mouse models of DNMT3l deficiency and germ cell–specific DNMT3a deficiency have undeniably demonstrated the functional importance of normal DNA methylation to spermatogenesis (Bourc'his *et al.*, 2001; Bourc'his & Bestor, 2004; Kaneda *et al.*, 2004). Although these mouse models established the essential nature of DNA methylation in the development of male germ cells, their phenotypes are quite severe; germ cells more mature than spermatocytes are not observed in either model. For our purposes, we were interested in effects of disrupting male germ cell DNA methylation on the development of sperm, but also on the ability of undermethylated sperm to fertilise and result in embryo development. Thus, I **hypothesised that perturbation, but not complete abrogation, of male germ cell DNA methylation could have deleterious consequences on both germ cell development and the ability of sperm to function in fertilisation and normal embryo development.** The role that altered epigenetics might play in infertility has not been rigorously examined. Thus, the aim of the studies in this thesis was to determine the effects of altering DNA methylation through both pharmacological (5-azaCdR) and genetic (DNMT1 and MTHFR) methods on spermatogenesis.

1.6.1 Choice of Animal Model

Previous work in our lab underscored the importance of normal methylation to male sperm development; chronic exposure of male rats to the hypomethylating agent 5-aza caused spermatogenic damage (Doerksen & Trasler, 1996; Doerksen *et al.*, 2000), but the type and mechanism of damage were unclear. In the rat, further mechanistic studies were impeded by the limited genetic information available and the relatively large size of the rat. An animal model much more amenable to genetic manipulation is the mouse. The mouse model enables us to take advantage of the numerous transgenic mouse models available, particularly those with altered components of the DNA methylation pathway. There are also well-established culture conditions for mouse embryos, allowing for

examination of embryonic effects of hypomethylated paternal DNA. Moreover, the small size of the mouse makes treatment with the DNA-specific hypomethylating 5-azaCdR analogue economically feasible, thus eliminating the non-specific effects of 5-aza incorporation into RNA. Because mice are easy to breed, have a short gestational period and produce large litters, these animals are ideal for mating and reproductive studies. Furthermore, the kinetics of spermatogenesis are well detailed.

In our studies, we used two models that challenge the methylation pathway from two different aspects: through reduction of DNMT activity (*Dnmt1*$^{c/+}$) and through limited availability of the methyl groups necessary for methylation of DNA, via a mutation in a crucial enzyme (MTHFR) in the folate pathway. Working in the mouse we were also able to combine the pharmacological and genetic models. Treatment of males deficient in one of the DNMTs with 5-azaCdR allows further suppression of the methylation machinery using lower doses of 5-azaCdR. These models will now be discussed in more detail.

1.6.1.1 5-AzaCdR and DNMT1 Deficiency

Treatment of the *Dnmt1*$^{c/+}$ males with 5-azaCdR provided a novel approach with which to further reduce methylation levels, *in vivo*, within the male germ line. This approach allowed us to genetically reduce the most predominant methyltransferase, DNMT1, while pharmacologically inhibiting the methyltransferase abilities of any catalytically active DNMTs (both known and unknown). It is likely that the various DNMTs can compensate, to some degree, for losses in each other, and simultaneous genetic reduction of all three of the known catalytically active DNMTs (DNMT1, DNMT3a and DNMT3b) and the cooperative enzyme, DNMT3l, would be very difficult. Therefore, treatment with 5-azaCdR offers a way in which to simultaneously inhibit the various DNMTs.

Because the DNMT1-deficient males have only 50% of wild-type enzyme levels,

there are two potential, but not mutually exclusive, outcomes when these mice are exposed to 5-azaCdR. Firstly, because of depleted DNMT1 enzyme levels, it may be easier to challenge and deplete enzyme stores in *Dnmt1*$^{c/+}$ males, and thus methylation may be perturbed to a greater extent than in *Dnmt1*$^{+/+}$ males. Secondly, if the primary toxicity of 5-azaCdR is mediated by formation of covalent adducts with DNMTs as proposed by Jüttermann *et al.* (1994) (Fig. 1.5C), heterozygous males should be more resistant to the cytotoxic effects of 5-azaCdR as they possess only half the amount of DNMT1 enzyme and therefore chances of adduct formation would be halved. Indeed, Laird *et al.* (1995) observed a greater extent of minor satellite hypomethylation in DNMT1-deficient mice relative to *Dnmt1*$^{+/+}$ mice after chronic treatment with 5-azaCdR.

1.6.2 Doses of 5-AzaCdR

In the studies described in this thesis, we employed a low dose chronic treatment with 5-azaCdR to effect changes in male germ cell methylation. Doses of 5-azaCdR employed in these studies were determined in a pilot study (Appendix I). Initial doses for the pilot study were based on the 5-aza doses previously used in the rat (Doerksen & Trasler, 1996; Doerksen *et al.*, 2000), but were scaled back to approximately one-fourth the initial dose to avoid severe reproductive effects. Because 5-azaCdR is approximately 10 times more potent than 5-aza, doses of 5-azaCdR were adjusted accordingly. It is interesting to note that the doses employed in these studies are equal to or considerably less than those used therapeutically in humans. Experiments describing the dose effects of the two drugs are described in Appendix I.

Low doses were employed in order to affect germ cell methylation without severe consequences on male germ cell development and thus sperm number. By minimising germ cell loss, we could examine the consequences of subtle perturbation of sperm methylation (through exposure to 5-azaCdR) on the function of sperm in fertilisation and

the effects of altered paternal DNA methylation on offspring. In order to maximally decrease methylation, males were treated three times a week with 5-azaCdR. This dose regimen ensures continual incorporation of the drug into replicating DNA while limiting toxicity to the mice and is similar to that employed by Doerksen and Trasler (1996) and Doerksen *et al.* (2000).

The kinetics of spermatogenesis in the mouse are tightly regulated and have been well elucidated (Clermont, 1972). This regulated nature lends itself well to pharmacological and toxicological studies of the testis because the timing of exposure to specific cell populations can be determined. Several investigators have used this detailed timing to their advantage in studies of drug action on the male reproductive system (Trasler *et al.*, 1985; Doerksen & Trasler, 1996). A treatment duration of 7 weeks (Fig. 1.8) was chosen because this is the time required for spermatogonia to develop into mature sperm and reach the ejaculate (Fig. 1.8).

1.6.3 Mating Regimen

To determine the fertilisation and progeny effects of male germ cell exposure to 5-azaCdR, treated males were mated, after 7 weeks of treatment, to untreated females. Sperm in the ejaculate at the time of these matings had been exposed to 5-azaCdR throughout development, from spermatogonia through epididymal transit to storage in the caudal epididymis. This mating design has been used previously to determine differential drug sensitivity amongst different stages of germ cell development (Trasler *et al.*, 1985). Initial matings were performed with C57BL/6 females (the same strain as *Dnmt1* males), but due to poor pregnancy rates (even in controls), all matings therein were completed with outbred CD1 females, which produce large litters even when unstimulated and are commonly used for early embryo analysis.

Mated females were examined for vaginal plugs, indicative of normal mating behaviour, and were sacrificed at 19 dpc (day of vaginal plug = 0.5 dpc), approximately

one day before birth. Pregnancy rate was calculated as the percent of plug positive females that became pregnant. Retrieval of fetuses just prior to birth allowed for examination of birth defects as female mice are known to cannibalise malformed young. More importantly, it also allowed for the determination of preimplantation loss, postimplantation loss and placental weights. Preimplantation loss represents oocytes that were either unfertilised or were fertilised but lost prior to implantation. Postimplantation loss can be observed as dead embryos or resorptions (necrotic tissue). A number of other pregnancy-related criteria were also determined, such as fetal weights, sex ratios and litter sizes.

1.6.4 Determination of Spermatogenic Defects in Mthfr$^{-/-}$ Males.

In contrast to the genetic and pharmacological depletion of DNA methyltransferase activity, the MTHFR mouse model allowed an opportunity to target the methylation pathway via the availability of methyl groups (Fig. 1.7). While MTHFR mice have been well-characterised, the effects of MTHFR-deficiency on the male reproductive system had not been investigated prior to our study. To determine the effects of MTHFR deficiency on testicular development, the testis was examined in males at 18.5 dpc, 6 dpp and 3 and 8 months of age, in order that various developmental time points could be investigated. Mating behaviour and fertility were tested for each male by mating them with two to four females each. Females were allowed to give birth and pups were counted, sexed, and examined for gross malformations on the day of birth.

Unlike homozygous DNMT knockouts, mice nullizygous for MTHFR are born at their expected Mendelian ratio, but their postnatal survival is poor (Schwahn *et al.*, 2003, 2004). Fortunately, survival can be substantially increased through provision of betaine, a choline derivative, to females throughout gestation until pup weaning (Schwahn *et al.*, 2004). Betaine acts as an alternative methyl donor for remethylation of homocysteine to methionine; supplementation of MTHFR nullizygotes *in utero* to weaning corrects, to a

limited degree (*i.e.,* not to wild-type levels), defects characteristic of severe MTHFR deficiency (Schwahn *et al.*, 2004). We therefore also investigated the effects of betaine supplementation on MTHFR deficiency-induced spermatogenic defects and provide evidence for nutritional amelioration of infertility. In the experiments reported in Chapter III, we examined the effects on spermatogenesis of MTHFR deficiency alone and in combination with short-term betaine (until weaning) and long-term betaine (lifetime) supplementation.

1.6.5 Specific Objectives

The overall aim of the work presented in this thesis was to establish a mouse model of hypomethylated DNA through both pharmacological and genetic manipulations, in order that present and future mechanistic studies of these effects could be completed. To this end, my specific objectives were:

1. To establish that results observed in the rat, after 5-aza treatment, are applicable to the mouse model. Doses of 5-aza appropriate to the mouse and 5-azaCdR doses of similar potency will be determined (Appendix I).

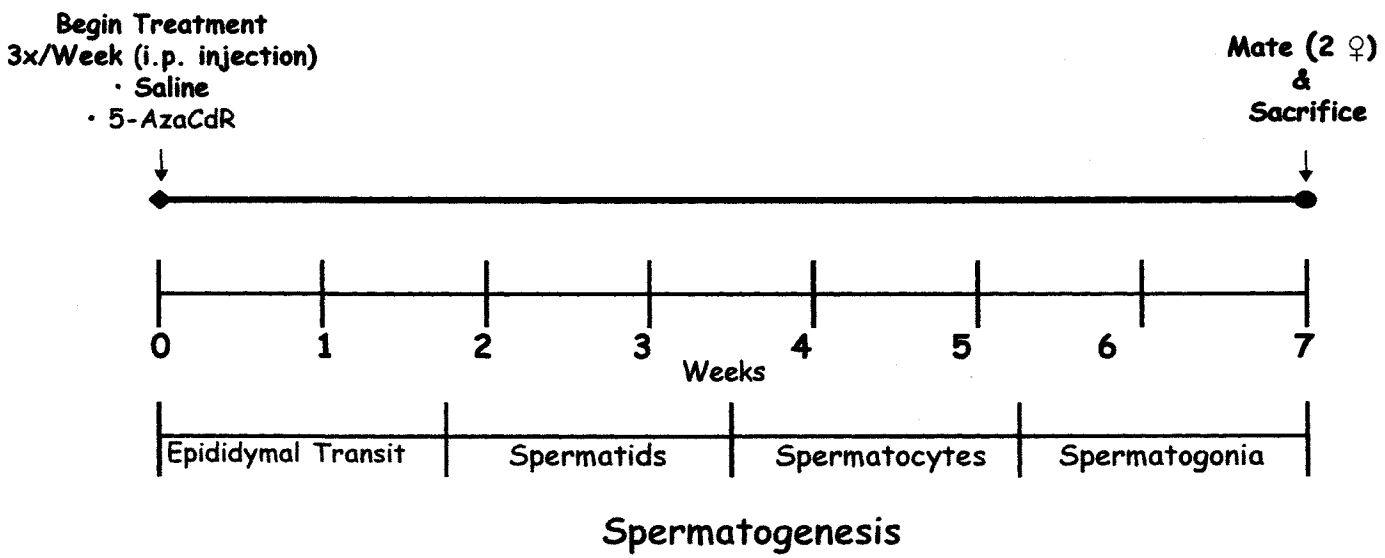
2. To examine the effects of depleting DNMT availability and activity, through genetic and pharmacological means, on male germ cell development and DNA methylation in the mouse. This includes evaluation of the genotypic differences in the reproductive response of *Dnmt1*$^{+/+}$ and *Dnmt1*$^{c/+}$ males to 5-azaCdR treatment (Chapters II and IV).

3. To determine the spermatogenic effects of depleting a gene, MTHFR; this can alter the pool of methyl groups within the DNA methylation pathway. This includes investigation of the potential therapeutic use of an alternate methyl donor in reversing MTHFR deficiency-induced spermatogenic defects (Chapter III).

4. To ascertain the mechanisms of early embryo loss and reduced fertility following chronic treatment of *Dnmt1*$^{+/+}$ and *Dnmt1*$^{c/+}$ males with 5-azaCdR (Chapter IV).

Figure 1.8. Treatment protocol employed to determine effects of 5-aza-2'-deoxycytidine (5-azaCdR) on male germ cell development. Male mice were treated for 7 weeks to expose germ cells throughout spermatogenesis. After treatment, males were mated with untreated females. The timing of drug exposure was calculated from the kinetics of spermatogenesis in the mouse (Clermont, 1972). Sperm from males treated for 7 weeks were initially exposed as spermatogonia and throughout the remainder of germ cell development. i.p., intraperitoneal injection.

5-AzaCdR Treatment Protocol



Spermatogenesis

CHAPTER II

5-AZA-2'-DEOXYCYTIDINE INDUCES ALTERATIONS IN MURINE SPERMATOGENESIS AND PREGNANCY OUTCOME

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2.1 ABSTRACT

Because of the ability of cytidine analogues, such as 5-aza-2'-deoxycytidine, to incorporate into DNA and lead to decreases in DNA methylation, there has recently been renewed interest in using these drugs in anticancer therapy. To determine the effects of paternal 5-aza-2'-deoxycytidine treatment on spermatogenesis and progeny outcome in the mouse, and whether these effects are modulated by decreased levels of the predominant DNA methyltransferase, DNMT1, adult *Dnmt1*$^{+/+}$ and DNMT1-deficient (*Dnmt1*$^{c/+}$) male mice were treated with 5-aza-2'-deoxycytidine for 7 weeks, which resulted in dose-dependent decreases in testicular weight, an increase in histological abnormalities, and a decline in sperm counts, with no apparent effect on androgen status. Testes of *Dnmt1*$^{c/+}$ mice, however, were less severely affected by 5-aza-2'-deoxycytidine than were those of wild-type mice. The exposure of *Dnmt1*$^{+/+}$ male mice to even low doses of 5-aza-2'-deoxycytidine followed by mating elicited significantly reduced pregnancy rates and elevated preimplantation loss in females. DNMT1 deficiency, however, protected against such drug-induced decreases in pregnancy rate but not preimplantation loss. Altered DNA methylation or DNMT1 activity may explain such adverse effects, because treatment resulted in dose-dependent decreases in the global methylation of sperm DNA. Thus, in the mouse, paternal administration of 5-aza-2'-deoxycytidine interferes with normal male germ cell development and results in reduced fertility, whereas lowering DNMT1 levels appears to partially protect the seminiferous epithelium from deleterious drug effects.

2.2 INTRODUCTION

DNA methylation of the mammalian genome occurs primarily within CpG dinucleotides and has been implicated in a number of processes, including X-chromosome inactivation, carcinogenesis, and the regulation of gene expression. During gametogenesis, patterns of DNA methylation are established that are then modified during early embryogenesis (Monk *et al.*, 1987; Chaillet *et al.*, 1991; Kafri *et al.*, 1992). In males, methylation of the repeat element intracisternal A particle (*IAP*; Walsh *et al.*, 1998) and the imprinted gene *H19* (Davis *et al.*, 1999) is initiated in prenatal germ cells and is subsequently maintained throughout postnatal spermatogenesis.

Various members of the (cytosine-5)-DNA methyltransferase (DNMT) family are capable of catalysing methylation in mammalian cells. *Dnmt1* encodes the predominant mammalian maintenance DNMT, and several other enzymes, including DNMT2, DNMT3a, DNMT3b, and DNMT3l, have recently been characterised (Okano *et al.*, 1998; Aapola *et al.*, 2001). Cytosine methylation is essential for normal development; mice homozygous for targetted partial ($Dnmt1^{n/n}$ or $Dnmt1^{s/s}$) (Li *et al.*, 1992) and complete loss-of-function ($Dnmt1^{c/c}$) mutations in *Dnmt1* have retarded growth and die by mid-gestation (Lei *et al.*, 1996). Levels of methylation in $Dnmt1^{c/c}$ embryos are only 5% of those in wild-type mice (Lei *et al.*, 1996). DNMT1-deficient embryos have biallelic expression of imprinted genes (Li *et al.*, 1993), ectopic X-chromosome inactivation (Panning & Jaenisch, 1996), hypomethylation and transcription of the normally silent endogenous retroviral *IAP* sequences (Walsh *et al.*, 1998), and increased levels of apoptosis (Li *et al.*, 1992). However, mice that are heterozygous for the null ($Dnmt1^{c/+}$) mutation survive and are phenotypically normal, although they possess only half the wild-type DNMT1 levels (Lei *et al.*, 1996).

Dnmt1 levels are highest in the testis and ovary, and expression is highly regulated throughout spermatogenesis in both the rat and the mouse (Trasler *et al.*, 1992; Benoit &

Trasler, 1994; Jue *et al.*, 1995). Both replicating and nonreplicating germ cells express DNMT1, although the enzyme is translationally down-regulated in pachytene spermatocytes (Trasler *et al.*, 1992; Jue *et al.*, 1995; Mertineit *et al.*, 1998). The presence of DNMT1 in mitotically dividing spermatogonia suggests that it may function in maintaining methylation patterns after replication, and during meiotic prophase it may play a role in repair. For the more recently described DNMT enzymes, testicular expression has not yet been fully characterised.

Previous studies in rats exposed to cytidine analogues have underscored the importance of DNA methylation in normal male germ cell development. The long-term administration of 5-azacytidine (5-aza) to male Sprague-Dawley rats, exposing both mitotic and/or meiotic developing male germ cells, was associated with decreased germ cell DNA methylation, reduced sperm production, and aberrant embryo development (Doerksen & Trasler, 1996; Doerksen *et al.*, 2000).

Cytidine analogues incorporate into replicating DNA, but, because of the presence of a nitrogen moiety at the fifth position of the pyrimidine ring, methylating DNMTs remain bound as covalent adducts (Gabbara & Bhagwat, 1995). DNMT adduct formation is thought to cause indirect genomic hypomethylation through the decreased activity of the DNMT enzymes (Gabbara & Bhagwat, 1995). 5-Aza is a nonselective analogue that is incorporated into both RNA and DNA. In rat studies (Doerksen & Trasler, 1996; Doerksen *et al.*, 2000), it was unclear whether 5-aza's effects were due to cytotoxicity or to decreased DNA methylation. In the present study, we used the more selective and clinically useful cytidine analogue 5-aza-2'-deoxycytidine (5-azaCdR). This analogue is incorporated only into DNA and thus should affect DNA methylation without the toxicity that arises from decreases in protein synthesis. Mice, rather than rats, were used, because the numerous gene-targetted mouse models may prove useful in unravelling the mechanisms underlying the effects of cytidine analogues on male germ cells. We used a

combination of pharmacological (5-azaCdR) and genetic (*Dnmt1* haploinsufficiency in *Dnmt1*$^{c/+}$ mice) manipulation to determine how experimental approaches known to alter DNA methylation affect spermatogenesis in the mouse. We show that paternal administration of 5-azaCdR interferes with normal male germ cell development, without affecting the general health of the mice, and results in reduced fertility or function of treated sperm. Furthermore, we show that *Dnmt1*$^{c/+}$ heterozygotes appear to be more resistant to 5-azaCdR–induced germ cell toxicity than their wild-type littermates.

2.3 MATERIALS AND METHODS

2.3.1 Animals

Dnmt1$^{+/+}$ and *Dnmt1*$^{c/+}$ males, heterozygous for a mutation in the catalytic domain of DNMT1 on a C57BL/6 background, have been described elsewhere (Lei *et al.*, 1996). Mice were genotyped using a polymerase chain reaction assay with primers amplifying a 146-bp fragment in *Dnmt1* exon 33, present only on wild-type alleles, and a 281-bp fragment indicative of the targetted allele. Primers exon 33S (5'-AGCTACTGTGACTAC-TACCGGC-3') and exon 33As (5'-ACCTGGAGCACAC-CAAAGGTGC-3') amplified the wild-type fragment, whereas primers intron 31S (5'-GTGGTGCGATGCATGTTTGAGCA-3') and pgkAS (5'- AAGTGCCAGCGGGGCTG-CTAAA-3') amplified a fragment of the targetted allele.

Adult virgin female C57BL/6 and CD1 mice were obtained from Charles River Canada (St Constant, Canada). They were maintained on a 12 : 12 hour light/dark cycle and were provided with food and water *ad libitum*. All animal studies were conducted in accordance with the principles and procedures outlined in the Guide to the Care and Use of Experimental Animals, by the Canadian Council on Animal Care.

2.3.2 Treatment and Mating

Male *Dnmt1*$^{+/+}$ (age 7 weeks) mice were randomly assigned to 1 of 4 treatment groups (n = 12/group) and, 3 times a week for 7 weeks, received intraperitoneal injections of saline or 5-azaCdR (0.05, 0.1, and 0.15 mg/kg) (Sigma Chemical Company, St Louis, Mo). *Dnmt1*$^{c/+}$ males were treated with either saline (n = 8) or 0.1 mg/kg 5-azaCdR (n = 9). Because alterations induced by this drug can be repaired, a dose regimen of 3 times per week was used to ensure continual incorporation of the drug into DNA while limiting toxicity to the mice. A treatment regimen of 7 weeks was chosen to target germ cells during their development; sperm collected after 7 weeks had been treated throughout spermatogenesis. All mice were weighed twice weekly. At the end of 7 weeks, each male was mated with 2 virgin CD1 females (age 8 weeks) that were checked daily for seminal plugs.

After treatment, male mice were killed, and blood was collected for white blood cell (WBC) counts and hemoglobin determination using a Coulter counter (Coulter Electronics, Hialeah, FL). Testes, epididymides, and seminal vesicles were removed, weighed, snap frozen, and stored at -80°C for further analyses. Caudal sperm were isolated (Alcivar *et al.*, 1989) and stored at -80°C for DNA methylation analysis.

2.3.3 Light Microscopy

For histological examination, the right testis was immersed in Bouin's fixative (BDH Inc, Toronto, Canada) for 12 to 24 hours, dehydrated, and embedded in paraffin. Sections (6 μm) were cut, mounted on glass slides, deparaffinised with xylene, and stained with hematoxylin and eosin. A Zeiss Axiophot photomicroscope was used to view the slides, and pictures were taken using a SPOT RT Slider digital camera (Diagnostic Instruments Inc, Sterling Heights, MI). Tubules were staged according to the method of Oakberg (1956), and abnormal seminiferous tubules were quantified. The number of

abnormal tubules was expressed as a percentage of total tubules examined (90 tubules counted/animal).

2.3.4 Sperm Counts

Hemocytometric counts of spermatozoa were done as described by Robb *et al.* (1978). To prepare for counting, a weighed portion of the left testis was homogenised (Polytron, setting 10; Brinkmann Instruments Inc, Westbury, NY) for three 15-second periods, separated by 10-second intervals, in 3 ml of 0.9% NaCl, 0.1% thimerosal, and 0.5% Triton X-100.

2.3.5 Analysis of Pregnancy Outcome

To examine the effect of paternal treatment on progeny outcome, each male, after 7 weeks of treatment, was mated with 2 untreated CD1 females over a period of 6 days. The success of mating was determined each morning by the presence of a vaginal plug; females were killed 19 days post-coitum (dpc). Ovaries were removed, and the number of corpora lutea, which is representative of the number of oocytes released, was counted. The uterus was then opened, and numbers of implantations, resorptions, and live fetuses were determined. The pregnancy rate is the number of plug-positive females that became pregnant. Preimplantation loss was calculated as the difference between the number of corpora lutea and implantations for each female. Thus, preimplantation loss is the number of oocytes that were either unfertilised or fertilised but were lost prior to implantation. The difference in the number of live fetuses and uterine implantations is a measure of postimplantation loss. Fetuses were weighed (as were resorption sites), sexed, and examined for gross malformations. Placentas and liver segments from 2 female and 2 male fetuses were snap frozen and stored at -80°C. All data concerning pregnancy outcome were expressed on a per-male basis.

2.3.6 DNA Methylation

Thin-layer chromatography (TLC) was used to examine global methylation of

CCGG sites of genomic sperm DNA as described elsewhere (Doerksen *et al.*, 2000). Quantification was done by phosphorimager.

2.3.7 Statistical Analyses

Data were examined statistically using analysis of variance with Dunnett's correction for pairwise comparisons or Student's two-sided t test. Fisher's exact test was used to analyse embryo data. The level of significance used was $p<0.05$ for all analyses (Sigma Stat; SPSS, Chicago, IL).

2.4 RESULTS

2.4.1 Effects on Body Weight and the Hematologic System

As a general gauge of health, body weight was monitored throughout the experiments; all animals survived and gained weight. No effects on weight or behaviour were seen after treatment initiation or cessation, and initial and final weights did not significantly differ between treatment groups (Table 2.1).

The effects of 5-azaCdR on the hematologic system are summarised in Table 2.1. Hemoglobin, a measure of red blood cell numbers, and WBC counts were not significantly altered by treatment with 5-azaCdR.

2.4.2 Effects on the Male Reproductive System

As an accessory sex organ, the seminal vesicles are very sensitive to changes in androgen status. The administration of 5-azaCdR did not appear to alter hormone levels: seminal vesicle weights were unaffected after 7 weeks of 5-azaCdR treatment.

As illustrated in Figure 2.1A, *Dnmt1*$^{+/+}$ testis weights declined by 35% and 55% after 7 weeks of treatment with 0.1 and 0.15 mg/kg 5-azaCdR, respectively. Although a significant decrease in testis weight (15%) was observed for *Dnmt1*$^{c/+}$ animals treated with 0.1 mg/kg 5-azaCdR, this decline was significantly less ($p<0.05$) than the decrease

seen in *Dnmt1*$^{+/+}$ mice treated with the same dose.

2.4.3 Effects on Testicular Histology

To assess the histological consequences of 5-azaCdR administration in mouse testis, detailed morphological examinations were conducted. A tubule was considered abnormal if it possessed any of the following characteristics: multinucleate/giant cells, degenerating germ cells, vacuole formation, disorganisation of germ cells, sloughing of immature germ cells, or a lack of at least one germ cell population.

Considerable histological abnormalities were first noted in the testes of *Dnmt1*$^{+/+}$ males treated with 0.1 mg/kg 5-azaCdR (Fig. 2.1B & 2.2C); tubules in mice given this dose displayed sloughing of germ cells as well as extensive vacuolisation and multinucleate cells, which are indications of ongoing germ cell death. Seventy-five percent of tubules were abnormal after 7 weeks of treatment with 0.15 mg/kg 5-azaCdR (Fig. 2.1B). Here, various germ cell populations were often absent, and sloughing of germ cells and vacuolisation were also observed (Fig. 2.2E). Of interest, saline-treated *Dnmt1*$^{c/+}$ males had a higher, but not significantly higher, baseline level of tubule abnormalities than their *Dnmt1*$^{+/+}$ counterparts (Fig. 2.1B); defects such as vacuolisation were at an increased frequency compared with wild-type saline control mice. In direct contrast to the effect observed in *Dnmt1*$^{+/+}$ males, there was no appreciable increase of histological abnormalities in *Dnmt1*$^{c/+}$ males given 0.1 mg/kg 5-azaCdR (Fig. 2.2D & F) compared with their saline controls (Fig. 2.2B).

2.4.4 Sperm Counts

Reduced testicular weights of *Dnmt1*$^{+/+}$ male mice were coupled with significant decreases of 33% (0.1 mg/kg) and 66% (0.15 mg/kg) in sperm number, per gram testis weight (Fig. 2.1C). In keeping with the histological results, sperm counts of *Dnmt1*$^{c/+}$ male mice treated with saline or 0.1 mg/kg 5-azaCdR were not significantly different.

Figure 2.1. Effects on **(A)** testis weight, **(B)** seminiferous tubule histology, and **(C)** sperm counts after 7 weeks of treatment with saline (*black*), 0.05 mg/kg 5-azaCdR (*vertical stripes*), 0.1 mg/kg 5-azaCdR (*diagonal stripes*), and 0.15 mg/kg 5-azaCdR (*crosshatch*). *Dnmt1*$^{c/+}$ mice were treated with saline (*grey*) and 0.1 mg/kg 5-azaCdR (*diagonal stripes on grey*). Bars represent means ± SEM. **p<0.05* vs. *Dnmt1*$^{+/+}$ control; †*p<0.05* vs. *Dnmt1*$^{c/+}$ control.

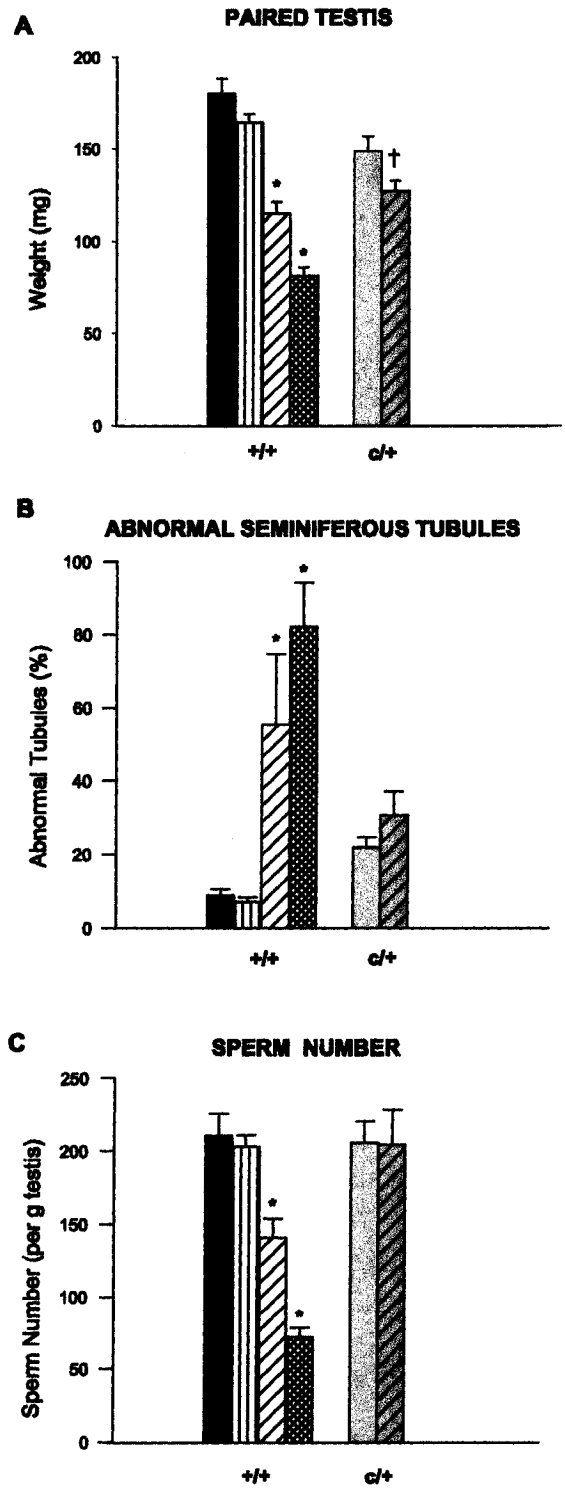

A
PAIRED TESTIS
Weight (mg)
200
150
100
50
0
*
*
†
+/+
c/+
B
ABNORMAL SEMINIFEROUS TUBULES
Abnormal Tubules (%)
100
80
60
40
20
0
*
*
+/+
c/+
C
SPERM NUMBER
Sperm Number (per g testis)
250
200
150
100
50
0
*
*
+/+
c/+

Figure 2.2. Examples of testicular histology after 7 weeks of treatment with 5-azaCdR. **(A)** *Dnmt1*$^{+/+}$ mice treated with saline (similar to 0.05 mg/kg 5-azaCdR); **(B)** *Dnmt1*$^{c/+}$ mice treated with saline; **(C)** *Dnmt1*$^{+/+}$ mice treated with 0.1 mg/kg 5-azaCdR; **(D)** and **(F)** *Dnmt1*$^{c/+}$ mice treated with 0.1 mg/kg 5-azaCdR; and **(E)** *Dnmt1*$^{+/+}$ mice treated with 0.15 mg/kg 5-azaCdR. The bar in panel **(E)** indicates 50 μm for panels **(A)** through **(F)**.

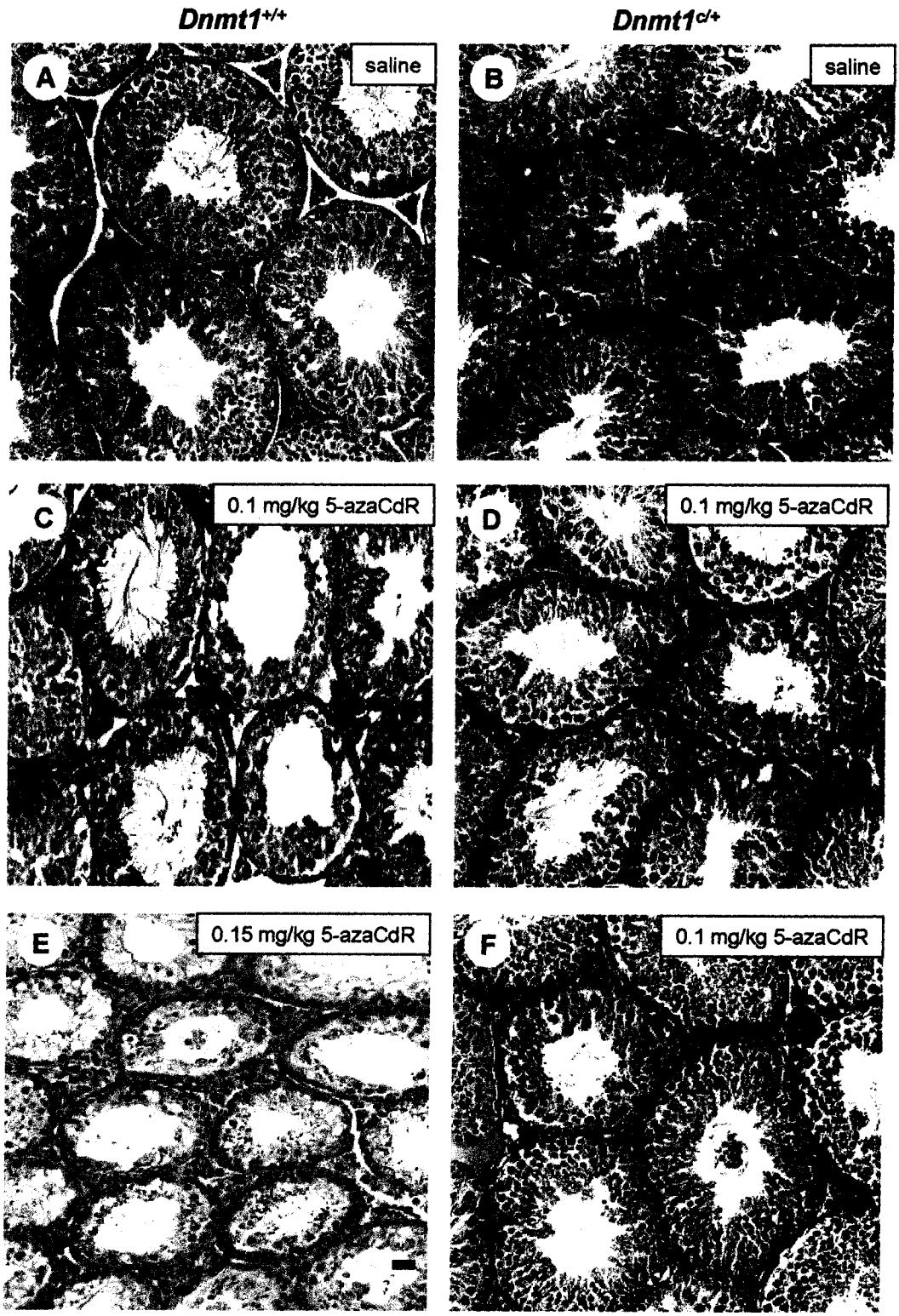

Dnmt1+/+
Dnmt1c/+
A
saline
B
saline
C
0.1 mg/kg 5-azaCdR
D
0.1 mg/kg 5-azaCdR
E
0.15 mg/kg 5-azaCdR
F
0.1 mg/kg 5-azaCdR

Table 2.1: Body and organ weights after 7 weeks treatment with 5-azaCdR.

	Dnmt1$^{+/+}$				*Dnmt1*$^{c/+}$	
Weight	**Saline**	**0.05 mg/kg**	**0.1 mg/kg**	**0.15 mg/kg**	***Dnmt1***$^{c/+}$ **saline**	***Dnmt1***$^{c/+}$ **0.1mg/kg**
Final (g)	28.05 ±0.34	27.63 ±0.24	27.56 ±0.34	27.59 ±0.35	23.4 ±0.70	24.02 ±0.66
Seminal Vesicles (mg)	48.03 ±3.73	47.54 ±2.93	47.44 ±2.45	53.62 ±3.17	42.56 ±2.88	48.27 ±3.13
WBC	13.11 ±1.87	11.92 ±1.26	10.55 ±1.19	8.86 ±0.96	10.7 ±0.71	9.85 ±1.36
Hgb	5.81 ±0.69	5.44 ±0.51	5.84 ±0.54	5.09 ±0.73	5.27 ±0.61	4.49 ±0.54

WBC represents white-blood cell count.
Hgb represents hemoglobin levels.

2.4.5 Effects on Progeny Outcome

Effects of 5-azaCdR treatment on pregnancy outcome were assessed by mating two CD1 female mice with each male. Mating behaviour was similar for all treatment groups; the number of sperm-positive females, per male, was not affected at any dose (data not shown). Although the pregnancy rate in the 0.05 mg/kg group remained the same as that in control group, it decreased in female mice mated with *Dnmt1*$^{+/+}$ males treated with 0.1 mg/kg (67%) and fell dramatically, to zero, in female mice mated with males exposed to 0.15 mg/kg 5-azaCdR (Fig. 2.3A). In direct contrast to the wild-type groups, the pregnancy rate did not differ between female mice mated with *Dnmt1*$^{c/+}$ males administered saline or 0.1 mg/kg 5-azaCdR (Fig. 2.3A); indeed, the pregnancy rate for both groups was identical (100%). Mating of female mice with *Dnmt1*$^{+/+}$ males exposed to 0.1 mg/kg 5-azaCdR resulted in a considerably lower pregnancy rate than in those females mated with *Dnmt1*$^{c/+}$ males given the same dose. In analysing full-term

pregnancies, a number of parameters were ascertained, including the number of corpora lutea, implantations, resorptions and live fetuses; litter size; sex ratios; fetal and placental weights (as well as those <75% or >125% of mean weight); and preimplantation and postimplantation loss. With the exception of preimplantation loss, none of these factors was significantly different than those for control mice. Regardless of genetic background, treatment with 0.1 mg/kg 5-azaCdR resulted in significantly increased preimplantation loss (27%) compared with saline controls (*Dnmt1*$^{c/+}$, 6% and *Dnmt1*$^{+/+}$, 8%) (Fig. 2.3B).

2.4.6 Methylation of Genomic Sperm DNA

The TLC/end-labelling assay is an established method to examine global methylation within CCGG sites of genomic DNA and was used to determine changes in overall methylation status caused by 7 weeks of treatment with 5-azaCdR (Fig. 2.4). Treatment with 5-azaCdR resulted in a dose-related decline in sperm DNA methylation; however, only treatment with the highest dose (0.15 mg/kg) elicited a significant decrease (29%) in sperm DNA methylation ($p<0.01$). Of interest, treated *Dnmt1*$^{+/+}$ and *Dnmt1*$^{c/+}$ males treated with 0.1 mg/kg 5-azaCdR exhibited similar decreases in DNA methylation of about 15% (Fig. 2.4).

Figure 2.3. Effects on **(A)** pregnancy rate at 19 dpc and **(B)** preimplantation loss after 7 weeks of paternal treatment with saline (*black*), 0.05 mg/kg 5-azaCdR (*vertical stripes*), 0.1 mg/kg 5-azaCdR (*diagonal stripes*), and 0.15 mg/kg 5-azaCdR (*crosshatch*). *Dnmt1*$^{c/+}$ mice were treated with saline (*grey*) and 0.1 mg/kg 5-azaCdR (*diagonal stripes on grey*). Bars represent means ± SEM. *$p<0.05$ vs. *Dnmt1*$^{+/+}$ control; †$p<0.05$ vs. *Dnmt1*$^{c/+}$ control. N/A indicates the 0.15 mg/kg dose of 5-azaCdR; no full term pregnancies were found at this dose.

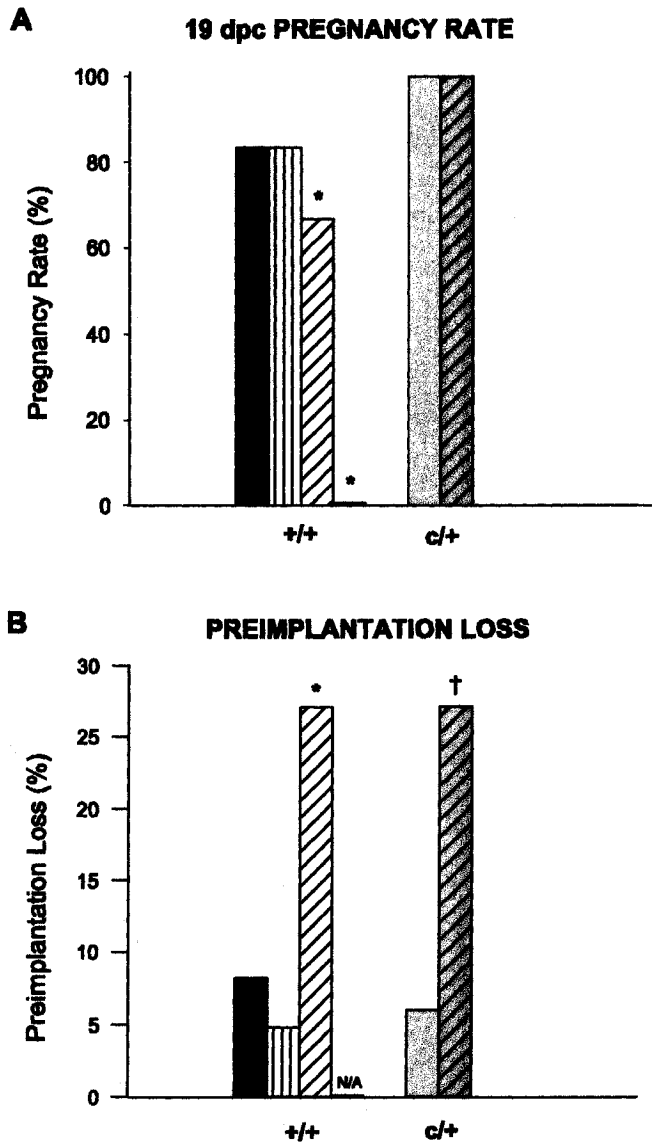
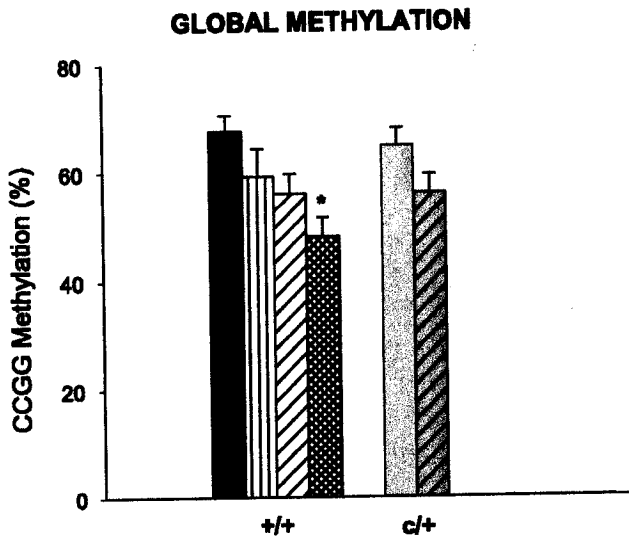

A
19 dpc PREGNANCY RATE
Pregnancy Rate (%)
100
80
60
40
20
0
*
*
+/+
c/+
B
PREIMPLANTATION LOSS
Preimplantation Loss (%)
30
25
20
15
10
5
0
*
†
N/A
+/+
c/+

Figure 2.4. Effect on global methylation of genomic sperm DNA as assessed by TLC after 7 weeks of paternal treatment with saline (*black*), 0.05 mg/kg 5-azaCdR (*vertical stripes*), 0.1 mg/kg 5-azaCdR (*diagonal stripes*), and 0.15 mg/kg 5-azaCdR (*crosshatch*). *Dnmt1*$^{c/+}$ mice were treated with saline (*grey*) and 0.1 mg/kg 5-azaCdR (*diagonal stripes on grey*). Bars represent means ± SEM. **p*<*0.05* vs. *Dnmt1*$^{+/+}$ control.


GLOBAL METHYLATION
CCGG Methylation (%)
80
60
40
20
0
*
+/+
c/+

2.5 DISCUSSION

The results of previous studies have pointed to the importance of proper methylation patterns in the development of male germ cells and their fertilisation competence (Doerksen & Trasler, 1996; Doerksen *et al.*, 2000). The long-term treatment of rats with 5-aza has been shown to result in decreased male germ cell DNA methylation and to be detrimental to both male germ cell maturation and early stage progeny of treated sires (Doerksen & Trasler, 1996; Doerksen *et al.*, 2000). Because similar exposure to the non-hypomethylating drug 6-azacytidine did not affect spermatogenesis or the progeny, we suggested that the observed effects of 5-aza were mediated, at least in part, by alterations in DNA methylation (Doerksen & Trasler, 1996). To facilitate more mechanistic studies of genomic methylation decreases on the function of male germ cells, we chose to pursue our studies in the mouse, an animal model that is more amenable to genetic studies than the rat. There are numerous advantages to using the mouse in germ cell DNA methylation studies, including the availability of transgenic animals and inbred strains, well-defined regulatory elements for many genes, and established preimplantation embryo culture techniques. Here, we demonstrate that the deleterious effects of the more DNA selective and clinically relevant cytidine analogue, 5-azaCdR, on male mouse germ cell development, DNA methylation, and preimplantation loss are similar to those seen elsewhere for the less selective 5-aza analogue in the rat. Furthermore, and of potential importance for the use of cytosine analogues in clinical practice, we show highly selective effects on mouse germ cells, in that doses of 5-azaCdR that affect spermatogenesis have only minimal effects on other tissues.

2.5.1 Germ Cells Affected

Because of the precise timing of spermatogenesis (Clermont, 1972), it is possible to determine the germ cell types exposed from the onset of drug treatment. After 7 weeks of treatment, mature spermatozoa had been exposed to 5-azaCdR throughout the course

of spermatogenesis. It was the ability of these sperm to fertilise that was tested when males were mated with untreated females. In keeping with our results, rats exposed throughout spermatogenesis, a regimen that targetted mitotic, meiotic, and postmeiotic germ cells simultaneously, exhibited significant abnormal germ cell development (Doerksen & Trasler, 1996; Doerksen *et al.*, 2000). Raman and Narayan (1995) demonstrated, with 5-day neonatal mice, that both 5-aza and 5-azaCdR inhibited the differentiation of spermatogonia to spermatocytes; experiments in the rat have also shown that 5-aza targets rapidly dividing cells (Doerksen *et al.*, 2000).

2.5.2 Effects of Cytosine Analogues on General Health

Similar to the results of rat studies with 5-aza, there were minimal effects of 5-azaCdR treatment on body weight in our study, which suggests that the general health of the mice was unaffected. The absence of treatment-induced reductions in seminal vesicle weights, together with apparently normal mating behaviour, as indicated by a similar number of plug-positive females in the treated and control groups, suggests that testosterone production was not significantly altered. Thus, it is likely that the dose-dependent reduction in testis weight resulted from germ cell loss rather than altered testosterone production, given that germ cells normally account for 50% to 70% of testicular and epididymal weights. Unlike the rat (Doerksen & Trasler, 1996), the treatment of male mice with low doses of 5-aza (Appendix I, unpublished data) or 5-azaCdR did not affect hemoglobin levels or WBC numbers. Bone marrow, like the testis, is a highly replicating tissue and is often affected by treatments that alter germ cell numbers. Together, the lack of treatment effects on body weight, weight gain, organ weights (other than the testis), and the hematologic system indicates that, at the doses we chose, the effects of cytosine analogues are highly selective for the mouse testis.

2.5.3 Progeny Outcome

To investigate the consequences of paternal treatment on progeny outcome, males treated for 7 weeks were mated, and females were killed at 19 dpc. Similar to rat studies, we observed a significant increase in preimplantation loss but not in postimplantation loss. Moreover, there was a sharp dose-related decline in the percentage of plug-positive females that became pregnant after mating with $Dnmt1^{+/+}$ males. Treatment with 0.1 mg/kg 5-azaCdR resulted in a three-fold increase in preimplantation loss in both the $Dnmt1^{+/+}$ and $Dnmt1^{c/+}$ groups. The etiology of pregnancy failure could have been due to the inability of treated sperm to fertilise or to the failure of normal embryo development after fertilisation. Transcription in mouse embryos is believed to initiate in the paternal pronucleus as early as the late 1-cell stage (Bouniol *et al.*, 1995); it follows that any defect in the paternal genome induced by 5-azaCdR could have deleterious consequences on embryo viability and normal development.

DNA methylation is intimately linked to gene expression. Alterations in methylation, such as those seen after the exposure of mouse male germ cells to 5-azaCdR, could affect gene expression in developing sperm and the resultant embryos. Although not statistically significant, perhaps the hypomethylation at the 0.1 mg/kg 5-azaCdR dose is adequate to compromise the ability of some sperm to participate in fertilisation or early embryo development. Imprinted genes have been implicated in embryogenesis, and it is likely that DNA methylation plays a role in the establishment or maintenance of genomic imprinting (Reik *et al.*, 2001). Thus, any disruption of the methylation patterns of imprinted genes could alter the expression of paternally imprinted genes and interfere with early embryo development. Current investigations examining the effect of paternal treatment with 5-azaCdR on preimplantation embryo development should shed some light on this issue (see Chapter IV).

2.5.4 Combination of Cytosine Analogues and DNMT1 Deficiency

We proposed that 5-azaCdR and DNMT1 deficiency might act synergistically to lower genomic methylation levels in the male germ line. Mice homozygous for deficiencies in one or more of the known DNA methyltransferases exist; however, these animals are inappropriate for studies of male germ line methylation, because most of them die before mid-gestation, prior to the onset of spermatogenesis (Li *et al.*, 1992; Okano *et al.*, 1999). Moreover, the existing DNMT-deficient mouse models may not reflect the full extent of the DNMT family; it has been suggested that additional enzymes may exist. Here, use of the hypomethylating drug 5-azaCdR offered an advantage. This agent is incorporated into replicating DNA and can then inhibit cytosine methylation by binding both known, and presumably unknown, methyltransferases. The combination of hypomethylating drugs with mice deficient in DNMT1, the principal mammalian methyltransferase, provides an interesting approach by which to reduce methylation levels and avoid toxicity of high-dose drug treatment.

Despite differing DNMT1 levels, both $Dnmt1^{+/+}$ and $Dnmt1^{c/+}$ mice treated with 0.1 mg/kg 5-azaCdR responded similarly with respect to sperm DNA methylation and preimplantation loss. Yet, treated $Dnmt1^{c/+}$ mice appeared to be resistant to other deleterious effects of 5-azaCdR. $Dnmt1^{c/+}$ males displayed a smaller reduction in testis weight and considerably less histological abnormalities; furthermore, the pregnancy rate was not affected. These results suggest less germ cell toxicity in the treated $Dnmt1^{c/+}$ male mice. Our results are consistent with the findings of Jüttermann *et al.* (1994), who demonstrated that 5-aza's primary toxicity may be mediated via the formation of "toxic" covalent adducts with DNMT1. $Dnmt1^{c/+}$ males may be more resistant to the cytotoxic effects of these drugs, because they possess only half the wild-type level of DNMT1, and the chances of adduct formation are thus reduced. Our findings here suggest that $Dnmt1^{c/+}$ mouse testes are more resistant to the toxic effects of cytosine analogues, possibly

because of the drug's mechanism of toxicity. We propose that the adverse consequences of 5-azaCdR in *Dnmt1*$^{+/+}$ mice may be due to a combination of drug toxicity (through the formation of toxic adducts) and hypomethylation (secondary to the inhibition of DNMTs bound to 5-azaCdR incorporated into DNA), whereas decreased methylation itself may explain the majority of the effects observed in *Dnmt1*$^{c/+}$ mice.

Furthermore, the dose-related effects of 5-azaCdR on mouse sperm DNA methylation were reminiscent of results seen in previous rat studies with 5-aza. CpG methylation occurs at 3×10^7 sites throughout the mammalian genome, and the TLC assay assesses only those CpGs within CCGG sites. CCGG sites represent only about 5% of the total number of CpGs that become methylated. To detect gene-specific alterations in methylation and effects at low doses, more sensitive techniques such as restriction landmark genome scanning (RLGS) may be needed (see Chapter IV). In a recent study that compared old and young rats, RLGS revealed alterations in DNA methylation that were not detected by the TLC assay (Oakes *et al.*, 2003).

2.5.5 Mechanisms of Germ Cell Damage by Cytosine Analogues

The mechanism of action of this cytosine analogue is complex and may involve many events associated with a reduction in DNA methylation, including altered gene expression, alterations in chromatin structure, chromosome rearrangements, the induction of apoptosis, and abnormal genomic imprinting. The treatment of cell cultures with 5-aza results in the expression of normally silent genes (Jones *et al.*, 1982; Jones, 1985; Eversole-Cire *et al.*, 1993), chromatin decondensation, and micronuclei formation (Schmid *et al.*, 1984; Davidson *et al.*, 1992; Stopper *et al.*, 1995). The reactivation of genes may involve the formation of a more open chromatin structure, as has been shown by increased sensitivity to nucleases in 5-aza–treated cultures (Litt *et al.*, 1997). Similarly, the treatment of cells with the histone deacetylase inhibitor trichostatin-A has been shown to increase the expression of imprinted genes (Cameron *et al.*, 1999). DNA

methylation and histone deacetylation are intrinsically linked and together help form the closed chromosomal conformation characteristic of silenced gene areas (heterochromatin) (Jones *et al.*, 1998; Nan *et al.*, 1998; Cameron *et al.*, 1999); if one of these elements is disrupted, so in turn may be the other. Understanding the mechanisms underlying the effects of cytosine analogues on male germ cells, with or without DNMT deficiency, will clearly require an examination of their effects on end points such as gene expression, chromatin structure, and chromosomal instability. The fact that the results of both rat and mouse studies have suggested that mitotic and meiotic male germ cells are affected by cytosine analogues is consistent with drug effects on any one of these end points. To date, most studies on the mechanisms of action of cytosine analogues have been done in cultured cells, and none have been done in germ cells. Our studies provide an *in vivo* model to study cellular effects of cytosine analogues in male germ cells.

2.5.6 Implications

Recently, there has been a resurgence of interest, and encouraging results, in the use of cytosine analogues to treat various diseases, including sickle cell anemia and malignant hematologic disease, such as acute myelogenous leukemia (Koshy *et al.*, 2000; Wijermans *et al.*, 2000; Silverman *et al.*, 2002). The results of the experiments described here have implications for the clinical use of such drugs. The treatment of these diseases involves chronic drug courses as either the primary therapy, or in conjunction with other drugs, at doses equal to or greater than those used in our study (Koshy *et al.*, 2000; Wijermans *et al.*, 2000). The fact that effects on spermatogenesis are similar in rats and mice suggests that adverse effects on male germ cells will also be seen in humans. With increasing implications of the role of epigenetic factors such as DNA methylation and chromatin structure in germ cell and embryo development, we suggest that a thorough study of the mechanisms underlying the germ cell effects of cytosine analogues is warranted.

2.6 ACKNOWLEDGEMENTS

We are grateful to Tonia Doerksen for her help in the conception of this project and to Daniel LeClerc and Liyuan Deng for establishing the assay to genotype the *Dnmt1*$^{c/+}$ mice. We thank Eric Simard and Xinying He for their superb technical assistance.

APPENDIX I

COMPARISON OF THE EFFECTS OF THE HYPOMETHYLATING CYTOSINE ANALOGUES 5-AZACYTIDINE AND 5-AZA-2'-DEOXYCYTIDINE ON MURINE SPERMATOGENESIS.

Tamara LJ Kelly

A1.1 INTRODUCTION

In Chapter II, the effects of chronic treatment with 5-azaCdR on the male mouse reproductive system were described (Kelly *et al.*, 2003). Original studies in our lab, however, had utilised the rat model and examined its response to the hypomethylating agent 5-aza. In the rat, chronic treatment with low doses (2.5 – 4.0 mg/kg) of 5-aza resulted in diminished testis and epididymal weights, decreased sperm DNA methylation and increased preimplantation loss (Doerksen & Trasler, 1996; Doerksen *et al.*, 2000). The rat model, however, is limited by its potential use to elucidate the underlying genetic mechanisms of 5-azaCdR's effects. In order to facilitate more mechanistic studies of decreases in male germ cell genomic DNA methylation, we chose to pursue our studies in the mouse model, which is more amenable to genetic studies than the rat. Advantages of the mouse model include: established and successful preimplantation embryo culture techniques, well-defined genetics, and the availability of transgenic mice with genetically reduced levels of one or more of the DNA methyltransferases (Li *et al.*, 1992; Okano *et al.*, 1999; Bourc'his *et al.*, 2001). Also because of the small size of the mouse use of the more DNA-specific cytosine analogue, 5-azaCdR is economically feasible.

Accordingly, before the work in Chapter II could be completed, a preliminary study was required, the purpose of which was threefold: to determine if changes elicited by 5-aza in the mouse were similar to those previously observed in the rat; to determine if effects of the more selective cytosine analogue, 5-azaCdR, corresponded to those seen at comparable potencies of 5-aza; and to determine doses at which future studies of 5-azaCdR could be carried out. This appendix details these initial studies and our findings.

A1.2 MATERIALS AND METHODS

A1.2.1 Animals, Treatment Regimen and Doses

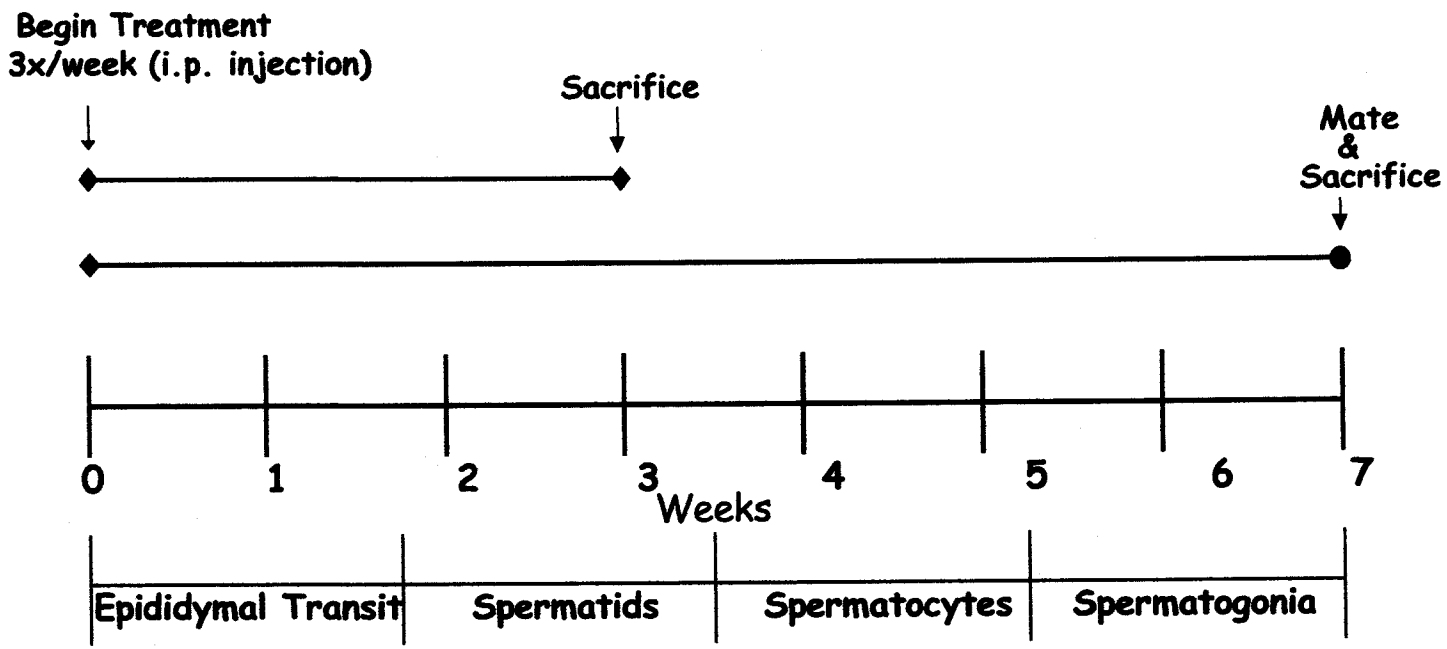
To answer these questions, adult C57BL/6 (7 weeks of age) male mice (Charles River, St. Constant, Canada) were treated for 3 or 7 weeks with saline, 5-aza or 5-azaCdR (Fig. A1.1). The doses of 5-aza used here were based on those previously used in the rat (2.5, 4.0 and 5.0 mg/kg) (Doerksen & Trasler, 1996; Doerksen *et al.*, 2000). Because 5-azaCdR is incorporated only into DNA, it is approximately 10 times more potent than 5-aza; doses of comparable potency were established based on this assumption (Flatau *et al.*, 1984; Momparler *et al.*, 1984; reviewed in Christman, 2002). The dosing regimen of 3 times per week has been used previously (Doerksen & Trasler, 1996; Doerksen *et al.*, 2000) and was designed to enable continual drug incorporation into DNA, whilst minimising drug toxicity.

Male mice were randomly assigned to one of five treatment groups (n = 8-12/group) and received intraperitoneal injections (i.p.) of either saline, 5-aza (1.0 mg/kg, 2.5 mg/kg, 4.0 mg/kg), or 5-azaCdR (0.1 mg/kg, 0.3 mg/kg) at a frequency of 3 times per week for 3 or 7 weeks. The 0.1 mg/kg 5-azaCdR dose was used only in the 7 week study, not the 3 week study. Following 7 weeks of treatment, each male was mated with two virgin C57BL/6 females.

A1.2.2 Tissue Collection

Male mice were killed post-treatment and blood was collected for white blood cell (WBC) and hemoglobin (Hgb) determination using a Coulter Counter (Coulter Electronics, Hialeah, FL). Testes, epididymides and seminal vesicles were removed, weighed, snap frozen (as was a section of liver) and stored at -80°C until further use. Seminal vesicles were emptied prior to weighing. Caudal sperm was isolated (Alcivar *et al.*, 1989) and stored at -80°C for future use.

Figure A1.1. Schematic representation of treatment protocol utilised, illustrating the targetting of specific germ cell populations based on treatment duration.



Spermatogenesis

3 Week Treatment
Saline
1.0 mg/kg 5-aza
2.5 mg/kg 5-aza
4.0 mg/kg 5-aza
0.3 mg/kg 5-azaCdR

7 Week Treatment
Saline
1.0 mg/kg 5-aza
2.5 mg/kg 5-aza
4.0 mg/kg 5-aza
0.1 mg/kg 5-azaCdR
0.3 mg/kg 5-azaCdR

A1.2.3 Light Microscopy

The right testis was immersed in Bouin's fixative for 12 to 24 hours, dehydrated and embedded in paraffin. Sections of 6 μm thickness were cut, mounted, deparaffinised with xylene, and stained with hematoxylin and eosin. Slides were viewed with a Zeiss Axiophot photomicroscope. Tubules were staged according to the method of Oakberg (1956). Abnormal tubules were quantified and expressed as a percentage of total tubules examined (~90 to 100 tubules/male).

A1.2.4 Matings

To determine the effects of paternal treatment with 5-aza or 5-azaCdR on fertility, each male, after 7 weeks treatment, was mated with 2 untreated C57BL/6 females (Charles River). Every morning, females were examined for the presence of a vaginal plug, an indication of successful mating. At 19 dpc, females were killed, their ovaries removed, and the number of corpora lutea counted – this represents the number of oocytes released from each ovary. The uterus was opened and the numbers of implantations, resorptions, and viable embryos were counted. As well, litter size, sex ratios, fetal and placental weights, resorptions weights, and pre- and post-implantation loss were determined. Preimplantation loss represents the number of oocytes that were either unfertilised or fertilised but lost prior to implantation; it was calculated by determining the difference between the number of corpora lutea and uterine implantations. Postimplantation loss is the difference between the number of live fetuses and the number of implantations; this can be observed as resorptions sites, the size of which are indicative of the time of death during gestation. Fetuses were examined for gross malformations, and placentas and liver segments from 2 female and 2 male fetuses were snap frozen and stored at -80°C. All data concerning pregnancy outcome were expressed on a per-male basis.

A1.2.5 Statistics

Data were analysed using Sigma Stat (Chicago, IL) software using One Way Anova with a post-hoc Tukey test. Fisher's exact test was used to analyse fertility and embryo data. The level of significance was $p<0.05$ for all analyses.

A1.3 RESULTS

This study was designed to compare the effects of chronic 5-aza administration in the rat and mouse models, as well as to determine doses of 5-azaCdR appropriate for future male germ cell studies in the mouse.

A1.3.1 Systemic Effects

Similar to the rat, all mice gained weight and appeared healthy and active throughout the course of the experiment although drug-treated males did not gain weight at the same rate as controls. Final body weights are shown in Table A1.1.

Unlike the rat, male mice experienced hematological alterations at only high doses of either drug (Table A1.1), at which testicular histology was also highly abnormal. Hemoglobin became significantly altered only after 3 weeks exposure to 4.0 mg/kg 5-aza, whereas white blood cell counts were affected by the end of 3 or 7 weeks treatment with 0.3 mg/kg 5-azaCdR or 4.0 mg/kg 5-aza, respectively.

A1.3.2 Effects on Male Reproductive System

As illustrated in Figure A1.2A, similar decreases in testis weights were achieved using comparable effective doses of the two drugs (*i.e.*, 5-azaCdR doses ~1/10 those of 5-aza). Both 3- and 7-week administration of 5-aza resulted in dose-dependent decreases in testis weight, although this was more prominent at 7 weeks than at 3 weeks. Significant reductions in testis weight, after 3 weeks of drug exposure, occurred with 5-aza doses of 2.5 mg/kg (35%) and 4.0 mg/kg (40%). With 7 weeks of treatment, testis weights of the 2.5 mg/kg and 4.0 mg/kg 5-aza-treated males dropped to 30% and 24% of controls,

Table A1.1: Effects of 3 and 7 weeks of treatment with 5-aza or 5-azaCdR on body weight, seminal vesicle weight and the hematopoietic system.

	3 WEEKS					7 WEEKS					
		5-aza			5-azaCdR		5-aza			5-azaCdR	
Weight	**Saline**	**1.0 mg/kg**	**2.5 mg/kg**	**4.0 mg/kg**	**0.3 mg/kg**	**Saline**	**1.0 mg/kg**	**2.5 mg/kg**	**4.0 mg/kg**	**0.1 mg/kg**	**0.3 mg/kg**
Final (g)	23.72 ±0.49	23.96 ±0.37	23.92 ±0.42	23.95 ±0.29	24.32 ±0.44	28.4 ±0.60	27.80 ±0.47	27.32 ±0.44	26.64 ±0.62	28.69 ±0.48	26.76 ±0.64
Seminal Vesicles (mg)	55.59 ±7.15	47.56 ±5.03	53.60 ±3.17	48.84 ±3.27	59.09 ±6.56	65.17 ±5.52	73.13 ±2.71	57.28 ±3.62	62.67 ±3.74	79.89 ±2.18	72.02 ±5.33
WBC	8.82 ±1.77	5.719 ±0.56	6.77 ±1.22	6.68 ±0.98	4.44* ±0.35	13.20 ±1.25	10.19 ±0.92	9.75 ±0.85	8.33* ±1.15	11.42 ±1.25	9.94 ±1.37
Hgb	12.46 ±0.73	12.25 ±0.63	10.76 ±0.41	10.46* ±0.41	11.15 ±0.29	4.44 ±0.74	4.70 ±0.90	2.40 ±0.41	2.34 ±0.26	4.33 ±0.43	2.97 ±0.53

* significantly different than control $p \leq 0.05$
WBC represents white-blood cell count.
Hgb represents hemoglobin levels.

respectively. Declines similar to those seen with the 4.0 mg/kg dose of 5-aza were observed after 3 and 7 weeks of treatment with 0.3 mg/kg 5-azaCdR (40% and 73%, respectively). Although much less severely than the reduction in the 0.3 mg/kg 5-azaCdR group, males treated for 7 weeks with 0.1 mg/kg 5-azaCdR also exhibited a significant reduction (27%) in testis weight.

Testicular histology was also evaluated and seminiferous tubules were scored for abnormalities. Similar to the study presented in Chapter II, a tubule was considered abnormal if it contained multinucleate/giant cells, degenerating germ cells, vacuoles, germ cell sloughing or had missing germ cell populations. In the saline and low dose groups for both drugs, all stages of the seminiferous epithelium were present and cellular associations were normal (Fig. A1.3A-B). However, there was a significantly higher incidence of tubule abnormalities, including giant cells and vacuoles in the low dose groups for both drugs (Fig. A1.2B) after 7 weeks of treatment. A marked and significant increase in the abnormal tubules was also observed in males treated for 3 weeks with 2.5 and 4.0 mg/kg 5-aza or 0.3 mg/kg 5-azaCdR (Fig. A1.2B). At these doses, degenerating and multinucleate cells were frequent and typically one or more germ cell populations were missing (Fig. A1.3C,E-F); these abnormalities were more extensive at the highest doses. Pachytene spermatocytes, as well as round spermatids, were often absent, particularly at the highest doses of each drug. After 3 weeks of treatment, the tubules of males treated with 4.0 mg/kg 5-aza and 0.3 mg/kg 5-azaCdR appeared to contain only elongated spermatids, Sertoli cells and a few spermatogonia. Effects on testicular histology were similar amongst males treated for 7 weeks with 2.5 or 4.0 mg/kg 5-aza, or 0.3 mg/kg 5-azaCdR; the seminiferous epithelium of these males was extremely abnormal in all tubules (Fig. A1.2B), with exceptionally few germ cells remaining (Fig. A1.3D).

A1.3.3 Progeny Outcome

The effects of paternal treatment on progeny outcome were examined after mating

males treated for 7 weeks with untreated females. Mating behaviour was similar for males in all treatment groups; neither drug appeared to affect the number of sperm-positive females per male (Table A1.2). Nonetheless, pregnancies were observed only in females mated to males in the control and low dose groups for both drugs. Pregnancy rates, shown on a per-male basis in Table A1.2, of the two low dose groups were significantly reduced compared to controls, but were similar to each other. A number of criteria related to fertility and progeny outcome were evaluated including: gross malformations; number of corpora lutea, implantations, resorptions and live fetuses; litter size; sex ratios; resorption weights; fetal and placental weights (including those <75% or>125% of mean weight); and preimplantation and postimplantation loss. Only one variable, preimplantation loss, was significantly altered after matings with treated males. At 0.1 mg/kg 5-azaCdR, preimplantation loss (29%) was significantly higher than in the control group (11%); there was no change in preimplantation loss in the 1.0 mg/kg 5-aza group. This increase in preimplantation loss in the 0.1 mg/kg 5-azaCdR group was similar to that described in Chapter II (Kelly *et al.*, 2003).

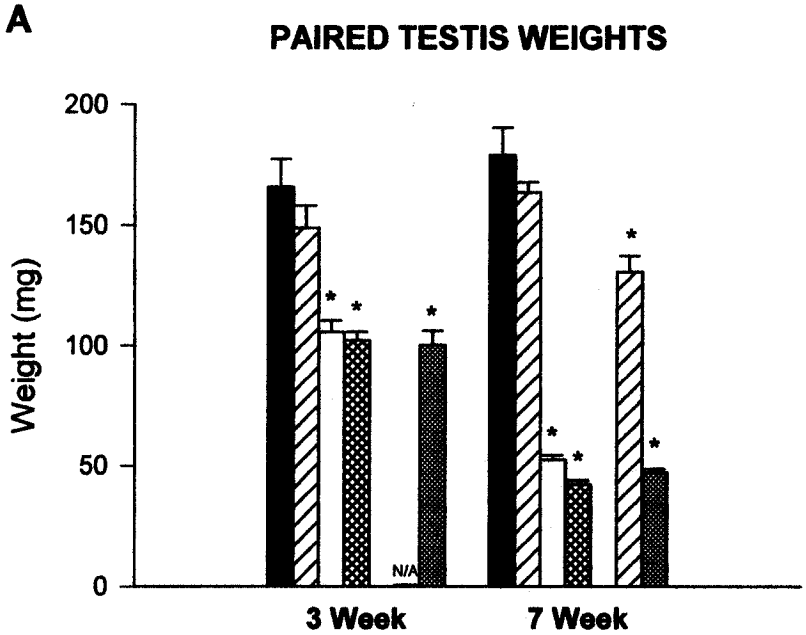
Table A1.2. Fertility measures for males exposed to 5-aza or 5-azaCdR for 7 weeks.

Treatment	# Females Mated	Mating Rate	Pregnancy Rate	Preimplantation Loss
Control	16	44%	71%	11%
1.0 mg/kg 5-aza	18	67%	42%*	10%
2.5 mg/kg 5-aza	18	44%	0%*	n/a
4.0 mg/kg 5-aza	17	47%	0%*	n/a
0.1 mg/kg 5-azaCdR	16	50%	37%*	29%*
0.3 mg/kg 5-azaCdR	16	56%	0%*	n/a

* significantly different than control $p \leq 0.05$
n/a, at this dose there were no full-term pregnancies

Figure A1.2. Effects of treatment on **(A)** the weights of testes and **(B)** seminiferous tubule histology, after 3 and 7 weeks of treatment with saline (*black*), 1.0 mg/kg 5-aza (*diagonal stripes*), 2.5 mg/kg 5-aza (*grey*), 4.0 mg/kg 5-aza (*cross-hatching*), 0.1 mg/kg 5-azaCdR (*diagonal stripes on grey*), and 0.3 mg/kg 5-azaCdR (*small hatching on grey*). Bars represent means ± SEM. N/A indicates the 0.1 mg/kg 5-azaCdR dose not followed up during the 3-week experiment. * $p<0.05$ from control.

A


PAIRED TESTIS WEIGHTS
Weight (mg)
200
150
100
50
0
N/A
3 Week
7 Week

B

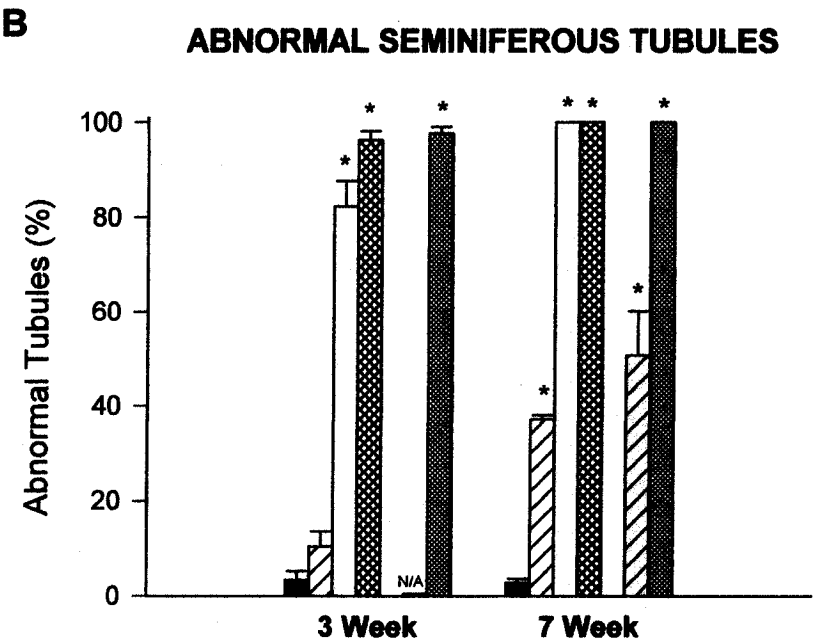
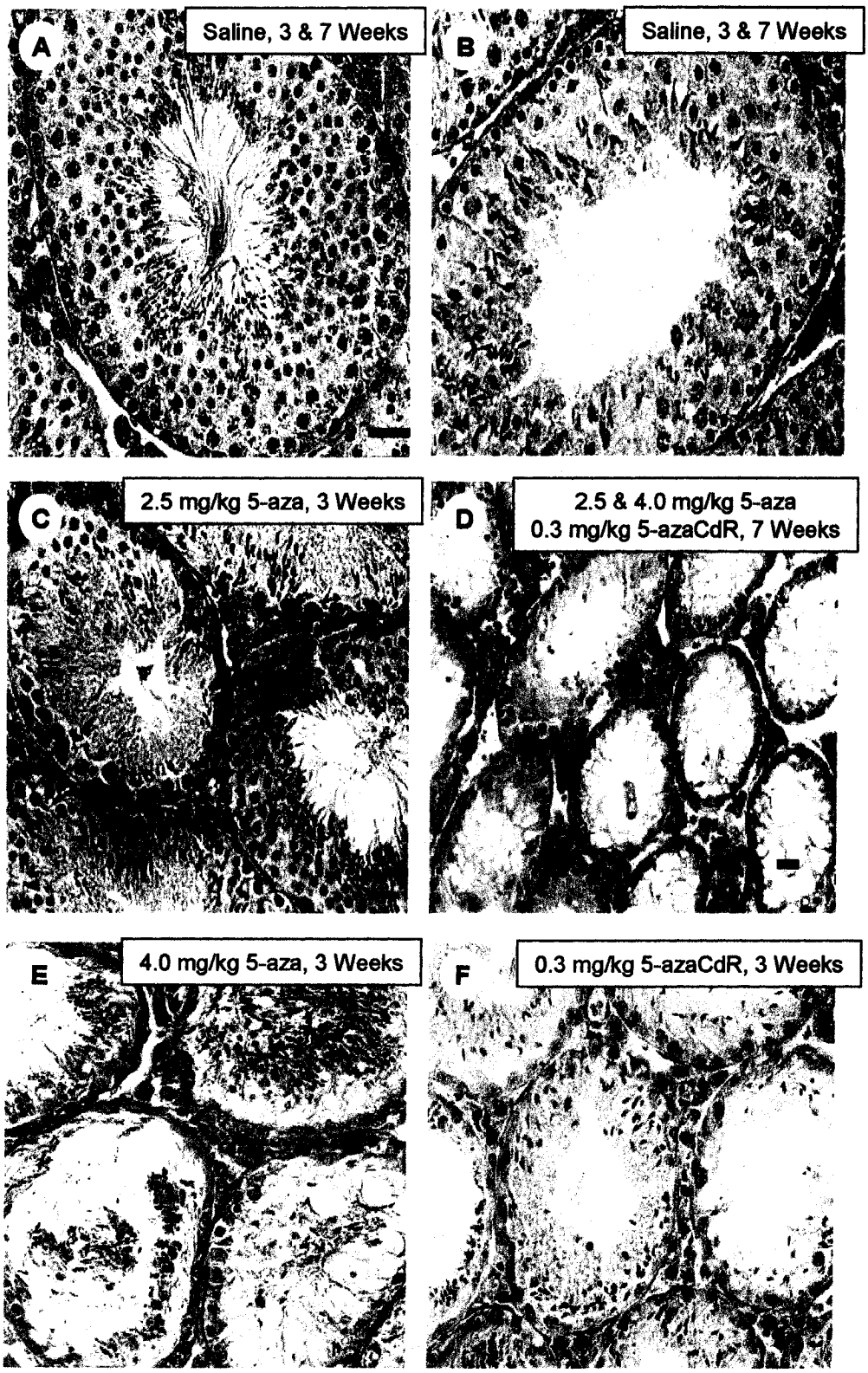

ABNORMAL SEMINIFEROUS TUBULES
Abnormal Tubules (%)
100
80
60
40
20
0
N/A
3 Week
7 Week

Figure A1.3. Examples of testicular histology after treatment for 3 and 7 weeks (first experiment). **(A)** saline, after 3 weeks (similar and normal after 3 and 7 weeks; similar to 3 weeks treatment with 1.0 mg/kg 5-aza or 0.1 mg/kg 5-azaCdR), Stage VII; **(B)** saline, after 7 weeks (similar and normal after 3 and 7 weeks; similar to 3 weeks treatment with 1.0 mg/kg 5-aza or 0.1 mg/kg 5-azaCdR), Stage XII; **(C)** 2.5 mg/kg 5-aza, after 3 weeks; **(D)** 2.5 mg/kg, after 7 weeks (similar to 4.0 mg/kg 5-aza and 0.3 mg/kg 5-azaCdR) **(E)** 4.0 mg/kg 5-aza, after 3 weeks; and **(F)** 0.3 mg/kg 5-azaCdR, after 3 weeks. Bar in **(A)**, 50μm, for A-C, E, F; bar in **(D)**, 50 μm for D only.


A
Saline, 3 & 7 Weeks
B
Saline, 3 & 7 Weeks
C
2.5 mg/kg 5-aza, 3 Weeks
D
2.5 & 4.0 mg/kg 5-aza
0.3 mg/kg 5-azaCdR, 7 Weeks
E
4.0 mg/kg 5-aza, 3 Weeks
F
0.3 mg/kg 5-azaCdR, 3 Weeks

A1.4 DISCUSSION

A1.4.1 Effects of 5-Aza on the Male Mouse Reproductive System

Treatment of male mice for 3 or 7 weeks with 5-aza doses equivalent and similar to those used in male rat treatments (Doerksen & Trasler, 1996; Doerksen *et al.*, 2000) results in significant dose- and time- dependent decreases in testis weight and fertility as well as increases in histological abnormalities, with no apparent effect on androgen status. Altered DNA methylation may explain such adverse effects because 5-aza is an established hypomethylating agent. The effects on testis weight at the higher doses of 5-aza (2.5 & 4.0 mg/kg) can be explained by germ cell loss as these cells comprise 50% to 70% of testis weight. Pachytene spermatocytes, which were absent from tubules exposed for 3 weeks, would have been mitotically dividing spermatogonia at the onset of treatment, whereas early spermatids would have been actively engaged in meiosis when treatment began. Sperm collected after 7 weeks of treatment had been exposed to 5-aza throughout the course of spermatogenesis. It is not surprising that any deleterious effects observed were in replicating germ cells, as 5-aza must be incorporated into its nucleic acid substrate to exert its biological effect. Studies in the male rat reproductive system have confirmed that 5-aza targets rapidly dividing cells (Doerksen *et al.*, 2000), and exposure of mitotically dividing spermatogonia to 5-aza and 5-azaCdR inhibits their differentiation to spermatocytes (Raman & Narayan, 1995) in neonatal mice.

After 7 weeks of treatment, mature spermatozoa had been exposed to 5-aza or 5-azaCdR throughout the entire course of spermatogenesis. Therefore, sperm present in the ejaculate, during matings with untreated females, would have been exposed initially as spermatogonia and then continually throughout spermatogenesis. At high doses of 5-aza the percent of sperm-positive females that were pregnant fell dramatically to zero. Sperm exposed to these high doses may not be able to fertilise oocytes or fertilisation may occur, but there may be an exceptionally high rate of early pregnancy loss. However,

pregnancies were observed, albeit at significantly reduced rates, in the low dose 5-aza group. It is unlikely that altered testosterone levels were responsible for the changes observed in spermatogenesis and fertility, because seminal vesicle weights were unaltered after 3- or 7-weeks treatment with 5-aza. Furthermore, treated mice exhibited normal mating behaviour, as evidenced by the similar number of plug-positive females in the treated and control groups. Of note, however, are the poor mating frequencies observed, even within the control group, between male and female C57BL/6 mice (Table A1.2). Furthermore, matings with C57BL/6 females produced only smaller litters (~5 pups), lending a degree of inaccuracy to statistical analyses. For these reasons, outbred CD1 females, which tend to have large litters (~10 pups) and mate at high frequencies with C57BL/6 males (personal observation), were used in all studies hereafter.

A1.4.2 Comparison of Rat and Mouse Models

Our earlier studies of paternal 5-aza administration were conducted in the rat, but due to the limited genetics information available and the size of the rat (which prohibited use of the more expensive DNA-specific 5-azaCdR), we wished to continue our studies in the male mouse. Here we demonstrate that effects similar to those of 5-aza on the male rat reproductive system can be obtained, but only at doses lower than those used in the rat. Treatment of male mice, for 3 or 7 weeks, with 5-aza doses similar to those used in the rat (Doerksen & Trasler, 1996; Doerksen *et al.*, 2000) results in significant effects on testis weight, the seminiferous epithelium and fertility. In contrast, the rat exhibits no changes in testis weight, histology or fertility after 4 or 6 weeks of treatment. Only after 11 weeks of 5-aza administration (exposing germ cells throughout their development) were effects on testis weight, histology and fertility observed (Doerksen & Trasler, 1996; Doerksen *et al.*, 2000). These data suggest that the seminiferous epithelium of mice may be more vulnerable to the effects of 5-aza than that of the rat. Indeed, at the lowest dose of 5-aza, an increase in histological abnormalities occurred after 7, but not 3, weeks of

treatment; this histology was similar to that observed in the rat after extended treatment with higher doses of 5-aza.

Despite the more severe effects of 5-aza on the mouse male reproductive system, mice, unlike rats, evidently do not exhibit significant changes in final body weight or seminal vesicle weights when treated. As well, mice only exhibited significant changes in white blood cell counts and hemoglobin levels at doses of 5-aza high enough to eliminate almost all germ cells. In contrast, hematological indices were altered in the rat at doses that significantly affected, but not severely so, testis weight and histology which suggests that the effects of 5-aza are more specific to the male reproductive system in the mouse than in the rat. The mouse is, therefore, an acceptable model for continued studies of paternal administration of 5-aza (and 5-azaCdR) because similar reproductive effects can be achieved with fewer drug-induced systemic alterations.

A1.4.3 Comparison of 5-Aza and 5-AzaCdR

Here we demonstrate that the deleterious effects of the hypomethylating agents 5-aza and 5-azaCdR on male mouse germ cell development are comparable at doses of similar potencies. Because 5-azaCdR is limited to DNA incorporation, it was hypothesised that at equally potent doses (~ 10 times less), 5-azaCdR would cause less severe effects than 5-aza. However, as observed here, at equally potent doses, the 5-azaCdR analogue elicits similar, to more, pronounced effects on gross male germ cell development, reproductive organ weights, and embryonic development. Pregnancy rates from matings with males treated with either 1.0 mg/kg 5-aza or 0.1 mg/kg 5-azaCdR were similar; however, only treatment with the 5-azaCdR analogue caused elevated preimplantation loss (3-fold increase). Nevertheless, it is believed that the 5-azaCdR analogue is the optimal drug choice for hypomethylation studies, as it specifically targets DNA and, therefore, any toxicity due to RNA incorporation can effectively be eliminated.

Thus, for our purposes, the 0.1 mg/kg dose of 5-azaCdR seemed appropriate for

future studies within the male mouse. At this dose, although there were significant changes to the seminiferous epithelium, males could produce mature sperm and were fertile; a necessity if the effects of paternal treatment on progeny development were to be explored in future experiments. Moreover, at this dose the level of preimplantation loss is similar to that seen in the rat at higher doses. Together, the lack of treatment effects on body weight, organ weights (other than the testis) and the hematological system indicate that, at the doses chosen here, effects of cytosine analogues are highly selective for the mouse testis.

A1.5 ACKNOWLEDGEMENTS

I would like to thank Jacquetta Trasler and Tonia Doerksen, as well as Eric Simard for his superb technical assistance.

APPENDIX II

ACUTE 5-AZA-2'-DEOXYCYTIDINE EXPOSURE INDUCES APOPTOSIS IN THE GERM CELLS OF MALE MICE

Tamara LJ Kelly

A2.1 INTRODUCTION

Chronic treatment of *Dnmt1*$^{+/+}$ male mice with low dose 5-azaCdR results in significant testis weight reduction and a corresponding increase in histological abnormalities (Kelly *et al.*, 2003). Males heterozygous for a null allele of *Dnmt1* (*Dnmt1*$^{c/+}$) appear to be more resistant to these drug effects. For example, although *Dnmt1*$^{c/+}$ males do experience a significant decrease in testis weight after 7 weeks of treatment with 0.1 mg/kg 5-azaCdR, this reduction is roughly half that observed in drug-exposed *Dnmt1*$^{+/+}$ males. Moreover, there is no accompanying rise in abnormalities of the seminiferous epithelium in *Dnmt1*$^{c/+}$ mice. Our studies to this point have examined the effects of extended exposure (7 weeks) to 5-azaCdR but have not pinpointed the testicular response at times soon after initiation of 5-azaCdR treatment. Furthermore, if the effects of 5-azaCdR are, as we believe, due to the hypomethylating action of 5-azaCdR and are epigenetic in nature, then they should, by definition, be reversible. Therefore, male *Dnmt1*$^{+/+}$ and *Dnmt1*$^{c/+}$ mice were treated acutely (3 days) with 5-azaCdR and testicular apoptosis was quantified the day after treatment cessation. In addition, some males were examined after being allowed to recover for up to four weeks after treatment, in order to determine if 5-azaCdR-induced effects on the seminiferous epithelium are reversible.

A2.2 MATERIALS AND METHODS

A2.2.1 Animals and Treatment

Male *Dnmt1*$^{+/+}$ and *Dnmt1*$^{c/+}$ mice were randomly assigned to one of two groups (n = 20-25/genotype/group) and treated daily, for three days with either saline or 0.3 mg/kg 5-azaCdR, via intraperitoneal injection. Four, 7, 14, 21 and 28 days after treatment initiation, four to five mice from each group were killed (Fig. A2.1).

After sacrifice, hemoglobin determination and white blood cell counts were

measured using a Coulter counter (Coulter Electronics, Hialeah, FA). Testes, epididymides, seminal vesicles and spleen were removed, weighed and snap frozen. Caudal sperm were isolated (Alcivar *et al.*, 1989) from males sacrificed on day 28 and stored at -80°C.

A2.2.2 Histology

The right testis was placed into Bouin's fixative (BDH Inc., Toronto, Canada) for 12 to 24 hours, then dehydrated, embedded in paraffin, mounted on glass slides, deparaffinised with xylene and stained with hematoxylin and eosin. Histology was examined using a Zeiss Axiphot photomicroscope and pictures taken with a SPOT RT Slider digital camera (Diagnostic Instruments Inc., Sterling Heights, MI).

A2.2.3 Sperm Counts

Hemocytometric counts of spermatozoa were done as described by Robb *et al.* (1978).

A2.2.4 Detection and Quantification of Apoptotic Cells

For analysis of apoptotic cells, the right testis was prepared as described but then mounted on glass Silane Prep slides. Four sections were analysed for each individual: two sets of two slides sampled from regions located 200 μm apart within the testis. The terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL) protocol was used for *in situ* detection of apoptotic DNA strand breaks according to manufacturer's instructions (Apoptag Kit, Intergen Co., Purchase, NY). Briefly, after deparaffinising with xylene and rehydration, slides were incubated with proteinase K (20 μg/ml) for 10 minutes at room temperature, washed with distilled water and then, in order to quench endogenous peroxidase activity, incubated with 3% H_2O_2 in PBS for five minutes. After washing, slides were incubated with terminal deoxynucleotidyl transferase (TdT) for 75 minutes (37°C) and then treated with anti-digoxegenin for 30 minutes at

room temperature in a humidified chamber. Slides were then incubated with diaminobenzidene (DAB) for 6 minutes, washed, counter-stained with hematoxylin (6 minutes), dehydrated in 100% n-butanol, cleared in xylene and mounted with Permount (Fisher Scientific Company, Fair Lawn, NJ). Tubules were staged according to Oakberg (1956), and TUNEL-positive cells were quantified and reported as the number of TUNEL-positive cells per 100 tubules. Each slide was counted three times and was blinded to the researcher performing the counts.

A2.2.5 Statistical Analyses

Statistical analyses were done using a two-way ANOVA, with a post-hoc Tukey test (SigmaStat 2.03, SPSS, Chicago, IL).

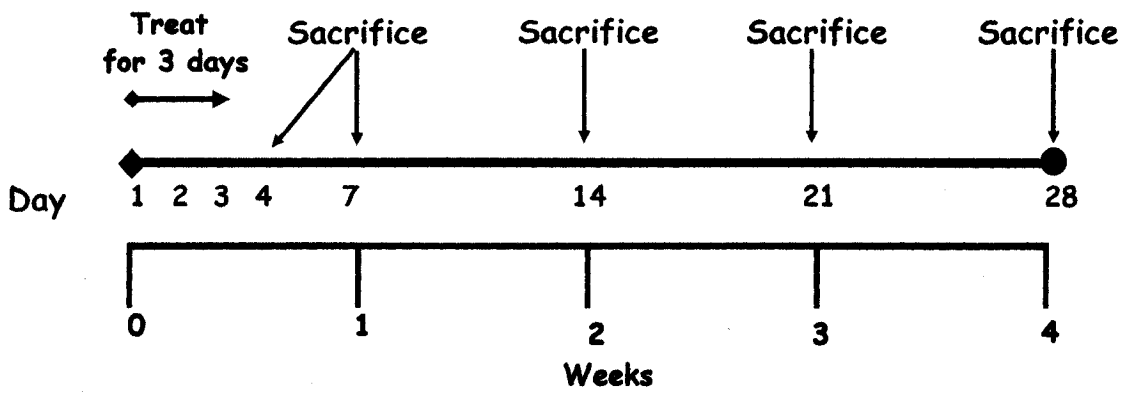
A2.3 RESULTS AND DISCUSSION

A2.3.1 Systemic and Male Reproductive Organ Effects

All animals appeared healthy throughout the experiment, but because body weights of *Dnmt1*$^{+/+}$ and *Dnmt1*$^{c/+}$ mice were significantly different (Table A2.1), all organ weights are reported per gram body weight. No changes in white blood cell counts, hemoglobin levels, or epididymal weights were observed (data not shown; Table A2.1).

A significant decrease in the relative testis weight of 5-azaCdR-treated *Dnmt1*$^{+/+}$ males was observed one week earlier in the treatment regime (at 21 days) than in *Dnmt1*$^{c/+}$ males (28 days) (Table A2.1). At 21 and 28 days after treatment initiation, relative testis weights of *Dnmt1*$^{+/+}$ males were 69% and 75% of control values, respectively. However, at no point did the testis weights of the two 5-azaCdR-treated groups substantially differ. As with earlier experiments, changes in testosterone levels are likely not responsible for decreases in testis weight; at no point were significant changes in seminal vesicle weights observed after 5-azaCdR treatment.

Figure A2.1: Treatment protocol for acute treatment (3 days) of *Dnmt1*$^{+/+}$ and *Dnmt1*$^{c/+}$ males with 0.3 mg/kg 5-azaCdR.



Dnmt1$^{+/+}$	saline
Dnmt1$^{+/+}$	0.3 mg/kg 5azaCdR
Dnmt1$^{c/+}$	saline
Dnmt1$^{c/+}$	0.3 mg/kg 5azaCdR

n = 4-5/group/day

Table A2.1: Mean body[a] and organ[bc] weights of *Dnmt1*$^{+/+}$ and *Dnmt1*$^{c/+}$ males treated acutely with saline or 5-azaCdR.

	DAY 4				DAY 7				DAY 14				DAY 21				DAY 28			
	Dnmt1$^{+/+}$		*Dnmt1*$^{c/+}$		*Dnmt1*$^{+/+}$		*Dnmt1*$^{c/+}$		*Dnmt1*$^{+/+}$		*Dnmt1*$^{c/+}$		*Dnmt1*$^{+/+}$		*Dnmt1*$^{c/+}$		*Dnmt1*$^{+/+}$		*Dnmt1*$^{c/+}$	
	Ctrl	0.3	Ctrl	0.3	Ctrl	0.3	Ctrl	0.3	Ctrl	0.3	Ctrl	0.3	Ctrl	0.3	Ctrl	0.3	Ctrl	0.3	Ctrl	0.3
Body weight	24.11 ± 0.45	23.3 ± 0.61	31.00[d] ± 1.98	26.70 ± 1.87	24.84 ± 0.30	24.64 ± 1.08	28.00 ± 1.46	27.56 ± 1.49	25.71 ± 0.74	24.60 ± 0.60	31.39[d] ± 2.04	29.35[f] ± 1.23	24.89 ± 0.40	27.15 ± 0.89	30.75[d] ± 1.27	25.95[e] ± 0.99	26.58 ± 0.41	26.17 ± 0.75	29.05 ± 2.16	31.39[f] ± 1.57
Testis weight	6.57 ± 0.28	6.48 ± 0.27	5.76 ± 0.36	6.17 ± 0.27	6.87 ± 0.29	6.56 ± 0.33	6.27 ± 0.47	6.19 ± 0.38	6.17 ± 0.20	5.72 ± 0.78	5.51 ± 0.20	5.35 ± 0.21	6.99 ± 0.22	4.87[d] ± 0.35	5.38[d] ± 0.15	5.14 ± 0.17	6.57 ± 0.22	4.95[d] ± 0.32	5.69 ± 0.47	4.51[e] ± 0.16
Epididymal Weight	1.06 ± 0.09	0.93 ± 0.06	1.03 ± 0.11	1.08 ± 0.04	1.02 ± 0.05	1.20 ± 0.26	1.10 ± 0.15	1.01 ± 0.05	1.10 ± 0.15	1.26 ± 0.14	0.94 ± 0.02	0.95 ± 0.02	1.09 ± 0.02	0.96 ± 0.06	0.96 ± 0.01	1.05 ± 0.02	1.10 ± 0.03	1.09 ± 0.11	0.94 ± 0.10	1.03 ± 0.09

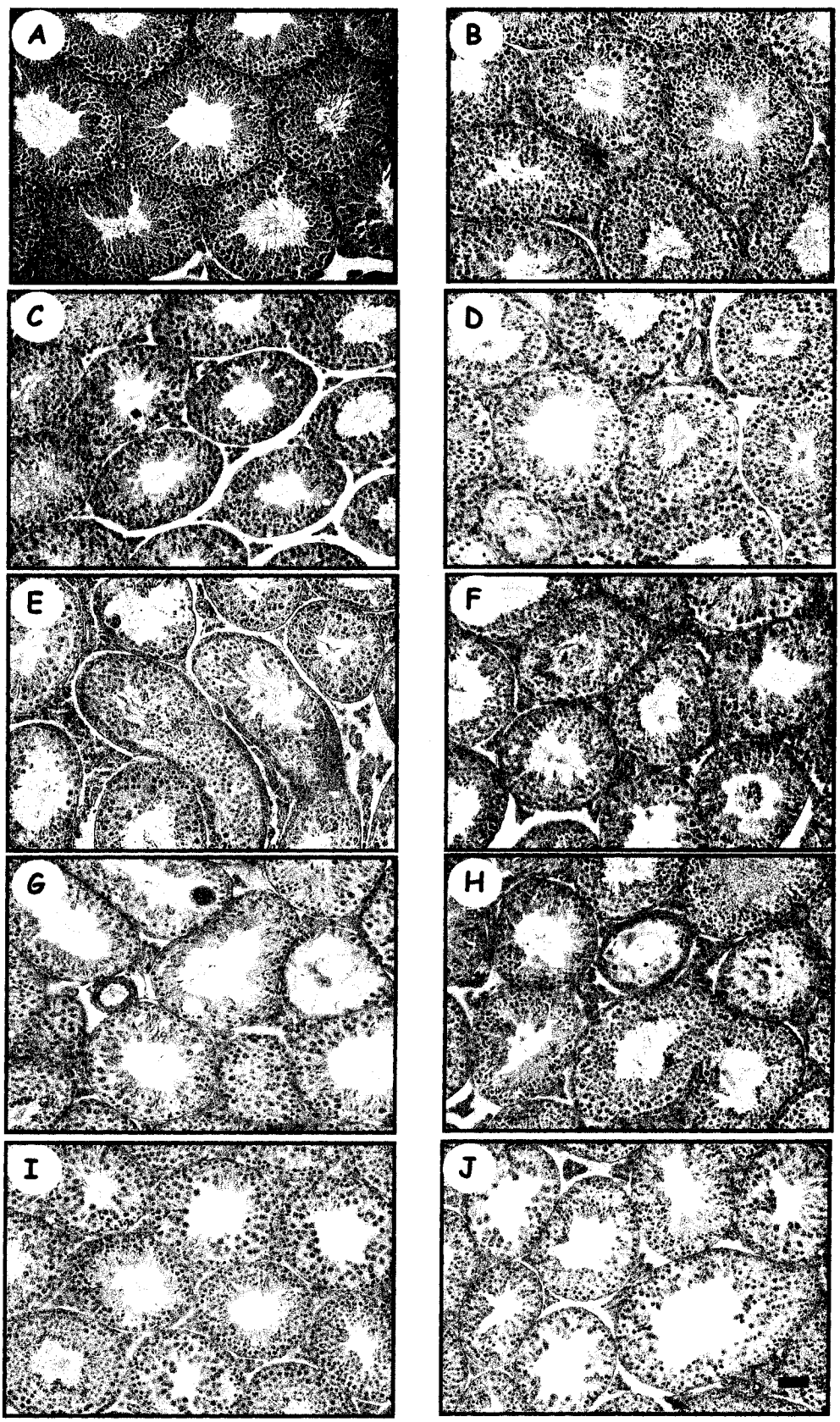
[a]Weight in g (± SEM)
[b]Relative weight of paired testis (mg/g body weight) (± SEM)
[c]Average weight of paired epididymides (mg/g body weight) (± SEM)
[d]$p<0.05$, vs. saline-treated *Dnmt1*$^{+/+}$
[e]$p<0.05$, vs. saline-treated *Dnmt1*$^{c/+}$
[f]$p<0.05$, vs. 5-azaCdR-treated *Dnmt1*$^{+/+}$

A2.3.2 Testicular Histology

As anticipated, based on the changes in relative testis weight, the seminiferous epithelium of *Dnmt1*$^{c/+}$ mice appeared to respond more slowly than that of *Dnmt1*$^{+/+}$ mice to 5-azaCdR insult. Four days after treatment initiation, there was an observable increase in vacuolisation of the seminiferous epithelium of 5-azaCdR-treated *Dnmt1*$^{+/+}$ males (Fig. A2.2C). As well, some sloughing was evident as were giant multinucleate cells. By day seven, vacuolisation and sloughing of immature germ cells into the lumen were more pervasive, with most vacuolisation occurring in premeiotic cells (Fig. A2.2D). The most severe histological abnormalities appeared 14 and 21 days after treatment initiation (Fig. A2.2E & G). At these time points, giant multinucleate cells were common, the majority of tubules had one or more germ cell populations missing (particularly pachytene and round spermatids), and in some cases there was complete atrophy of tubules. The significant drop in testis weight at day 21, therefore, is presumably due to a considerable loss of germ cells. By day 28, however, there was an improvement in testicular histology (Fig. A2.2I). Although some vacuolisation remained, sloughing was minimal, as was the presence of multinucleate cells. A few tubules were also missing round and elongating spermatids, likely because earlier germ cell populations had been lost.

Whereas 5-azaCdR-treated *Dnmt1*$^{+/+}$ males exhibited abnormalities within four days of initial 5-azaCdR exposure, the seminiferous epithelium of *Dnmt1*$^{c/+}$ males, with the exception of a small rise in vacuolisation, was relatively normal at four and seven days and did not display significant changes until day 14 (not shown, Fig. A2.2B & F). At this point, there was an increase in sloughing, multinucleate cells, and tubules lacking one or more germ cell populations. At 21 days, defects were similar to those observed in 5-azaCdR-treated *Dnmt1*$^{+/+}$ males, although less vacuolisation, fewer multinucleate cells and less tubule atrophy were apparent (Fig. A2.2H). Increased vacuolisation at day 28 (Fig. A2.2J) corresponded with a 20% decrease in testis weight (Table A2.1).

Figure A2.2: Examples of testicular histology from *Dnmt1*$^{+/+}$ and *Dnmt1*$^{c/+}$ male mice treated for 3 days with saline or 0.3 mg/kg 5-azaCdR. **(A)** saline-treated *Dnmt1*$^{+/+}$ males, at 14 days **(B)** saline-treated *Dnmt1*$^{c/+}$ males, at 14 days, similar to *Dnmt1*$^{c/+}$ histology 4 and 7 days after treatment initiation. **(C)** and **(D)** are examples of some defects observed in testis of *Dnmt1*$^{+/+}$ males 4 and 7 days, respectively after 5-azaCdR treatment. Histology of 5-azaCdR-treated *Dnmt1*$^{+/+}$ males **(E, G, I)** and *Dnmt1*$^{c/+}$ males **(F, H, J)** at 14, 21 and 28 days respectively. The bar in **J** represents 100μm for **(A-J)**.


A
B
C
D
E
F
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H
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J

Consistent with observations of pachytene germ cell loss at 14 days, testicular sperm production ($\times 10^6$) at 28 days was significantly reduced in 5-azaCdR-treated *Dnmt1*$^{+/+}$ relative to controls ($p<0.05$) (*Dnmt1*$^{+/+}$: saline, 27.08 ± 3.86; 5-azaCdR, 14.16 ± 3.11. *Dnmt1*$^{c/+}$: saline, 25.15 ± 5.64; 5-azaCdR, 21.07 ± 2.59). Although there was some reduction in *Dnmt1*$^{c/+}$ sperm production after 5-azaCdR treatment, it was not statistically significant (p=0.482).

Improvements in *Dnmt1*$^{+/+}$ testicular histology at 28 days suggest that the effects of acute 5-azaCdR exposure are reversible. This reversibility is consistent with an epigenetic rather than a genetic mode of action for 5-azaCdR. However, further studies of histology after a longer recovery period are necessary in order to unequivocally conclude that 5-azaCdR's effects are reversible. Although our results indicate a recovery in germ cell populations, we have not addressed whether sperm exposed acutely (during premeiotic phase) are functional, or whether recovery is possible from chronic 5-azaCdR treatment effects. Studies of this nature are relevant to understanding the effects of 5-azaCdR clinical treatments on male fertility.

A2.3.3 Apoptotic Response

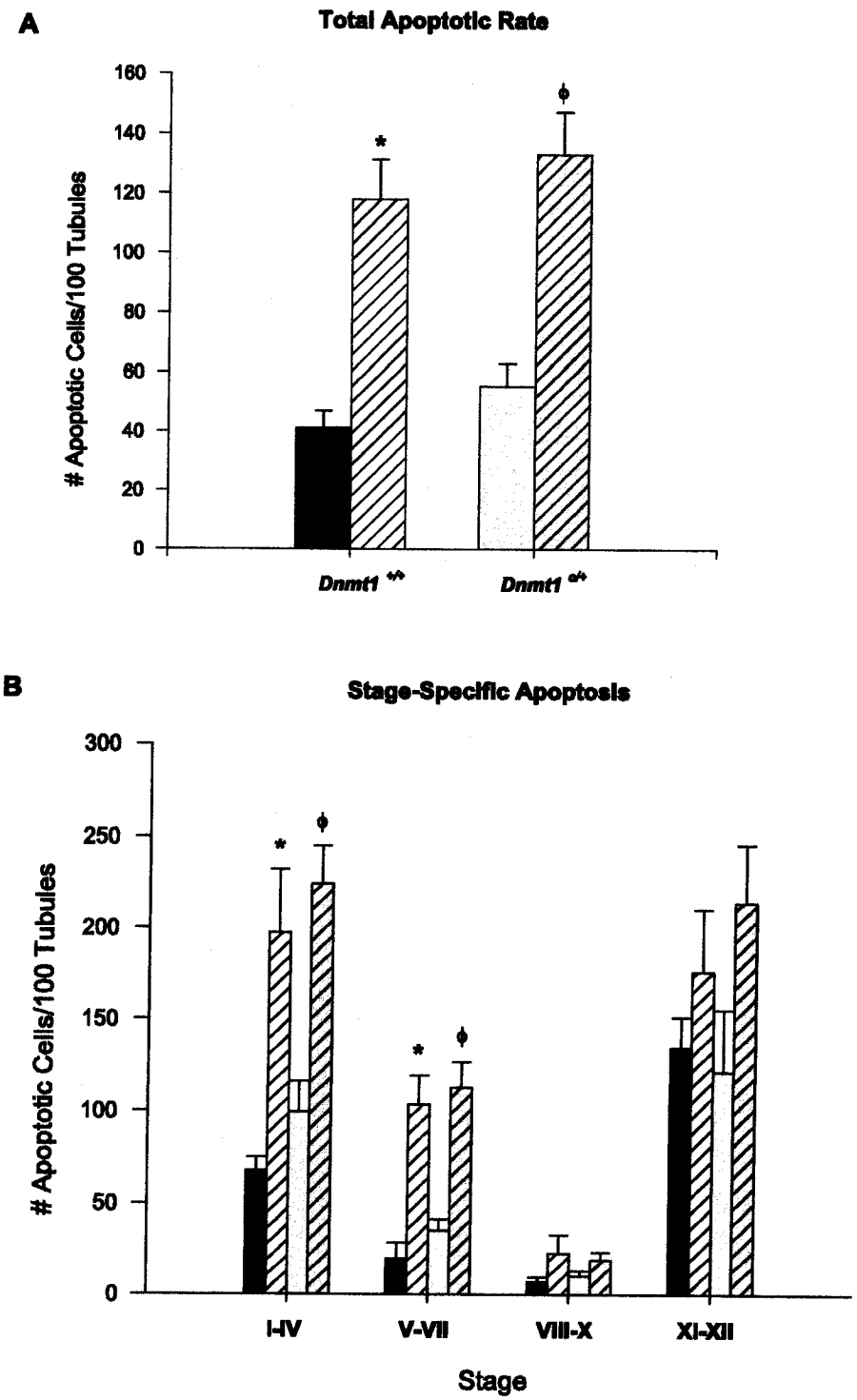
5-Aza and 5-azaCdR are both known to induce apoptosis (reviewed in Haaf, 1995), perhaps through induction of apoptosis genes via promoter demethylation. Alternatively, covalent drug-DNMT adduct formation may cause destabilisation of germ cell chromatin and induce DNA strand breaks, particularly in the germ cells of *Dnmt1*$^{+/+}$ males, resulting in activation of the apoptotic response. To gauge the apoptotic response of male germ cells to 5-azaCdR exposure, we used the TUNEL method, which has been used previously in our lab (Doerksen *et al.*, 2000), to detect apoptotic cell death. It is interesting to note that despite differences in timing of testicular weight reduction and histological abnormalities appearance, both *Dnmt1*$^{+/+}$ and *Dnmt1*$^{c/+}$genotypes displayed

similar increases (2.5- to 3-fold) in total germ cell apoptosis at four days (Fig. A2.3A). The extent of apoptosis in the testes of treated *Dnmt1*$^{c/+}$ males was surprising, since histology of these testes looked normal at this time point. It is possible that an induction of apoptosis occurs in germ cells of treated *Dnmt1*$^{c/+}$ males, which eliminates abnormal germ cells and coincides with an increase in spermatogonial proliferation in an attempt to maintain normal germ cell populations. For both genotypes, the vast majority of apoptotic cells (≥98%) were the dividing germ cells – spermatogonia and spermatocytes; this is consistent with 5-azaCdR targetting rapidly dividing cells. Unlike observations made after chronic treatment of rats with 5-aza (Doerksen *et al.*, 2000), no significant increase in spermatid apoptosis was observed in our present study of mice. However, our study was based on acute drug treatment and therefore might not be easily compared to results of a chronic regimen.

In Figure A2.3B, the incidence of apoptotic cells has been categorised by stage. The early (I-IV) and late (XI-XII) stages of mouse spermatogenesis show the highest incidence of spontaneously occurring apoptosis, similar to the rat (Blanco-Rodríguez & Martinez-García, 1996; Cai *et al.*, 1997; Doerksen *et al.*, 2000). However, 5-azaCdR-induced apoptosis was significantly increased only in stages I-IV. Surprisingly, apoptosis in stages V-VII, which normally do not have high levels of apoptosis, increased five-fold ($p<0.05$) in treated *Dnmt1*$^{+/+}$ males and three-fold in treated *Dnmt1*$^{c/+}$ males; this suggests that these stages may be more vulnerable to cytosine analogue incorporation in mice than they are in the rat (Doerksen *et al.*, 2000).

We have demonstrated that with respect to testicular histology, the response kinetics of *Dnmt1*$^{+/+}$ and *Dnmt1*$^{c/+}$ mice to acute 5-azaCdR exposure may differ as a function of genotype. However, levels of apoptotic response were similar in the two genotypes immediately following acute 5-azCdR treatment; both groups experienced

Figure A2.3: Quantification of apoptotic germ cells in the testes of *Dnmt1*$^{+/+}$ and *Dnmt1*$^{c/+}$ males at four days, after acute treatment with 5-azaCdR. **(A)** Total apoptotic rate, **(B)** incidence of apoptotic germ cells by stage, as calculated per 100 tubules counted in *Dnmt1*$^{+/+}$ males treated with saline (*black*) or 0.3 mg/kg 5-azaCdR (*diagonal stripes*) and *Dnmt1*$^{c/+}$ males with saline (*grey*) or 0.3 mg/kg 5-azaCdR (*diagonal stripes on grey*). Bars represent means ± SEM. *$p<0.05$ vs. *Dnmt1*$^{+/+}$ control; †$p<0.05$ vs. *Dnmt1*$^{c/+}$ control.


A
Total Apoptotic Rate
Apoptotic Cells/100 Tubules
160
140
120
100
80
60
40
20
0
*
ϕ
Dnmt1 +/+
Dnmt1 -/-
B
Stage-Specific Apoptosis
Apoptotic Cells/100 Tubules
300
250
200
150
100
50
0
*
ϕ
I-IV
V-VII
VIII-X
XI-XII
Stage

significant increases in apoptosis in stages I-IV and V-VII. This timing indicates that these cells were premeiotic and meiotic when exposed to 5-azaCdR and that alterations in methylation may interfere with normal meiotic progression in the male germ line. However, in order to determine conclusively which cell types are most vulnerable to the immediate effects of 5-azaCdR, a shorter time-course study following administration of one high dose would need to be conducted (*e.g.*, Cai *et al.*, 1997). Additionally, it is probable that the histopathology observed following exposure to 5-azaCdR is a combination of apoptosis and necrosis (Russell *et al.*, 1990).

A2.4 ACKNOWLEDGEMENTS

The author is grateful to Xinying He for excellent technical assistance, Andrea Lawrance for help in tweaking the TUNEL protocol, and Jacquetta Trasler and Bernard Robaire for discussions.

CONNECTING TEXT – CHAPTER II TO III

The results of Chapter II established that administration of the DNA-specific cytosine analogue 5-aza-2'-deoxycytidine has severe effects on male germ cell development in the mouse at doses similar to, or less than, those used clinically. These experiments also demonstrated that haploinsufficiency of DNMT1, the principal mammalian methyltransferase, modulates the effect of 5-azaCdR and is, to a certain degree, protective against the cytotoxic drug effects observed in wild-type (*Dnmt1*$^{+/+}$) males. The combination of 5-azaCdR treatment and DNMT1 haploinsufficiency provides a mouse model with which to potentially lower DNA methylation in the male germ line through the genetic and pharmacological reduction of methyltransferase activity.

Another approach to altering DNA methylation in the male germ cells is to use mice deficient in the pool of methyl groups necessary for methylation of DNA. Methylenetetrahydrofolate reductase (MTHFR) is a crucial enzyme within the folate pathway, which is the major cellular source of S-adenosylmethionine (SAM), the principal methyl donor for a range of transmethylation reactions including methylation of DNA. Deficiency in MTHFR results in lowered levels of methionine, SAM and consequently, DNA methylation. Moreover, MTHFR is postulated to have a role in spermatogenesis; tissue-specific activity of this enzyme is highest in the adult testis of mice (Chen *et al.*, 2001). For these reasons, we were interested in examining the potential spermatogenic effects of MTHFR deficiency within the male mouse.

CHAPTER III

INFERTILITY IN MTHFR-DEFICIENT MALE MICE IS PARTIALLY ALLEVIATED BY LIFETIME DIETARY BETAINE SUPPLEMENTATION

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Biology of Reproduction. (2005) 72: 667-677.

3.1 ABSTRACT

Metabolism of folate is essential for proper cellular function. Within the folate pathway, methylenetetrahydrofolate reductase (MTHFR) reduces 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, a methyl donor for remethylation of homocysteine to methionine, the precursor of S-adenosylmethionine. S-adenosylmethionine is the methyl donor for numerous cellular reactions. In adult male mice, MTHFR levels are highest in the testis; this finding, in conjunction with recent clinical evidence, suggest an important role for MTHFR in spermatogenesis. Indeed, we show here that severe MTHFR deficiency in male mice results in abnormal spermatogenesis and infertility. Maternal oral administration of betaine, an alternative methyl donor, throughout pregnancy and nursing, resulted in improved testicular histology in *Mthfr*$^{-/-}$ offspring at postnatal day 6, but not at 8 months of age. However, when betaine supplementation was maintained post-weaning, testicular histology improved and sperm numbers and fertility increased significantly. We postulate that the adverse effects of MTHFR deficiency on spermatogenesis, may, in part, be mediated by alterations in the transmethylation pathway and suggest that betaine supplementation may provide a means to bypass MTHFR deficiency and its adverse effects on spermatogenesis by maintaining normal methylation levels within male germ cells.

3.2 INTRODUCTION

The folate cycle is essential for normal cell function and is involved in such processes as methionine production, and purine and pyrimidine synthesis. One crucial enzyme within the folate pathway is methylenetetrahydrofolate reductase (MTHFR). MTHFR irreversibly reduces 5, 10-methylenetetrahydrofolate (5, 10-methylene-THF) to 5-methyltetrahydrofolate (5-methyl-THF), the primary methyl donor for remethylation of

homocysteine to methionine (Fig. 3.1). In turn, methionine provides the methyl group necessary for the formation of S-adenosylmethionine (SAM), which is involved in numerous cellular reactions including DNA, RNA and histone methylation. One of the these methylation reactions, that of cytosine residues within DNA, is essential for development; the majority of mice deficient in any one of the DNA methyltransferase (DNMT) enzymes (DNMT1, DNMT3a or DNMT3b) die before or soon after birth (Li *et al.*, 1992; Lei *et al.*, 1996; Okano *et al.*, 1999).

If MTHFR activity is eradicated in mice by gene-targetting, cellular levels of 5-methyl-THF, methionine and SAM fall and hypomethylation of DNA occurs (Chen *et al.*, 2001). Conversely, there are increased levels of homocysteine and S-adenosylhomocysteine (SAH), a potent inhibitor of DNA methylation (Cantoni, 1985). Inhibition of MTHFR activity may also cause shunting of 5, 10-methyleneTHF toward the DNA synthesis pathway.

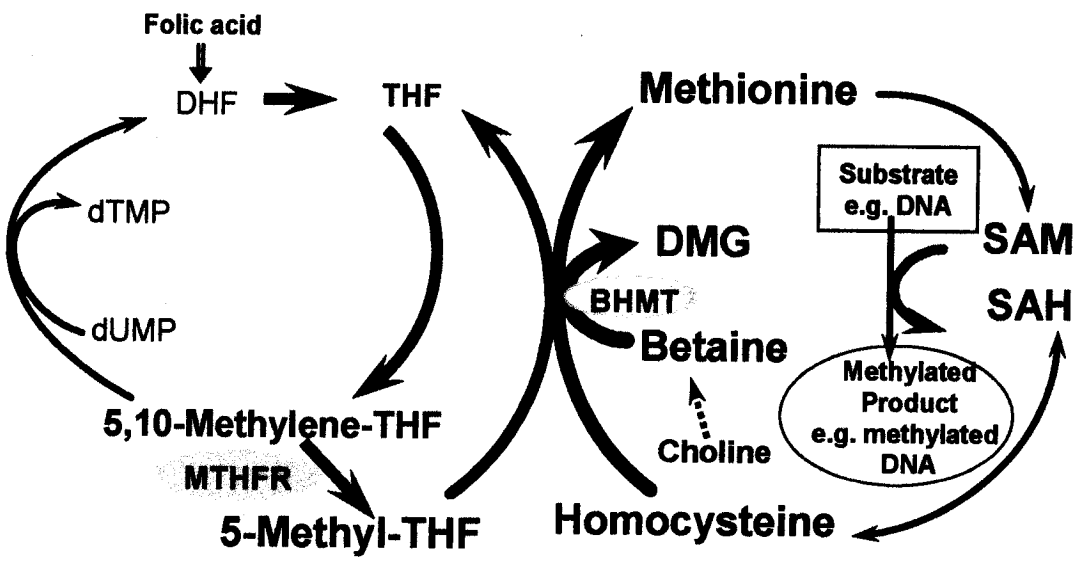
In humans, the common 677C→T polymorphism of *MTHFR* encodes a thermolabile enzyme with decreased activity that results in mild hyperhomocysteinemia when folate status is low (Frosst *et al.*, 1995; Jacques *et al.*, 1996; Christensen *et al.*, 1997); the frequency of the homozygous mutant genotype is about 12% in the general Caucasian population (Frosst *et al.*, 1995; Harmon *et al.*, 1996; Jacques *et al.*, 1996; Gudnason *et al.*, 1998). While individuals homozygous for this mutation are at increased risk of vascular disease they appear to be protected against colon cancer and acute lymphocytic leukemia (Ma *et al.*, 1997; Skibola *et al.*, 1999). Moreover, clinical evidence suggests that nearly 20% of males presenting at infertility clinics are homozygous for the *MTHFR* 677C→T polymorphism (Bezold *et al.*, 2001); this rate was almost double the prevalence found in the control group. The latter findings suggest that mutations affecting MTHFR activity may underlie the pathology of some cases of male infertility. Furthering the hypothesis that MTHFR plays an active and important role in

spermatogenesis is the observation that MTHFR activity is nearly five times higher in the adult testis than in other major organs (Chen *et al.*, 2001). Indeed, as we show here, MTHFR deficiency results in abnormal spermatogenesis and male infertility.

One obstacle to studying male germ cell development in *Mthfr*-deficient mice is their limited survival to adulthood. *Mthfr*$^{-/-}$ mice have severely compromised survival, with less than 20% surviving beyond three weeks of age (Schwahn *et al.*, 2004). However, dietary supplementation of betaine, provided to the mother throughout mating, pregnancy and nursing, produces a substantial increase in *Mthfr*$^{-/-}$ pup survival, from 17% to 74%, and results in substantial improvement of various health and metabolite indices (Schwahn *et al.*, 2004). Betaine, a choline derivative that has been used clinically to treat MTHFR-deficient patients with some success (Ogier de Baulny *et al.*, 1998; Bönig *et al.*, 2003), is a substrate for betaine-homocysteine methyltransferase (BHMT), and serves as an alternate methyl donor for remethylation of homocysteine (Fig. 3.1) in the liver and kidney (Finkelstein, 1990). It is thought that deficiency in MTHFR may result in increased reliance on betaine-dependent remethylation; thus, provision of supplemental betaine may supply greatly needed additional methyl groups.

The purpose of the current study was to determine the role of MTHFR in spermatogenesis by examining the testes of *Mthfr*$^{-/-}$ mice at different ages. Effects of short- and long-term betaine administration on sperm counts and fertility were also assessed. While MTHFR deficiency resulted in abnormalities in general development, severe oligospermia and infertility, long-term, but not short-term, betaine treatment partially restored both spermatogenesis and fertility in *Mthfr*$^{-/-}$ male mice.

Figure 3.1. The folate pathway, depicting the relationship between methylenetetrahydrofolate reductase, methionine, betaine and DNA methylation. DHF, dihydrofolate; THF, tetrahydrofolate; DMG, dimethylglycine; BHMT, betaine-homocysteine methyltransferase; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine.


Folic acid
DHF
THF
Methionine
Substrate
e.g. DNA
dTMP
DMG
SAM
BHMT
SAH
dUMP
Betaine
Methylated
Product
e.g. methylated
DNA
5,10-Methylene-THF
Choline
MTHFR
5-Methyl-THF
Homocysteine

3.3 MATERIALS & METHODS

3.3.1 Animals

Mice were maintained on a 12L: 12D cycle and provided with food and water *ad libitum*. Mice were generated in our own breeding facility on a BALB/c background (Charles River Canada Inc, St. Constant, QC, Canada). Mice were from N6 to N8 generations, as a result of at least six generations of backcrosses to the BALB/c strain. All animal experimentation was conducted in accordance with the principles and procedures outlined by the Canadian Council for Animal Care. *Mthfr* genotypes were determined using a PCR-based assay (Chen *et al.*, 2001).

3.3.2 MTHFR and Spermatogenesis – Experiment 1: Short-Term Betaine Treatment

In the first experiment the effects of MTHFR-deficiency and short-term betaine supplementation on spermatogenesis were examined. *Mthfr*$^{+/-}$ males and females were mated to obtain male offspring of all genotypes (*Mthfr*$^{+/+}$, *Mthfr*$^{+/-}$, *Mthfr*$^{-/-}$); 50% of mating females were assigned a diet of water and regular mouse chow (Purina Laboratory Rodent Diet 5001, Purina Mills) and the remaining females were provided with regular mouse chow and 2% betaine-supplemented water (Sigma), during the time of mating, pregnancy and nursing (short-term betaine). At weaning, all pups were given water and regular mouse chow. The 2% oral betaine concentration resulted in an intake of approximately 3 grams per kilogram body weight of the dams. This dosage was based on previous studies (Schwahn *et al.*, 2003; Schwahn *et al.*, 2004) where increasing the oral betaine beyond 2% improved phenotypes and metabolic indices only minimally.

Male offspring were sacrificed at 6 days post-partum (dpp) (control: *Mthfr*$^{+/+}$, n = 4; *Mthfr*$^{+/-}$, n = 3; *Mthfr*$^{-/-}$, n = 4. short-term betaine: *Mthfr*$^{+/+}$, n = 5; *Mthfr*$^{+/-}$, n = 7; *Mthfr*$^{-/-}$, n = 4) and 8 months of age (control: *Mthfr*$^{+/+}$, n = 5; *Mthfr*$^{+/-}$, n = 9; *Mthfr*$^{-/-}$, n = 4. short-term betaine: *Mthfr*$^{+/+}$, n = 3; *Mthfr*$^{+/-}$, n = 3; *Mthfr*$^{-/-}$, n = 5), with the day of birth designated as 0 dpp. Blood was collected and the testes were removed and weighed.

The left testis was snap frozen and stored at -80°C, and the right testis immersed in formalin or Bouin's fixative (BDH Inc., Toronto, ON) for 5 to 24 hours, dehydrated and embedded in paraffin for histological analysis. Sections (5μm) were cut, mounted on glass slides, de-paraffinised with xylene, and stained with haematoxylin and eosin. Slides were viewed with a Zeiss Axiophot photomicroscope and pictures were taken with a SPOT RT Slider digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI).

Additionally, the effects of MTHFR deficiency on early spermatogenesis were also examined at 18.5 days post-coitum (dpc; day of plug = 0.5 dpc). Pregnant females, all given supplemental betaine, were killed at 18.5 dpc and caesarean sections were performed. Testes of male embryos were collected in Bouin's fixative, processed, cut into serial sections (5μm), and used for germ cell quantification. DNA from embryo head samples was used to genotype embryos.

3.3.2.1 Detection of Germ Cells

The monoclonal germ-cell nuclear antigen antibody, GCNA1 specifically recognises germ cells and was used to identify germ cells on Bouin's- or formalin-fixed testes collected at 18.5 dpc and 6 dpp (Enders & May, 1994). Sections from 18.5 dpc testes were cut into serial sections, with every fifth section used for germ cell quantification. For both 18.5 dpc and 6 dpp testes, sections were incubated overnight with undiluted primary antibody at 4°C. The Elite ABC Kit protocol (Vector Laboratories, Burlington, ON) was used for blocking and primary antibody detection, with the exception that the biotinylated antibody and VECTASTAIN Elite ABC reagent incubations were extended to 2 hours on 6 dpp testes fixed in formalin. For 18.5 dpc testes, germ cells were counted by an individual blinded to the slide identities and were reported per testis and per 2000 Sertoli cells as described by Nadler and Braun (2000).

3.3.2.2 Detection of Apoptotic Germ Cells

Germ cell apoptosis at 6 dpp was examined using the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL) detection protocol (Apoptag Kit, Intergen Co., Purchase, NY) according to manufacturer's directions, on formalin- or Bouin's-fixed testes. The following modifications were used for formalin-fixed testes: terminal deoxynucleotidyl transferase (TdT) enzyme incubation, 95 minutes; anti-peroxidase incubation, 60 minutes; DAB developing time, 15 minutes; and haematoxylin staining, 45 seconds. TUNEL-positive cells were quantified and reported as the number of TUNEL-positive cells per 100 tubules.

3.3.2.3 Quantification of Abnormal Seminiferous Tubules

Slides were viewed by light microscopy and tubules were staged according to the method described by Oakberg (1956). Abnormal tubules were quantified and expressed as a percent of total tubules examined (100 tubules counted per animal).

3.3.3 Betaine & Maintenance of Spermatogenesis – Experiment 2: Short-Term Versus Long-Term Betaine Treatment

Betaine has been shown to substantially improve the health and phenotype of *Mthfr*$^{-/-}$ mice (Schwahn *et al.*, 2003; Schwahn *et al.*, 2004). Thus, we hypothesised that betaine would have a similar positive effect on male germ cell development in *Mthfr*$^{-/-}$ males. To do so, a second experiment was conducted in which all mating females were provided with a 2% oral betaine solution during the time of mating, pregnancy and nursing and betaine treatment was then continued post-weaning in half of the mice. Pups were weaned at 24 days and males of each genotype were randomly assigned to an amino acid-defined dry diet (control; TD 01463, Harlan Teklad, Madison, WI) with no betaine, or the same diet but containing 2.93 g/kg supplemental betaine (betaine; TD 01462, Harlan Teklad), resulting in an oral betaine intake of approximately 300 to 350 mg/kg body weight (short-term betaine: *Mthfr*$^{+/+}$, n = 8; *Mthfr*$^{+/-}$, n = 11; *Mthfr*$^{-/-}$, n = 3. long-

term betaine: *Mthfr*$^{+/+}$, n = 6; *Mthfr*$^{+/-}$, n = 7; *Mthfr*$^{-/-}$, n = 5). At 3 months of age, all males were mated. Following mating, males were killed and testes, epididymides, seminal vesicles, spleen and liver were removed, weighed, snap frozen and stored at -80°C. The right testis was placed in Bouin's fixative and prepared for histological analysis.

3.3.3.1 Sperm Counts

Hemocytometric counts of testicular spermatozoa were performed according to Robb *et al.*, (1978), with modifications (Kelly *et al.*, 2003).

3.3.3.2 Fertility Analysis

The effect of MTHFR-deficiency and extended betaine supplementation on male fertility was examined by mating each male to two virgin CD1 females (Charles River Canada, Inc., St. Constant, Quebec, Canada) over the course of five days. Females were examined daily for vaginal plugs. The following week, mating was repeated with another two virgin CD1 females. Mating behaviour, pregnancy rate (the number of plug-positive females that became pregnant), litter size, and sex ratio were determined. All fertility data were calculated and are presented on a per male basis.

3.3.4 Statistical Analyses

Data were examined statistically using two-way analysis of variance (ANOVA), followed by the Tukey test (Sigma Stat; SPSS, Chicago, IL). Non-parametric tests were applied when sample sizes were too small to formally test for normal distribution. Fishers Exact Test was used to analyse fertility data. The level of significance for all analyses was $p<0.05$.

3.4 RESULTS

3.4.1 General Effects

In addition to examining the role of MTHFR in spermatogenesis, the first experiment was designed to determine if betaine, while improving pup survival and liver and brain phenotype (Schwahn *et al.*, 2004), could remedy any MTHFR-deficiency-mediated effects on male germ cell development. As previously reported, there were no differences in litter size between supplemented and unsupplemented females and withdrawal of betaine at weaning did not affect *Mthfr*$^{-/-}$ pup survival (Schwahn *et al.*, 2004). In both experiments reported here, body weight was monitored as a general gauge of health. For body weight and other end-points evaluated, *Mthfr*$^{+/+}$ or *Mthfr*$^{+/-}$ males did not differ, both within and between diets, unless specified.

As shown in Table 3.1, the average body weight of untreated 6 dpp *Mthfr*$^{-/-}$ males was only 37% ($p<0.001$) of untreated *Mthfr*$^{+/+}$ males. The body weight of *Mthfr*$^{-/-}$ mice was also significantly lower than that of betaine-supplemented *Mthfr*$^{-/-}$ males, but otherwise, control-diet *Mthfr*$^{-/-}$ males appeared healthy. In adults, mean body weight of control *Mthfr*$^{-/-}$ males was not different from that of control-diet *Mthfr*$^{+/+}$ males ($p=0.449$), yet *Mthfr*$^{-/-}$ males given short-term betaine weighed significantly less ($p<0.05$) than their *Mthfr*$^{+/+}$ counterparts. White blood cell counts and hemoglobin levels did not differ across genotype or diet (data not shown).

3.4.2 MTHFR-Deficiency and Betaine to Weaning on Male Reproductive System (Experiment 1: Short-Term Betaine)

3.4.2.1 Effects on Male Reproductive Organ Weights at 6 dpp and 8 Months.

The effects of MTHFR deficiency on testis weight in neonates and adults are shown in Figure 3.2A and 3.2B. Despite lowered body weights, the mean relative testis weights of *Mthfr*$^{-/-}$ pups did not differ with either diet, from their *Mthfr*$^{+/+}$ controls (Fig. 3.2A). In adult males, however, MTHFR-deficiency resulted in considerably reduced

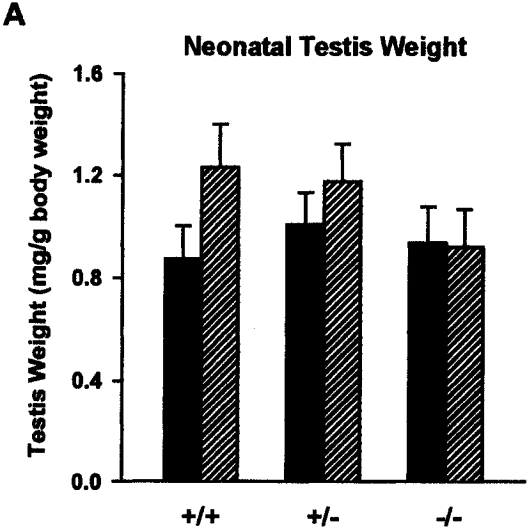
Table 3.1: Mean body[a] and organ[b] weights of males[c] from experiments 1 and 2.

		Control Diet			Betaine-to-Weaning (Short-term Betaine)			Betaine Beyond Weaning (Long-term Betaine)		
		+/+	+/-	-/-	+/+	+/-	-/-	+/+	+/-	-/-
Expt. 1	**6 dpp body**	3.93 ±0.25	2.94 ±0.88	1.46 ±0.12[d]	3.65 ±0.23	3.66 ±0.20	2.65 ±0.55[e]	-	-	-
	Adult (8 mos) body	31.98 ±0.87	28.77 ±1.08	25.47 ±1.26	31.04 ±0.68	29.97 ±1.29	23.68 ±2.15[f]	-	-	-
Expt. 2	**Adult (3 mos) body**	-	-	-	19.68 ±0.84	21.46 ±0.61	21.73 ±1.60	22.59 ±0.61	21.42 ±0.93	23.10 ±0.95
	Epididymides	-	-	-	2.44 ±0.07	2.47 ±0.06	1.26[f] ±0.03	2.43 ±0.13	2.46 ±0.08	1.55[g] ±0.09
	Seminal Vesicles	-	-	-	1.68 ±0.14	1.51 ±0.13	1.27 ±0.42	2.16[f] ±0.18	1.86 ±0.11	1.96[h] ±0.15

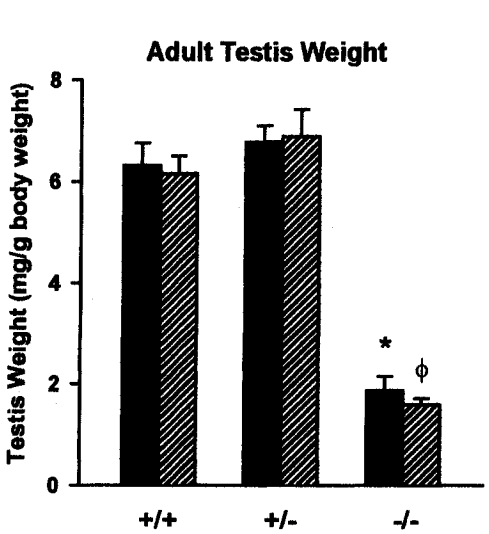
[a] Weight in g (± SEM)
[b] Relative weight of paired organs (mg/g body weight) (± SEM)
[c] Adult males in experiments 1 and 2 were 8 and 3 months (mos) old, respectively.
[d] $p<0.001$, vs. control-diet *Mthfr*$^{+/+}$
[e] $p<0.05$, vs. control-diet *Mthfr*$^{-/-}$
[f] $p<0.05$, vs. betaine-to-weaning *Mthfr*$^{+/-}$
[g] $p<0.05$, vs. betaine-beyond-weaning *Mthfr*$^{+/-}$
[h] $p<0.05$, vs. betaine-to-weaning *Mthfr*$^{-/-}$

Figure 3.2. Effect of MTHFR deficiency and betaine supplementation to weaning (short-term betaine), on testis weights at **(A)** 6 dpp and **(B)** 8 months of age. Black bars represent males never exposed to supplemental betaine (6 dpp: *Mthfr*$^{+/+}$, n = 4; *Mthfr*$^{+/-}$, n = 3; *Mthfr*$^{-/-}$, n = 4; 8 months: *Mthfr*$^{+/+}$, n = 5; *Mthfr*$^{+/-}$, n = 9; *Mthfr*$^{-/-}$, n = 4) ; hatched bars represent males provided with supplemental betaine, via their mother (6 dpp: *Mthfr*$^{+/+}$, n = 5; *Mthfr*$^{+/-}$, n = 7;*Mthfr*$^{-/-}$, n = 4; 8 months: *Mthfr*$^{+/+}$, n = 3; *Mthfr*$^{+/-}$, n = 3; *Mthfr*$^{-/-}$, n = 5). Testis weight is shown relative to body weight. Bars represent means ± SEM. * $p< 0.001$ vs. control-diet *Mthfr*$^{+/+}$; ϕ $p<0.001$ vs. betaine-supplemented *Mthfr*$^{+/+}$.

A


Neonatal Testis Weight
Testis Weight (mg/g body weight)
1.6
1.2
0.8
0.4
0.0
+/+
+/-
-/-

B


Adult Testis Weight
Testis Weight (mg/g body weight)
8
6
4
2
0
*
ϕ
+/+
+/-
-/-

testis weights (Fig. 3.2B). The relative testis weights of *Mtfhr*$^{-/-}$ mice of either diet, were less than one-third of their *Mthfr*$^{+/+}$ controls.

3.4.2.2 Identification of Germ Cell Effects at 6 dpp

Relative testis weights of *Mthfr*$^{-/-}$ pups were normal at 6 dpp, but were severely reduced relative to *Mthfr*$^{+/+}$ males when evaluated at 8 months of age. We therefore examined the testicular histology at both time points. The germ-cell specific antibody GCNA1 was used to characterise the relative quantity of germ cells in wild-type and MTHFR-deficient males at 6 dpp. GCNA1 is present in all prospermatogonial cells, from E11.5 and throughout spermatogonial development until 14 dpp (Enders & May, 1994). Staining of *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ testes revealed numerous gonocytes (Fig. 3.3A), but in the testes of *Mthfr*$^{-/-}$ males there were relatively few gonocytes, regardless of diet; in fact, many cords completely lacked gonocytes (Fig. 3.3B & C).

Given the dramatic reduction in germ cells in *Mthfr*$^{-/-}$ testes, we proposed that germ cell death could account for the observed loss of gonocytes. To examine this possibility, testicular apoptosis was assessed by TUNEL immunostaining at 6 dpp. TUNEL-positive cells were quantified and reported as an incidence per 100 tubules (control: *Mthfr*$^{+/+}$, 7.15 ± 2.11; *Mthfr*$^{-/-}$, 31.25 ± 11.93; betaine: *Mthfr*$^{+/+}$, 5.90 ± 2.40; *Mthfr*$^{-/-}$, 4.68 ± 1.33). Apoptotic cells were rare in the testes of unsupplemented and supplemented *Mthfr*$^{+/+}$ males and supplemented *Mthfr*$^{-/-}$ males, but increased significantly ($p<0.05$) in unsupplemented *Mthfr*$^{-/-}$ males. The increase in apoptotic cells was subtle with only one to two TUNEL-positive cells per tubule, but these cells were consistently observed throughout the whole testis. Although the apoptotic cells were similar in shape and size to germ cells, we cannot be absolutely certain that some were not Sertoli cells.

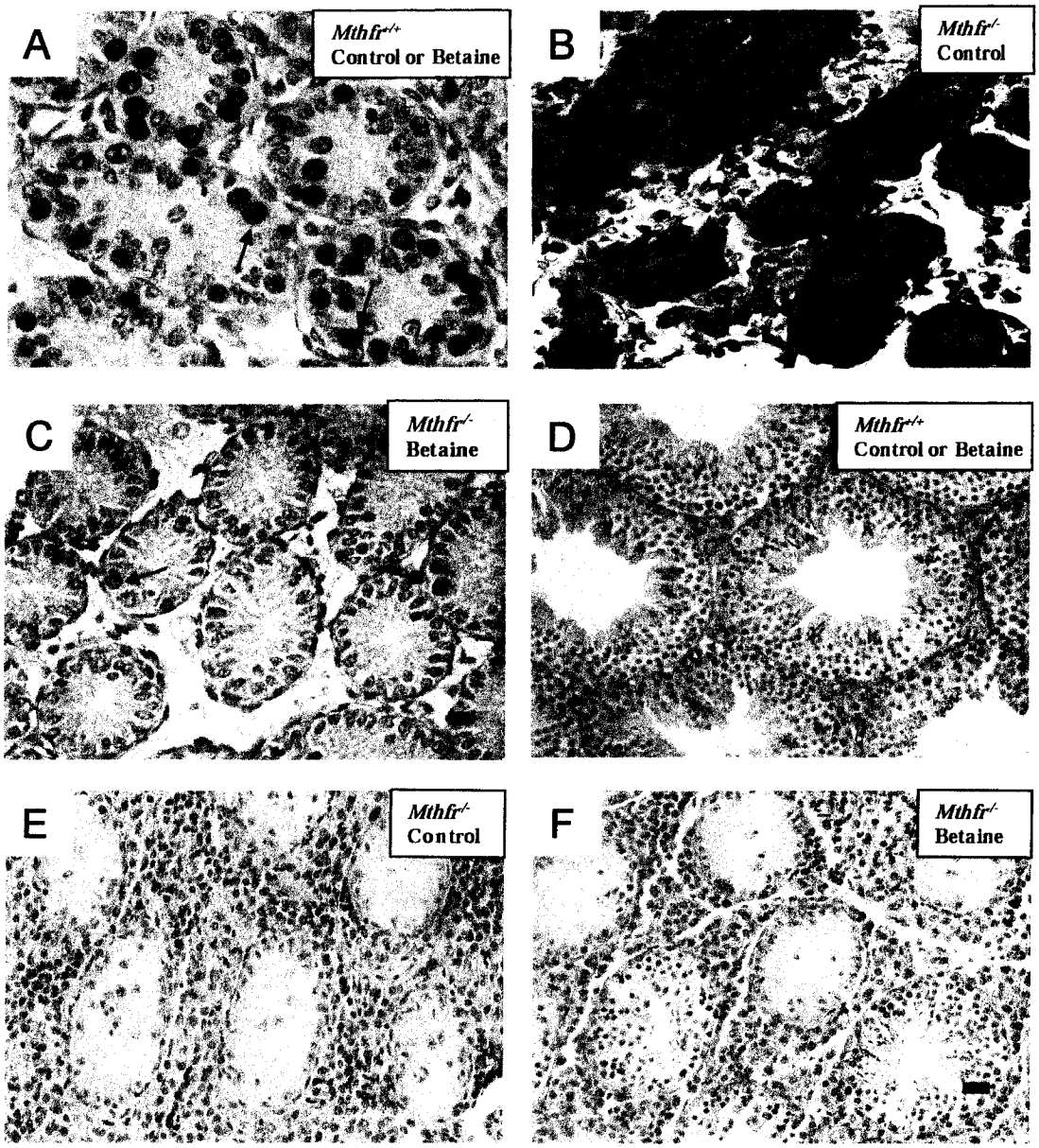
The increase in apoptosis at 6 dpp in control-diet *Mthfr*$^{-/-}$ males cannot however, completely account for the low germ cell numbers observed. Although betaine-diet *Mthfr*$^{-/-}$ males had normal levels of apoptosis, these mice had germ cell numbers similar

to control-diet *Mthfr*$^{-/-}$ males. We postulated that loss of germ cells in *Mthfr*$^{-/-}$ males may have occurred prenatally. To test this hypothesis, testes of 18.5 dpc male embryos were analysed quantitatively for germ cell number after immunostaining with GCNA1. Because germ cell numbers appeared similar between the two *Mthfr*$^{-/-}$ dietary groups at 6 dpp, and *Mthfr*$^{-/-}$ pups were difficult to obtain without maternal supplementation, prenatal gonocyte numbers were only analysed in betaine-supplemented pups. At 18.5 dpc, the fetal seminiferous cord is made up of mitotically-arrested gonocytes and Sertoli cells. Gonocytes are found in the central area of the cord, and in addition to staining positively for GCNA1, are easily distinguished from Sertoli cells by their large nucleus and several nucleoli, which stain light pink with haematoxylin and eosin. Quantification of GCNA1-stained germ cells revealed similar gonocyte numbers amongst all three genotypes when analysed on a per testis (betaine: *Mthfr*$^{+/+}$, 43780 and 35310; *Mthfr*$^{+/-}$, 39615 and 36221; and *Mthfr*$^{-/-}$, 44875 and 35310) and per 2000 Sertoli cell basis (betaine: *Mthfr*$^{+/+}$, 687 and 511; *Mthfr*$^{+/-}$, 453 and 487; and *Mthfr*$^{-/-}$, 667 and 411) indicating that gonocyte mitosis is normal before birth and suggesting that the decrease in gonocyte number occurs soon after birth.

3.4.2.3 Germ Cell Effects in Adult Males

Adult testes were analysed histologically and tubules were scored as abnormal if one or more of the following defects was observed: giant or multinucleate cells, vacuolisation, missing germ cell population, or sloughing. *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ testes appeared normal. All spermatogenic stages were present, with normal cellular associations; no differences could be attributed to diet (Fig. 3.3D). Conversely, all tubules of both control-diet and short-term betaine treated *Mthfr*$^{-/-}$ males were abnormal and an average of 80% to 90% of tubules within each group appeared to lack germ cells (Fig. 3.3E & F). Where possible tubules were staged (<10%); however the lack of elongating spermatids, frequent appearance of degenerating pachytene spermatocytes, and

Figure 3.3. Short-term betaine: examples of testicular histology at 6 dpp and 8 months of age. GCNA1 staining, at 6 dpp, of **(A)** *Mthfr*$^{+/+}$ males without betaine supplementation (similar to *Mthfr*$^{+/+}$ males with betaine and *Mthfr*$^{+/-}$ males, with or without betaine supplementation); **(B)** control-diet *Mthfr*$^{-/-}$ males; and **(C)** betaine-supplemented *Mthfr*$^{-/-}$ males. Arrows in **(A – C)** indicate germ cells. The bar in **(C)** represents 20μm for **(A – C)**. Testicular histology at 8 months of age in **(D)** *Mthfr*$^{+/+}$ males without betaine supplementation to weaning (similar to *Mthfr*$^{+/+}$ males with betaine supplementation to weaning and *Mthfr*$^{+/-}$ males, with or without betaine supplementation to weaning). **(E)** Control-diet *Mthfr*$^{-/-}$ males, and **(F)** *Mthfr*$^{-/-}$ males, provided with betaine supplementation to weaning. The bar in **(F)** represents 50μm for panels **(D – F)**.


A
Mthfr$^{+/+}$
Control or Betaine
B
Mthfr$^{-/-}$
Control
C
Mthfr$^{-/-}$
Betaine
D
Mthfr$^{+/+}$
Control or Betaine
E
Mthfr$^{-/-}$
Control
F
Mthfr$^{-/-}$
Betaine

the disruptive effects of pervasive vacuolisation made staging quite difficult and inexact. In tubules that could be staged, germ cell associations were appropriate, but tubules were often missing one or more germ cell populations, had extensive vacuolisation, or exhibited sloughing. All stages appeared to be equally affected in *Mthfr*$^{-/-}$ males, regardless of diet, and specific abnormalities did not correspond to particular stages. In those tubules that had germ cells yet could not be staged, abnormalities were similar to those in staged tubules, but in these cases disrupted the germ cell arrangement to such an extent that staging was not possible. Round spermatids were generally the most mature germ cell population; elongating spermatids were observed in less than 2% of tubules.

The tubules of individual MTHFR-deficient males were varied in appearance such that the timing of the MTHFR defect within spermatogenesis was difficult to pinpoint. Some tubules lacked germ cells entirely, whereas others had pachytene spermatocytes, but not germ cells that were more mature. Still other tubules, although more rare, exhibited elongating spermatids, but had considerable vacuolisation. Spermatogenesis did not appear to halt at any one particular stage or point during germ cell development. Not one particular germ cell population was affected and when vacuolisation was present it was not limited to one part of the seminiferous epithelium.

There was considerable variation amongst individual males within each *Mthfr*$^{-/-}$ treatment group. Of the three control-diet *Mthfr*$^{-/-}$ males, one had almost no germ cells and the vast majority of tubules appeared to contain only Sertoli cells; another male had germ cells in 34% of tubules, the majority of which could be staged, while the remaining tubules lacked germ cells; and the third male (shown in Fig. 3.3E) had only a few tubules that could be staged and in 94% of tubules, germ cells were absent. In those few tubules with germ cell differentiation, sloughing was often evident, as was vacuolisation.

At 8 months, testicular histology of betaine-supplemented *Mthfr*$^{-/-}$ males was similar to control-diet *Mthfr*$^{-/-}$ males, with the exception that elongated spermatids were

seen more frequently, in an average of 10% of tubules (Fig. 3.3E & F). Variability was also prevalent in the supplemented *Mthfr*$^{-/-}$ males. Three of five males had almost no germ cells and had histology resembling that shown in Figure 3.3E, but approximately 20% of the tubules in the remaining two males could be staged (Fig. 3.3F). As with unsupplemented *Mthfr*$^{-/-}$ males, abnormalities were predominantly vacuolisation, sloughing and missing germ cell populations and did not vary by stage.

With the decrease in germ cells was an apparent increase in the interstitial cell population in both nullizygous groups. This may be a visual consequence of decreased germ cell numbers and thus decreased tubule diameter.

3.4.3 *Betaine Supplementation Beyond Weaning and MTHFR Deficiency (Experiment 2: Short-Term Versus Long -Term Betaine)*

3.4.3.1 Effects on Male Reproductive Organ Weights

In the first experiment, betaine-supplemented *Mthfr*$^{-/-}$ males exhibited decreased apoptosis at 6 dpp, but once weaned from this diet, germ cell development became abnormal to nonexistent. In the second experiment, we therefore considered the positive effects that continued supplementation of betaine, beyond weaning and throughout adulthood, might have on spermatogenesis in *Mthfr*$^{-/-}$ males.

Unlike the first experiment, there were no deviations in body weight amongst genotypes or diets (Table 3.1). Additionally, spleen weight, white blood cell count, and haemoglobin level did not change with respect to genotype or diet (data not shown). However, upon examination of the male reproductive system, several effects became apparent.

Relative epididymal weights (Table 3.1) were severely decreased in both *Mthfr*$^{-/-}$ groups although this reduction was somewhat remedied, but not significantly, by the continuation of betaine beyond weaning. Similarly, genotype had a dramatic effect on testis weight, although as seen throughout both experiments, there was no observed

heterozygote effect. Testis weights of *Mthfr*$^{-/-}$ males were severely reduced in both dietary groups, yet the mean relative testis weight of long-term betaine *Mthfr*$^{-/-}$ males was twofold higher than those given betaine only until weaning (Fig. 3.4). The 76% decrease in the testis weight of *Mthfr*$^{-/-}$ males, relative to *Mthfr*$^{+/+}$ males, was similar to that seen in the first experiment. This decrease was slightly, although not significantly, alleviated by the addition of betaine post-weaning; in these males the decrease in relative testis weight was only 60% (Fig. 3.4).

One possible explanation for changes in testicular weight is altered testosterone levels. The seminal vesicles are accessory sex organs sensitive to changes in androgen status; weights of these organs often indicate changes in testosterone. Long-term betaine supplementation resulted in significantly higher seminal vesicle weights in both *Mthfr*$^{+/+}$ and *Mthfr*$^{-/-}$ males when compared with males of the same genotype provided with betaine only until weaning (Table 3.1). Within the same diet, however seminal vesicle weights were similar across genotypes, suggesting that the decrease in *Mthfr*$^{-/-}$ testis weights relative to those of *Mthfr*$^{+/+}$ males were not the result of altered testosterone levels.

3.4.3.2 Positive Effects of Betaine on Spermatogenesis

Mthfr$^{-/-}$ males provided with short-term betaine in this study displayed testicular abnormalities identical to those observed in adults of the first study (Fig. 3.3F, 3.5A & B). Again, there was considerable variation amongst *Mthfr*$^{-/-}$ members of the dietary groups. All tubules of one male appeared to lack germ cells (Fig. 3.5A), while germ cell development was evident in 14% and 76% of tubules in the other two males. The majority of tubules with germ cells could be staged, but all tubules were scored as abnormal because of pervasive vacuolisation, sloughing, and missing germ cell populations. Of note, considerably more tubules could be staged in this *Mthfr*$^{-/-}$ group compared to the first study. This increase, however, was because of one male (Fig. 3.5B), in which nearly

76% of tubules could be staged; the remaining males had levels similar to the first experiment. As in the first experiment, those tubules that could be staged contained normal germ cell associations although vacuolisation, multinucleate cells and sloughing were generally evident and frequently one or more germ cell populations were missing. Defects were not typical to one stage, and there was no relationship between the tubule stage and missing germ cell population. Elongating spermatids were observed in an average of less than 10% of tubules.

Remarkably, while some tubules of *Mthfr*$^{-/-}$ males supplemented with long-term betaine resembled those of males who received betaine only to weaning (Fig. 3.5D), the majority displayed much improved spermatogenesis (Fig. 3.5E). Indeed, quantification of abnormal tubules – those tubules with multinucleate/giant cells, degenerating germ cells, sloughing of immature germ cells, or lacking one or more germ cell populations – revealed that all (100% ± 0) tubules in *Mthfr*$^{-/-}$ males given betaine only to weaning were abnormal, however significantly less tubules (71% ± 12) from *Mthfr*$^{-/-}$ males given long-term betaine were abnormal. Normal spermatogenesis was present in the remaining tubules of *Mthfr*$^{-/-}$ males given long-term betaine and elongating spermatids were observed, on average, in more than 25% of tubules (Fig. 3.5E). As with the other dietary *Mthfr*$^{-/-}$ groups, there was considerable variation within the long-term betaine *Mthfr*$^{-/-}$ group. Four of the five males within this group exhibited normal spermatogenesis, but to differing extents (percent normal tubules: 14%, 29%, 32%, and 67%). Testicular histology of the male with 14% normal spermatogenesis is shown in Figure 3.5D; Figure 3.5E is an example of the histology from the male with 67% normal spermatogenesis. Despite provision of long-term betaine, one *Mthfr*$^{-/-}$male had no normal tubules and histology similar to that of Figure 3.5B. Again, similar defects were observed across all stages and when abnormal and normal tubules were staged, a comparable stage distribution was evident in both normal and abnormal tubules.

Figure 3.4. Effect of MTHFR deficiency and long-term betaine supplementation on relative testis weight (shown per gram body weight). Hatched bars represent males given betaine only to weaning (*Mthfr*$^{+/+}$, n=8; *Mthfr*$^{+/-}$, n=11; *Mthfr*$^{-/-}$, n=3) and white bars represent males provided with lifelong betaine supplementation (pre-weaning and post-weaning) (*Mthfr*$^{+/+}$, n=8; *Mthfr*$^{+/-}$, n=7; *Mthfr*$^{-/-}$, n=5). Bars represent means ± SEM. *$p<0.001$ vs. control-diet *Mthfr*$^{+/+}$; ϕ $p<0.001$ vs. betaine-diet *Mthfr*$^{+/+}$.

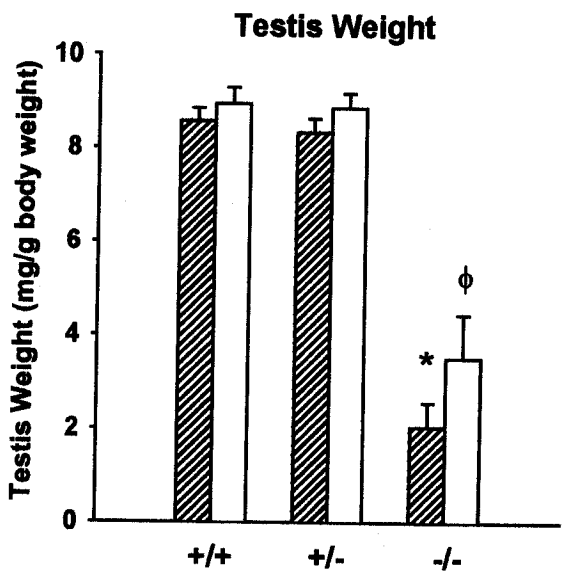
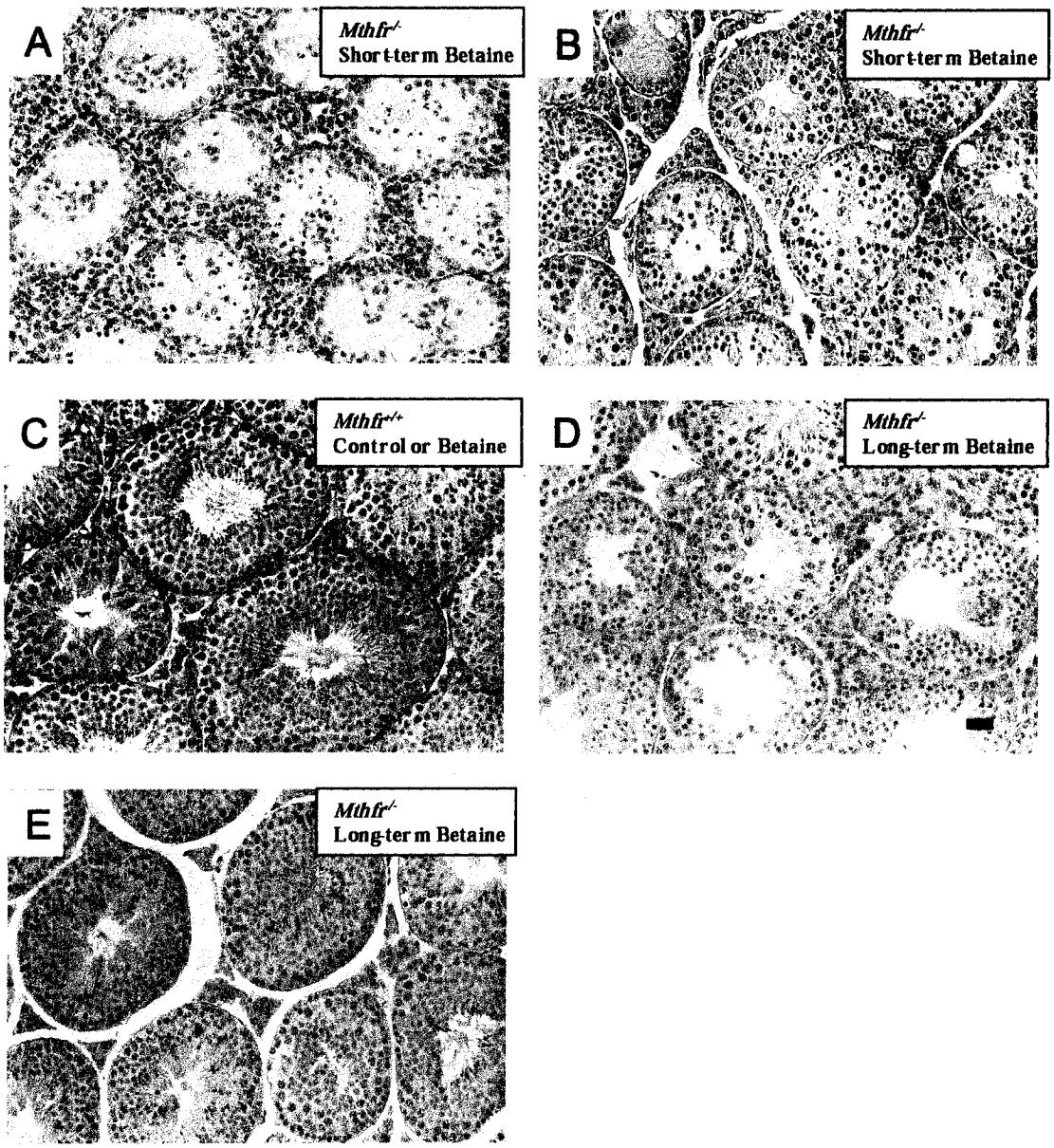

Testis Weight
Testis Weight (mg/g body weight)
10
8
6
4
2
0
*
ϕ
+/+
+/-
-/-

Figure 3.5. Long-term betaine: examples of testicular histology at 3 months of age. **(A)** and **(B)** depict the variability in testicular histology seen in *Mthfr*$^{-/-}$ males exposed to betaine only until weaning. **(C)** Testicular histology of *Mthfr*$^{+/+}$ males given lifelong betaine supplementation (similar to *Mthfr*$^{+/+}$ males given betaine only to weaning, and *Mthfr*$^{+/-}$ males provided with short- and long-term betaine supplementation). **(D)** and **(E)** depict variability in testicular histology seen in *Mthfr*$^{-/-}$ males provided with betaine up to and beyond weaning. In *Mthfr*$^{-/-}$ males receiving long-term betaine approximately 28% of all tubules (mean of all males within the group) display normal spermatogenesis. The bar in D indicates 50μm for **(A – E)**.


A
Mthfr-/-
Short-term Betaine
B
Mthfr-/-
Short-term Betaine
C
Mthfr+/+
Control or Betaine
D
Mthfr-/-
Long-term Betaine
E
Mthfr-/-
Long-term Betaine

3.4.3.3 Effects on Testicular Sperm Production

Mean testicular sperm count per gram testis weight, was severely decreased in *Mthfr*$^{-/-}$ males given betaine only to weaning and was only 6% of genotypic controls (Fig. 3.6A). Betaine treatment beyond weaning resulted in higher sperm production, per gram testis weight, in *Mthfr*$^{-/-}$ males. Although still significantly lower than their genotypic controls, this mean relative sperm production was a significant improvement over sperm counts in *Mthfr*$^{-/-}$ males given betaine only to weaning ($p<0.01$).

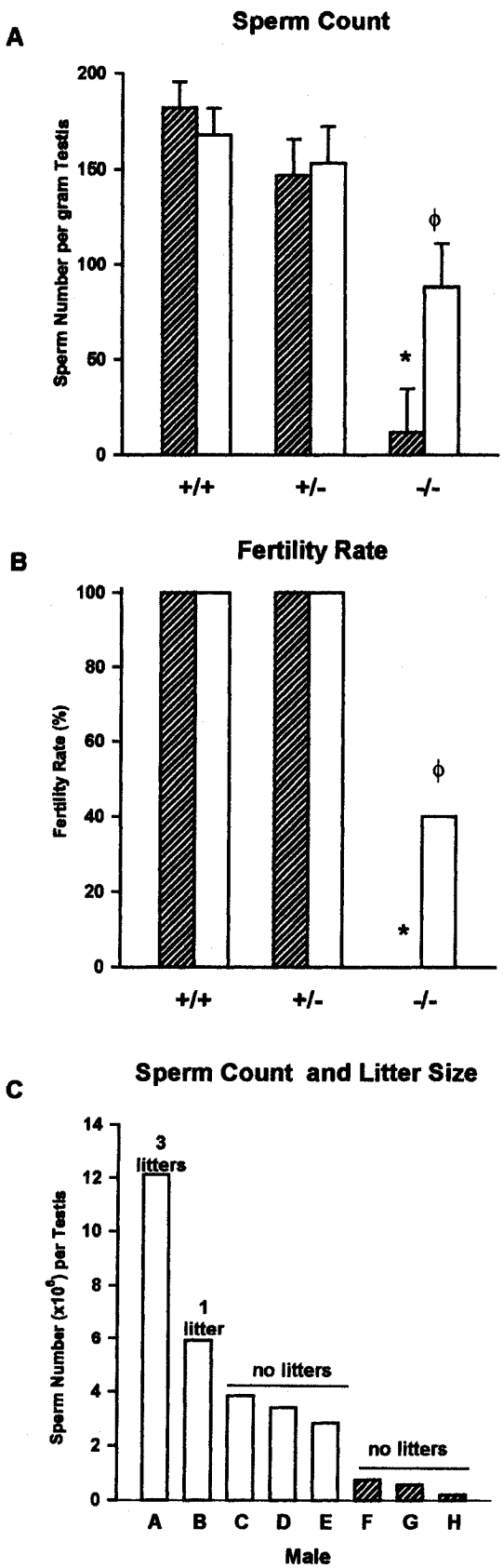
3.4.3.4 MTHFR & Fertility

Because of the observed improvements in testicular histology and sperm numbers, we postulated that betaine supplementation into adulthood would improve *Mthfr*$^{-/-}$ male fertility. Males from each group were mated over a period of two weeks with four females and mating success was evident by the presence of a vaginal plug. All data were calculated, and are shown, on a per male basis. Regardless of genotype or diet, mating performance was similar for all groups (data not shown). Fertility in *Mthfr*$^{-/-}$ males given short-term betaine, however, was completely compromised. Pregnancy rate, calculated as the percent plug-positive females that gave birth, was zero in females mated to males given betaine only until weaning (Fig. 3.6B). In contrast, two of the five *Mthfr*$^{-/-}$ males (40%) given long-term betaine were capable of siring offspring, a fertility rate significantly higher ($p<0.05$) than in the *Mthfr*$^{-/-}$ group given betaine only to weaning. One male given long-term betaine sired three litters and another male, one litter (Fig. 3.6C). When litters were analysed for pup number and sex ratio there were no differences amongst genotypes or diets (data not shown).

Only two of the five *Mthfr*$^{-/-}$ males provided with the long-term betaine were able to sire litters, even though relative sperm counts were increased in all these males compared to *Mthfr*$^{-/-}$ males given betaine only until weaning. Moreover, there was no relationship between an individual's relative testis weight or relative sperm count and

fertility. Total sperm production, however, was severely decreased in those males that were infertile (Fig. 3.6C). As illustrated in Figure 3.6C, the *Mthfr*$^{-/-}$ male that sired three litters had a total sperm production that was twice that of the *Mthfr*$^{-/-}$ male that sired one litter. Also of note, is that infertile long-term betaine *Mthfr*$^{-/-}$ males had total sperm counts that were at least three and one-half times the counts of *Mthfr*$^{-/-}$ males given betaine only until weaning. There appears to be a threshold of sperm production in MTHFR-deficient males, below which males are infertile. Male C had a total sperm count that was only 35% less than Male B, yet was unable to sire any offspring. Also, overall sperm production did not increase correspondingly with increased testicular weight. Relative testis weights were similar for some males given long-term betaine and males given short-term betaine. Despite this, those *Mthfr*$^{-/-}$ males given long-term betaine each had total sperm production that was at least three and one-half times higher than *Mthfr*$^{-/-}$ mice given betaine only to weaning, suggesting that although testis weight may not increase significantly with continued betaine supplementation, the ability to maintain spermatogenesis increases with prolonged betaine supplementation.

Figure 3.6. Effect of MTHFR deficiency and long-term betaine supplementation on **(A)** testicular sperm counts, per gram testis weight, and **(B)** male fertility. **(C)** Relationship between total sperm counts of *Mthfr*$^{-/-}$ males, with or without betaine post-weaning and the number of litters sired. Hatched bars represent males given betaine only to weaning (for sperm counts: *Mthfr*$^{+/+}$, n=3; *Mthfr*$^{+/-}$, n=3; *Mthfr*$^{-/-}$, n=3) (for matings: *Mthfr*$^{+/+}$, n=8; *Mthfr*$^{+/-}$, n=11; *Mthfr*$^{-/-}$, n=3) and white bars represent males provided with lifelong betaine supplementation (pre-weaning and post-weaning) (for sperm counts: *Mthfr*$^{+/+}$, n=3; *Mthfr*$^{+/-}$, n=3; *Mthfr*$^{-/-}$, n=5) (for matings: *Mthfr*$^{+/+}$, n=8; *Mthfr*$^{+/-}$, n=7; *Mthfr*$^{-/-}$, n=5). Bars represent means ± SEM. * $p< 0.001$ vs. control-diet *Mthfr*$^{+/+}$; ϕ $p<0.001$ vs. betaine-diet *Mthfr*$^{+/+}$; ‡ $p<0.001$ vs. control-diet *Mthfr*$^{-/-}$.


A
Sperm Count
Sperm Number per gram Testis
200
150
100
50
0
*
ϕ
+/+
+/-
-/-
B
Fertility Rate
Fertility Rate (%)
100
80
60
40
20
0
*
ϕ
+/+
+/-
-/-
C
Sperm Count and Litter Size
Sperm Number (x10⁶) per Testis
14
12
10
8
6
4
2
0
3 litters
1 litter
no litters
no litters
A
B
C
D
E
F
G
H
Male

3.5 DISCUSSION

3.5.1 MTHFR and Spermatogenesis

The association between mutations/polymorphisms in MTHFR and other folate pathway genes and decreased fertility or pregnancy success in females has been exhaustively examined (reviewed in Nelen, 2001). However, the role that MTHFR may play in male fertility has not yet been examined in detail. Here, we show, for the first time, that male mice lacking MTHFR suffer severe reproductive consequences; spermatogenesis in these mice fails during early postnatal development and results in complete infertility.

In the original study of the MTHFR knockout (Chen *et al.*, 2001), mice were on a mixed genetic background and *Mthfr*$^{-/-}$ males were reportedly fertile. However, our study indicates that after multiple generations of backcrossing into the BALB/c strain, MTHFR deficiency results in male infertility, with extensive testicular histological abnormalities. Presumably, the mixed genetic background of these early generations provided a degree of "hybrid vigour", resulting in less deleterious effects of the MTHFR mutation. Although Chen *et al.* (2001) and others (Schwahn *et al.*, 2004) have described a heterozygous phenotype in MTHFR-deficient mice, there was no observed heterozygote effect in our studies of male reproduction.

3.5.1.1 Neonatal Testis

Despite normal relative testis weights, there were significant histological differences in seminiferous cord morphology at 6 dpp between *Mthfr*$^{+/+}$ and *Mthfr*$^{-/-}$ mice. *Mthfr*$^{-/-}$ males had extremely reduced gonocyte populations and unsupplemented *Mthfr*$^{-/-}$ males displayed significantly increased levels of apoptosis. At 6 dpp the seminiferous cords normally contain only Sertoli cells and gonocytes, with the latter comprising only 16% of the total cellular content of the tubules (Bellvé *et al.*, 1977). Because gonocytes make up such a small portion of the cellular population, even a substantial reduction in

germ cells would likely not manifest as a significant decrease in testis weight. Prior to birth, gonocytes stop proliferating (Vergouwen *et al.*, 1991; Nagano *et al.*, 2000) and do not resume replication until about 1.5 dpp. One possible explanation for the dramatically reduced gonocyte numbers at 6 dpp is that some gonocytes in *Mthfr*$^{-/-}$ males fail to resume mitosis postnatally. It has been suggested that maternal folate pools may protect *Mthfr*$^{-/-}$ pups throughout gestation, but once born, these mice are unable to meet the increased need for methyl groups that elevated cellular proliferation demands (Schwahn *et al.*, 2004). However, if failure to proliferate postnatally were the only cause for declining germ cell numbers, we would expect similar numbers of gonocytes at 18.5 dpc and 6 dpp. That there is a dramatic reduction in gonocyte number between 18.5 dpc and 6 dpp suggests that there is likely another reason, perhaps in addition to non-proliferation, for the reduced gonocyte numbers observed at 6 dpp in *Mthfr*$^{-/-}$ males.

Shortly after birth gonocytes begin relocating from the central area of the seminiferous cord, where the lumen later develops, to the basement membrane (McGuinness & Orth, 1992a; Nagano *et al.*, 2000). Relocation is typically complete by 6 dpp (Nagano *et al.*, 2000). Relocation to the periphery of the tubule is not necessary for reproliferation and vice versa (Orth & Boehm, 1990; McGuiness & Orth, 1992b; Orth & Jester, 1995), however, it has been postulated that gonocytes failing to undergo relocation apoptose or are removed with the formation of the lumen (Roosen-Runge & Leik, 1968; Kluin & De Rooij, 1981); hence, movement to the cord periphery may be necessary to avoid apoptosis or phagocytosis by Sertoli cells (Roosen-Runge & Leik, 1968). In our study, apoptotic cells were typically located toward the central area of the cord. We suggest that the reduced germ cell number seen at 6 dpp and in adults may be the result of apoptosis of gonocytes that fail to replicate postnatally or are improperly aligned along the basal aspects of the seminiferous tubule. Our results indicate that such an event occurs sometime before 6 dpp and may occur soon after birth when relocation

and reproliferation are initiated (2-4 dpp). Unfortunately, the difficulty of obtaining *Mthfr*$^{-/-}$ pups without betaine supplementation impedes studies of these early postnatal days.

3.5.1.2 Adult Testis

Mthfr$^{-/-}$ males exhibited extremely abnormal testicular histology, with tubules that were nearly devoid of germ cells, and rarely were the more mature germ cell populations observed. The exact nature and timing of the MTHFR defect within spermatogenesis is unclear. Spermatogenesis did not terminate at any one specific stage or point in development and it was not one particular stage, germ cell population, or layer of the seminiferous epithelium that was affected by MTHFR deficiency. Also striking was the degree of heterogeneity amongst *Mthfr*$^{-/-}$ males of the same diet. Furthermore, it is unclear at this time whether the testicular defect observed with MTHFR deficiency is due to abnormalities within the somatic cells of the testis or the germ cells themselves. Spermatogonial stem cell transplantation from *Mthfr*$^{-/-}$ males into germ-cell deficient mice or the use of *Mthfr*$^{-/-}$ males as the recipients of stem cells from normal mice would aid in this discrimination. This method has been used previously to localise defects within the testis (Boettger-Tong *et al.*, 2000) and could be used to great advantage here to examine the role of MTHFR in testicular development.

Mthfr$^{-/-}$ males appeared to have greatly expanded populations of interstitial cells. Increased Leydig cell proliferation could result from a rise in luteinising hormone, but would likely manifest as increased testosterone. That seminal vesicle weights, a well-accepted proxy for testosterone changes, were similar across genotypes within the same diet suggests that the apparent increase in interstitial cells may simply be the result of decreased germ cell numbers and a concomitant reduction in tubule diameter. However, we cannot exclude the possiblility that local testosterone levels may have been altered.

MTHFR is crucial to the folate pathway and disruption of this enzyme

compromises mouse development in several ways. Schwahn and colleagues (2004) postulated that accumulation of homocysteine and impaired transmethylation reactions (Fig. 3.1) may be primary factors in MTHFR pathology. These factors may also underlie the spermatogenic defects seen with MTHFR deficiency. MTHFR deficiency is thought to impede the methylation of a wide variety of substrates including proteins, DNA, RNA, and histones through decreased methionine supply. This condition is potentially exacerbated by accumulation of SAH, which can directly inhibit methylation reactions through strong binding of SAM-dependent methyltransferases (Hoffman *et al.*, 1980). One transmethylation reaction, DNA methylation, has been shown to be crucial for normal spermatogenesis (Doerksen *et al.*, 2000; Kelly *et al.*, 2003) and thus may be particularly vulnerable to the changes in methyl pools brought about by deficiency in MTHFR. Moreover, inactivation of MTHFR can also disrupt cellular nucleotide pools, effectively enhancing cellular proliferation. If transmethylation processes are not able to keep pace with increased cell division, diminished cell survival may result.

3.5.2 Betaine and Spermatogenesis

In severe MTHFR deficiency, provision of a methyl donor can restore remethylation of homocysteine to methionine (Schwahn *et al.*, 2003). As such, betaine has proven clinically useful in treating MTHFR deficiency (Haworth *et al.*, 1993; Al Tawari *et al.*, 2002; Bönig *et al.*, 2003). Betaine is the substrate for BHMT (Fig. 3.1), which typically acts in concert with methionine synthetase to keep homocysteine levels in check via remethylation of this amino acid (Finkelstein, 1990; Millian & Garrow, 1998). Because administration of a 2% oral betaine solution to females during mating, pregnancy and nursing results in a four-fold increase in pup survival and improved liver and brain phenotypes (Schwahn *et al.*, 2004), we postulated that betaine supplementation might also improve spermatogenesis in *Mthfr*$^{-/-}$ males.

In rodents, BHMT activity dramatically increases soon after birth, indicating a

pronounced need for this enzyme (Finkelstein *et al.*, 1971), perhaps because of increased cellular proliferation at this time. Homocysteine remethylation is responsible for elimination of 50% of homocysteine levels; and this remethylation is equally divided between the BHMT pathway and 5-methyltetrahydrofolate, provided by MTHFR (Finkelstein, 1974). Under typical dietary conditions, remethylation provides more than half of normal methionine requirements (Mudd & Poole, 1975). In MTHFR-deficient mice, there is an increased reliance on remethylation via the BHMT pathway, perhaps because of lowered methionine availability (Finkelstein *et al.*, 1982; Park *et al.*, 1997; Park & Garrow, 1999; Schwahn *et al.*, 2004). Although gonocyte number was not different in control-diet and betaine-diet 6 dpp *Mthfr*$^{-/-}$ males, there was some improvement as indicated by decreased levels of apoptosis. The precise mechanism by which betaine improves spermatogenesis is unclear. Because betaine supplemented *Mthfr*$^{-/-}$ pups have significantly reduced homocysteine levels (Schwahn *et al.*, 2004), they may be able to maintain, to a limited extent, the higher levels of transmethylation that increased cellular proliferation, soon after birth, requires. As such, betaine supplementation may help these mice to maintain a minimal proliferating gonocyte population that may be spared from apoptosis. Hence, provision of betaine during this period may help to maintain a gonocyte population at this time that can later populate the testis. Although the localisation of BHMT has not been examined in the gonads (Sunden *et al.*, 1997; Chadwick *et al.*, 2000), members of the solute carrier (SLC) 6 family, responsible for betaine transport have been identified within the testis (Chen *et al.*, 2004). Alternatively, higher circulating levels of SAM, observed with betaine supplementation (Schwahn *et al.*, 2004) may be taken up by the testis and thus enable transmethylation reactions to continue within the testis.

It is clear from our studies though, that any benefits derived from early betaine exposure did not carry through to adulthood if betaine was removed at weaning. Adult

Mthfr$^{-/-}$ males given betaine to weaning exhibited a testicular phenotype similar to control-diet *Mthfr*$^{-/-}$ males. Decreased apoptosis at 6 dpp however, raised the question of whether continued betaine administration to *Mthfr*$^{-/-}$ males, beyond weaning, might have positive effects on the testis development and spermatogenesis. Indeed, with long-term betaine provision came improved testis weights, histology and remarkably, a small but significant increase in fertility.

Our most intriguing finding was that fertility, in a subset of *Mthfr*$^{-/-}$ males, could be restored upon provision of long-term betaine. Fertility appeared to be related to overall testicular sperm production. Thus, we have produced an interesting model for further studies of dietary/nutritional amelioration of genetic infertility. In mice, sperm production must be reduced to approximately 20% of normal levels before low sperm counts manifest as infertility (M. Handel, personal communication). Extremely low sperm counts may be the reason for the infertility observed in *Mthfr*$^{-/-}$ males given betaine only until weaning. However, in long-term betaine supplemented *Mthfr*$^{-/-}$ males, only two of the five were able to sire litters, despite all males having sperm counts 20% of normal values. Therefore, a threshold, above which a minimum number of normal sperm are produced, may exist. However, it remains to be seen if betaine is constantly required, from gestation through adulthood, to promote spermatogenesis, or if it could be used acutely in adults to renew spermatogenesis. Such acute treatments have been shown to partially rescue other defects of MTHFR deficiency (Schwahn *et al.*, 2003). It is possible that betaine may only be necessary until the testis is fully developed. Future studies are needed to determine the interval and timing of betaine supplementation necessary to recoup male fertility.

That betaine supplementation did not result in complete amelioration of the testicular phenotype of *Mthfr*$^{-/-}$ males agrees with Schwahn *et al.* (2004), who demonstrated that increased betaine corrected some characteristics of severe MTHFR

deficiency but not to wild-type levels. Although homocysteine and its metabolite, SAH, were significantly decreased in betaine-supplemented *Mthfr*$^{-/-}$ mice these levels were still substantially higher than those in *Mthfr*$^{+/+}$ males. These amounts of homocysteine and SAH may be adequate to inhibit transmethylation reactions. Moreover, correction did not occur to the same extent in all males provided with long-term betaine. It is currently unknown why some males would respond, to such a high degree, to treatment while others would not. Perhaps variations in neonatal or early post-natal homocysteine or methylation levels may explain the degree of response to betaine supplementation and the variation seen later in life.

3.5.3 Implications

Our finding that lack of MTHFR impairs spermatogenesis provides further support for the hypothesis that the 677C→T mutation in MTHFR may explain some degree of human male infertility. We have shown a substantial improvement in spermatogenesis in *Mthfr*$^{-/-}$ mice with continual provision of dietary betaine. Our findings suggest that infertile men with MTHFR mutations may benefit from diets supplemented with alternative methyl donors. Indeed, infertile men provided with folic acid report improved fertility and decreased round cell azoospermia (Bentivoglio *et al.*, 1993); such men may have defects in MTHFR or other enzymes involved in the folate pathway.

Many other mutations have been identified within the human *MTHFR* gene, among these a common 1298A→T variant. The decrease in enzyme activity associated with the 677C→T polymorphism is greater than that seen with the 1298 variant. Other mutations in folate pathway enzymes may have roles in hampering male fertility. However, as in the *Mthfr*$^{-/-}$ model, spermatogenesis is extremely difficult to study in these models due to complex systemic effects. Inactivation of these enzymes in a testis-specific manner (*e.g.*, TNAP-Cre-mediated deletion) may help to elucidate the role of these enzymes in testicular health.

3.6 ACKNOWLEDGEMENTS

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CONNECTING TEXT – CHAPTER III TO IV

Within the previous two chapters, I have described the spermatogenic effects of perturbing two key aspects of the DNA methylation pathway: through genetic and pharmacological inhibition of DNMT activity (5-azaCdR and *Dnmt1*$^{c/+}$) and through genetic reduction of methyl group availability (*Mthfr*$^{-/-}$). Both models resulted in compromised fertility and/or a rise in preimplantation loss. These studies, however, did not address the reasons for altered fertility indices, although they did suggest that alterations in sperm DNA methylation might underlie the infertility and preimplantation loss. Therefore, we wished to examine, in more detail, the possible mechanisms by which effects on fertility occurred, including altered sperm motility, preimplantation development, and DNA methylation of sperm. However, because the inhibition of MTHFR leads to a decreased methyl group supply, disruption of this enzyme could potentially affect any or all of the transmethylation reactions fed by S-adenosylmethionine, not just methylation of DNA. For this reason, in Chapter IV, we investigated changes in the motility and DNA methylation of sperm of 5-azaCdR-treated *Dnmt1*$^{+/+}$ and *Dnmt1*$^{c/+}$ males, but not MTHFR-deficient males, as well as *in vitro* preimplantation development of their progeny.

CHAPTER IV

MECHANISMS OF 5-AZA-2'-DEOXYCYTIDINE EFFECTS ON MALE MOUSE FERTILITY: ALTERED SPERM MOTILITY, DNA METHYLATION AND EMBRYO DEVELOPMENT

Tamara L.J. Kelly, Christopher C. Oakes and Jacquetta M. Trasler

4.1 ABSTRACT

Chronic low dose treatment of wild-type male mice with 5-aza-2'-deoxycytidine (5-azaCdR), a hypomethylating agent used in cancer treatment, results in decreased testicular weight, sperm count and reduced fertility. However, males deficient in DNMT1, the primary mammalian methyltransferase, appear to be more resistant to the cytotoxic effects of 5-azaCdR; these males experience less severe reductions in testis size and escape alterations in sperm count and fertility. Despite these differences, after matings with untreated females there is a significant increase in preimplantation loss in both genotypic groups. It is unclear whether this preimplantation loss is the result of an inability of 5-azaCdR treated sperm to fertilise or a loss of embryos during the preimplantation period. Our earlier studies suggested that one of the mechanisms underlying the effects of 5-azaCdR could be a reduction in DNA methylation. To gain a better understanding of the etiology of this elevated preimplantation loss, we have investigated the movement characteristics, fertilisation ability, and DNA methylation state of caudal sperm from 5-azaCdR treated *Dnmt1*$^{+/+}$ and *Dnmt1*$^{c/+}$ male mice. As in our previous studies, sperm from *Dnmt1*$^{+/+}$ males appear to be more vulnerable to the effects of 5-azaCdR; at clinical doses, significantly reduced sperm motility and fertilisation ability were observed in these males, as well as a substantial loss of embryos at the blastocyst stage. While *Dnmt1*$^{c/+}$ males exhibit significant changes in fertilisation ability only, greater changes in methylation, as measured by the restriction landmark genome scanning (RLGS) technique, were observed in the sperm of *Dnmt1*$^{c/+}$ mice. We propose that the reduced fertility of *Dnmt1*$^{+/+}$ male mice exposed to 5-azaCdR is the result of both cytotoxicity and hypomethylation, with the former playing a larger role, whereas the changes observed after treatment of *Dnmt1*$^{c/+}$ males are attributed more to hypomethylation and its consequences.

4.2 INTRODUCTION

An essential modification of mammalian DNA, methylation at cytosine residues is catalysed by a family of DNA methyltransferases (DNMTs) and occurs at 60 to 80% of CpG dinucleotides throughout the mammalian genome. DNA methylation is known to play a pivotal role in numerous processes and is often associated with transcriptional repression and genomic stability (Bird, 2002). In cancer, dysregulation of DNA methylation is often observed (Das & Singal, 2004; Egger *et al.*, 2004).

Around the time primordial germ cells (PGCs) enter the gonad, somatic patterns of DNA methylation are erased. In the male mouse, the new germ line-specific patterns of methylation are initiated before birth in the prospermatogonia, but are not completed until after birth when the cells reach the pachytene phase of meiosis I (Davis *et al.*, 1999; Davis *et al.*, 2000). The highly regulated nature of methylation and the tightly controlled dynamics of the various methyltransferases throughout spermatogenesis (Trasler *et al.*, 1992; Benoit & Trasler, 1994; Jue *et al.*, 1995; La Salle *et al.*, 2004) point to an important role for methylation in the process of male germ cell development. Accordingly, we propose that disruption of methylation may have adverse effects on developing sperm and impair their ability to function normally.

Two types of methylation act to initiate and propagate methylation patterns: *de novo* methylation and maintenance methylation, respectively. These two processes are accomplished by different members of the methyltransferase family. The known *de novo* methyltransferases are DNMT3a and DNMT3b (Okano *et al.*, 1999), and the maintenance methyltransferase is DNMT1 (Li *et al.*, 1992). Levels of the predominant mammalian methyltransferase, DNMT1, are highest in the gonads of adult mice, and *Dnmt1* mRNA and protein are expressed at high levels in mitotic and early meiotic germ cells (Singer-Sam *et al.*, 1990; Trasler *et al.*, 1992; Numata *et al.*, 1994). Mice homozygous for a targetted gene mutation in the catalytic domain of DNMT1 are growth retarded, die prior to mid-gestation and have extremely hypomethylated DNA (<5%), which indicates that

DNA methylation is essential in development (Li *et al.*, 1992; Lei *et al.*, 1996). Because of the embryo lethal nature of this knockout, it is inappropriate for studies of the spermatogenic effects of hypomethylation. On the other hand, germ cell-specific deficiency in DNMT3a or DNMT3l, but not DNMT3b, results in severe spermatogenic defects without the confounding systemic effects of whole animal knockouts (Bourc'his *et al.*, 2001; Bourc'his & Bestor, 2004; Kaneda *et al.*, 2004). Deficiency in either DNMT3a or DNMT3l produces infertile males with dramatically reduced testis weights; these testes lack mature germ cells (*e.g.*, spermatids) and methylation analysis of early postnatal germ cells revealed disrupted paternal imprints (*e.g.*, *H19*). While these DNMT germ cell-specific knockouts do demonstrate the importance of normal methylation in male germ cell development, their phenotypes are severe. As such, they do not allow for examination of the more subtle effects on male germ cell development and function induced by lowering, but not completely eradicating, DNA methylation.

To this end, it is promising to note that DNA methylation may also be modified pharmacologically. 5-Aza-2'-deoxycytidine (5-azaCdR) is a cytosine analogue that incorporates into replicating DNA. The presence of a nitrogen atom at the fifth position of the pyrimidine ring blocks methylation by impeding transfer of the methyl group from S-adenosylmethionine (SAM); ultimately, the DNMT remains bound to the DNA as a covalent adduct (recall mechanism in Fig. 1.5C). 5-AzaCdR inhibits methylation directly by blocking methylation at drug-incorporated sites via the above mechanism, and indirectly through depletion of cellular enzymes by covalent trapping. One advantage to using 5-azaCdR is that it effectively inhibits all of the known or potentially unknown methyltransferases with methylating capabilities.

A model of hypomethylation in the male germ line has been created by treating male mice haploinsufficient for *Dnmt1* (*Dnmt1*$^{c/+}$) with 5-azaCdR (Kelly *et al.*, 2003). These mice have only 50% of wild-type levels of DNMT1, but are otherwise healthy and

are indistinguishable from their wild-type littermates. Previous studies by other groups have indicated that, in other tissues, this combination of 5-azaCdR treatment and *Dnmt1* heterozygosity results in hypomethylated DNA with less cellular toxicity than when wild-type mice are treated with this drug (Jüttermann *et al.*, 1994; Laird *et al.*, 1995). These results led us to believe that the combination of these two approaches offers a method by which to stress the methylation dynamics of male gametes to a greater extent than in wild-type males.

Indeed, our previous studies show that 5-azaCdR treatment of $Dnmt1^{+/+}$ males elicits more severe effects than observed with $Dnmt1^{c/+}$ males. At clinical doses of 5-azaCdR, $Dnmt1^{+/+}$ mice experience significant changes in testis weight, sperm count, and fertility, whereas $Dnmt1^{c/+}$ males suffer only decreased testis weight; this indicates genotype-specific differences in response to 5-azaCdR. Thus, sperm of $Dnmt1^{c/+}$ mice appear to suffer less cytotoxic damage via accumulation of covalent adducts (Jüttermann *et al.*, 1994) and may overall be less damaged by treatment than those of $Dnmt1^{+/+}$ males. Despite these differences in response to treatment, both genotypic groups experienced a significant increase in preimplantation loss after matings with untreated females. As described earlier, preimplantation loss represents either the inability of sperm to fertilise oocytes or the death of an embryo during the preimplantation period. The aim of the present study was to ascertain the nature of the increased preimplantation loss and to determine if there were genotypic differences underlying this loss. To answer these questions, we examined sperm motility and DNA methylation as well as *in vitro* development of embryos sired by treated fathers.

4.3 MATERIALS AND METHODS

4.3.1 Animals

Male $Dnmt1^{+/+}$ and $Dnmt1^{c/+}$ mice, heterozygous for a deletion within the catalytic domain of the primary mammalian DNA methyltransferase, *Dnmt1* (Lei *et al.*,

1996), were bred and raised in our own facilities (McGill University – Montreal Children's Hospital Research Institute) on a C57BL/6 background. Male mice of both genotypes were obtained through crosses of *Dnmt1*$^{c/+}$ males and C57BL/6 females. PCR genotyping of mice was performed as described by Kelly *et al.* (2003). Adult virgin C57BL/6 and CD1 females were obtained from Charles River, Canada (St. Constant, Canada). All mice were maintained on a 12:12 hour light/dark cycle and were provided with food and water *ad libutum*. Animal experiments were carried out according to the principles and procedures detailed in the Guide to the Care and Use of Experimental Animals, by the Canadian Council on Animal Care.

4.3.2 Treatment

Dnmt1$^{+/+}$ and *Dnmt1*$^{c/+}$ males (age 7 to 10 weeks) were randomly assigned to one of two treatment groups (Saline: *Dnmt1*$^{+/+}$, n = 13; *Dnmt1*$^{c/+}$, n = 15. 5-AzaCdR: *Dnmt1*$^{+/+}$, n = 14; *Dnmt1*$^{c/+}$, n = 15). Males were treated 3 times a week for 7 weeks, by intraperitoneal injection, with either saline or 0.1 mg/kg 5-azaCdR to expose male germ cells throughout their development. Throughout treatment, males were weighed twice per week. After 7 weeks of treatment, males were mated with four virgin superovulated CD1 females (age 8 weeks) and then sacrificed. The testes, epididymides, seminal vesicles and spleen were removed, weighed, snap-frozen and stored at -80°C. A section of liver was also removed and frozen. Spermatozoa from the caudal epididymides were isolated and purified as described by Alcivar *et al.* (1989) with modifications by Kelly *et al.* (2003); sperm were stored at -80°C until further use.

4.3.3 Southern Blotting

Southern blots were performed as described previously (Trasler *et al.*, 1990) and visualised by autoradiography. Major and minor satellite probes were constructed by PCR amplification of mouse genomic DNA using primers (MajF1) 5'-GACGACTTGAAAAATGACGAATC-3', (MajR1) 5'CATATTCCAGGTCCTTCAGT-GTGC-3', (MinF1) 5'-CATGGAAAATGATAAAAACC-3' and (MinR1) 5'-

CATCTAATATGTTCTACAGTGTGG-3' (Lehnertz *et al.*, 2003). The IAP probe was a gift of T. Bestor and has been used previously (Michaud *et al.*, 1994; Walsh *et al.*, 1998) High molecular weight genomic DNA from treated male caudal sperm was obtained according to Okazaki *et al.* (1995) with modifications by Oakes *et al.* (2003). DNA was then completely digested with either *MspI* or its methylation-sensitive isoschizomer, *HpaII* (Invitrogen, Burlington, ON). The membrane was stripped and reprobed according to the manufacturer's recommended conditions (Hybond, Amersham BioSciences, Baie d'Urfé, QC). Each lane represented an individual animal.

4.3.4 Restriction Landmark Genome Scanning

High molecular weight genomic DNA was obtained from caudal sperm of treated *Dnmt1*$^{+/+}$ and *Dnmt1*$^{c/+}$ mice as described by Okazaki *et al.* (1995) with modifications (Oakes *et al.*, 2003). Restriction landmark genome scanning (RLGS) was then performed as described by Okazaki *et al.* (1995) using the sperm of 3 males per treatment group. Sperm were not pooled, so that each RLGS profile represented an individual animal. The criterion for an observed change was differential methylation in two of the three individuals.

The number of hypermethylated loci present within the analysed region of the RLGS profile was calculated by *in silico* digestion of the published mouse genomic sequence (Mouse Genome Sequencing Consortium, October 2002). Using a two-dimensional spot mobility/fragment size relationship, the fraction of genomic fragments that would migrate into the analysed region was determined. The number of loci visible on the actual RLGS profile, which are biologically hypomethylated, was then subtracted from those present in the virtual profile to calculate the number of hypermethylated loci.

4.3.5 Sperm Motility Analyses

Sperm motility of treated *Dnmt1*$^{+/+}$ and *Dnmt1*$^{c/+}$ male mice (n = 6/group) was analysed using an IVOS semen analyser (Hamilton-Thorne Research, Beverly, MA) with parameters determined by the Jackson Laboratory (courtesy of Hamilton-Thorne). All

dishes and slides were kept at 37°C during all steps. Briefly, the cauda epididymidis was tied off, both proximally and distally, removed from the epididymis and rinsed in 3 ml of warmed M199 medium with Hank's salts (Sigma, St. Louis, MO) supplemented with 0.5% w/v BSA, pH 7.4 (GIBCO, Mississauga, ON) in a 35 mm Petri dish at 37°C. The cauda epididymidis was then moved to a new Petri dish containing 3 ml of warm supplemented M199 medium, then minced and the epididymal tissue removed. Sperm were allowed to disperse for 5 minutes. The sperm suspension was diluted 1:10 in warm supplemented medium prior to motility analyses such that concentration did not impair motility. The diluted suspension (20μl) was loaded into a pre-warmed 2X-CEL Sperm Analysis Chamber (80μm deep) (Hamilton-Thorne Research). Movement characteristics analysed were: percent motility – motile sperm divided by the sum of the motile and immotile sperm within the analysis field; percent progressive motility – progressively motile sperm divided by the sum of motile and immotile sperm within the field; average path velocity (VAP) – the average velocity of the smoothed cell path; progressive/straight line velocity (VSL) – the average velocity measured in a straight line from the beginning to end of the track; curvilinear velocity (VCL) – the sum of the distances moved in each frame along the sampled path divided by the time taken to cover the track; amplitude of lateral head displacement (ALH); beat cross frequency (BCF) – the frequency with which the sperm track crosses the sperm path; straightness (STR) – the departure of the cell path from a straight line; and linearity (LIN) – the departure of the cell track from a straight line.

Tracks were digitally recorded at 60Hz under 4X dark-field illumination. Analysis was completed using the following IVOS settings: stage temperature, 37°C; frames acquired, 30; frame rate, 60 Hz; minimum contrast, 30; minimum cell size, 4 pixels; magnification, 0.81; cell intensity, 75; static size, 0.13-2.43; static intensity, 0.10-1.52. Five slides were analysed for each mouse and each slide was sampled five times such that a minimum of 300 sperm were analysed per slide. The mean of the five slides

was calculated for each mouse.

4.3.6 Mating and Embryo Culture

Adult female CD1 mice aged 8 weeks were superovulated by administration of 5 IU of pregnant mare serum gonadotropin (PMSG; Sigma) followed by 5 IU of human chorionic gonadotropin (hCG; Sigma) 48 hours later. To obtain fertilised embryos, each male (n = 7 – 9/treatment group), after 7 weeks of treatment, was mated overnight with 1 virgin superovulated CD1 female per night for 4 nights, for a total of 4 females per male. The next morning females were examined for the presence of a vaginal plug. One-cell embryos and unfertilised oocytes were isolated at 27 hours post-hCG and cumulus cells removed by hyaluronidase treatment (1mg/ml) (Sigma) in HEPES-buffered M2 medium (Sigma). Oocytes were examined for the presence of two pronuclei indicating that fertilisation had taken place. Oocytes were classified as fertilised, unfertilised or fragmented; the majority of fragmented oocytes could not be evaluated as to fertilisation status, and thus, were not subcategorised. Fertilised embryos were washed three times, using a mouth-controlled drawn-out glass pipette, and placed into pre-equilibrated bicarbonate-buffered kSOM medium (Erbach *et al.,* 1994) with gentomycin, under oil, and cultured under an atmosphere of 5%O_2, 5%CO_2, in nitrogen at 37°C in a humidified modular incubator (Billups-Rothenberg, Del Mar, CA). Embryos were examined daily, on a heated stage, and scored for development through to the blastocyst stage. Because discrepancies exist in linking cytofragmentation with poor embryo viability, embryos with cytofragmentation were not removed from culture. Data are presented on a per male basis; to avoid skewing of data males were removed from all data sets if less than 10 eggs, in total, were collected from females mated to that male; only two males were removed, one saline *Dnmt1*$^{+/+}$ male and one 5-azaCdR-treated *Dnmt1*$^{c/+}$ male.

4.3.7 Statistical Analyses

Statistical analysis was performed using SigmaStat 2.03 software (SPSS, Chicago, IL). Significant differences ($p<0.05$) between treatment groups with respect to the various

motility parameters were detected using two-way ANOVA, with a post-hoc Tukey test. Embryo data are expressed on a per male basis and were evaluated for significance ($p<0.05$) using three-way ANOVA, with a post-hoc Tukey test.

4.4 RESULTS

4.4.1 General Results

As observed in our previous study (Kelly *et al.*, 2003), treatment with 5-azaCdR elicited no obvious changes in behaviour and weight, although initial and final body weights of the two genotypes were significantly different ($p<0.05$). Again, testis weights were significantly decreased in treated males, regardless of genotype ($p<0.001$), and similar to our previous studies, the extent of reduction was considerably less in *Dnmt1*$^{c/+}$ males than in *Dnmt1*$^{+/+}$ males ($p<0.05$).

4.4.2 Methylation of Repetitive Sequences

The majority (30% – 40%) of methylcytosines (m^5C) within the mammalian genome are located in repeat sequences (Yoder *et al.*, 1997). To address whether chronic treatment of *Dnmt1*$^{+/+}$ and *Dnmt1*$^{c/+}$ males with the hypomethylating agent 5-azaCdR results in altered methylation of sperm DNA, the methylation status of three repetitive mouse sequences were determined by Southern blotting. Genomic DNA was isolated, digested with the methylation insensitive restriction enzyme *MspI* or its isoschizomer, the methylation-sensitive *HpaII*, and then hybridised to probes specific for the dispersed *IAP* elements or the pericentromeric minor and major satellite sequences. As demonstrated by the lack of digestion with *HpaII*, our results indicate that neither DNMT1 deficiency nor 5-azaCdR treatment results in significantly altered methylation at *IAP*, minor satellite or major satellite sequences as demonstrated by the lack of digestion with *HpaII* (Fig. 4.1A-C).

4.4.3 Methylation at Individual Loci

Although methylation was not altered at highly repetitive elements we wondered if DNA methylation might be impaired at single-copy loci. To examine the methylation of individual loci, we used the restriction landmark genome scanning (RLGS) technique. RLGS is a sensitive method that reveals the methylation status of multiple sites located throughout the genome via genomic DNA digestion with the methylation-sensitive restriction enzyme, *NotI*. The presence of a spot on the RLGS profile indicates that *NotI* could cut, and is indicative of a lack of methylation at that specific locus. Because more than 90% of *NotI* restriction sites reside within CpG islands (Lindsay & Bird, 1987), this method produces a methylation profile that focuses primarily on the methylation status of transcribed sequences.

RLGS profiles of the two treatment extremes, saline-treated $Dnmt1^{+/+}$ sperm and 5-azaCdR-treated $Dnmt1^{c/+}$ sperm, are shown in Figure 4.2A. To determine changes in methylation state, all treatment groups were compared to the saline-treated $Dnmt1^{+/+}$ profile. Haploinsufficiency of *Dnmt1* resulted in demethylation at only one locus, whereas 5-azaCdR treatment of $Dnmt1^{+/+}$ males was associated with demethylation of five loci different than the locus altered in the $Dnmt1^{c/+}$ saline group (Table 4.1). In addition to those sites altered with *Dnmt1* haploinsufficiency or 5-azaCdR alone, six other loci were also demethylated in the sperm of $Dnmt1^{c/+}$ males exposed to 5-azaCdR (Table 4.1; Fig. 4.2A). Paradoxically, in addition to the 11 demethylated loci, three sites were hypermethylated in the 5-azaCdR-treated $Dnmt1^{c/+}$ group (Table 4.1; Fig. 4.2A-B); hypermethylation was not observed in any other treatment group.

Figure 4.1. DNA methylation of repeat sequences in sperm of *Dnmt1*$^{+/+}$ and *Dnmt1*$^{c/+}$ males treated with saline or 5-azaCdR. Genomic DNA was digested with *MspI* or *HpaII* and hybridised to probes specific for **(A)** *IAP* repeats (LTR), **(B)** major satellite and **(C)** minor satellite repetitive sequences. **(A-C)** represent the same blot after being stripped and hybridised to each respective probe. +/+, *Dnmt1*$^{+/+}$. c/+, *Dnmt1*$^{c/+}$.

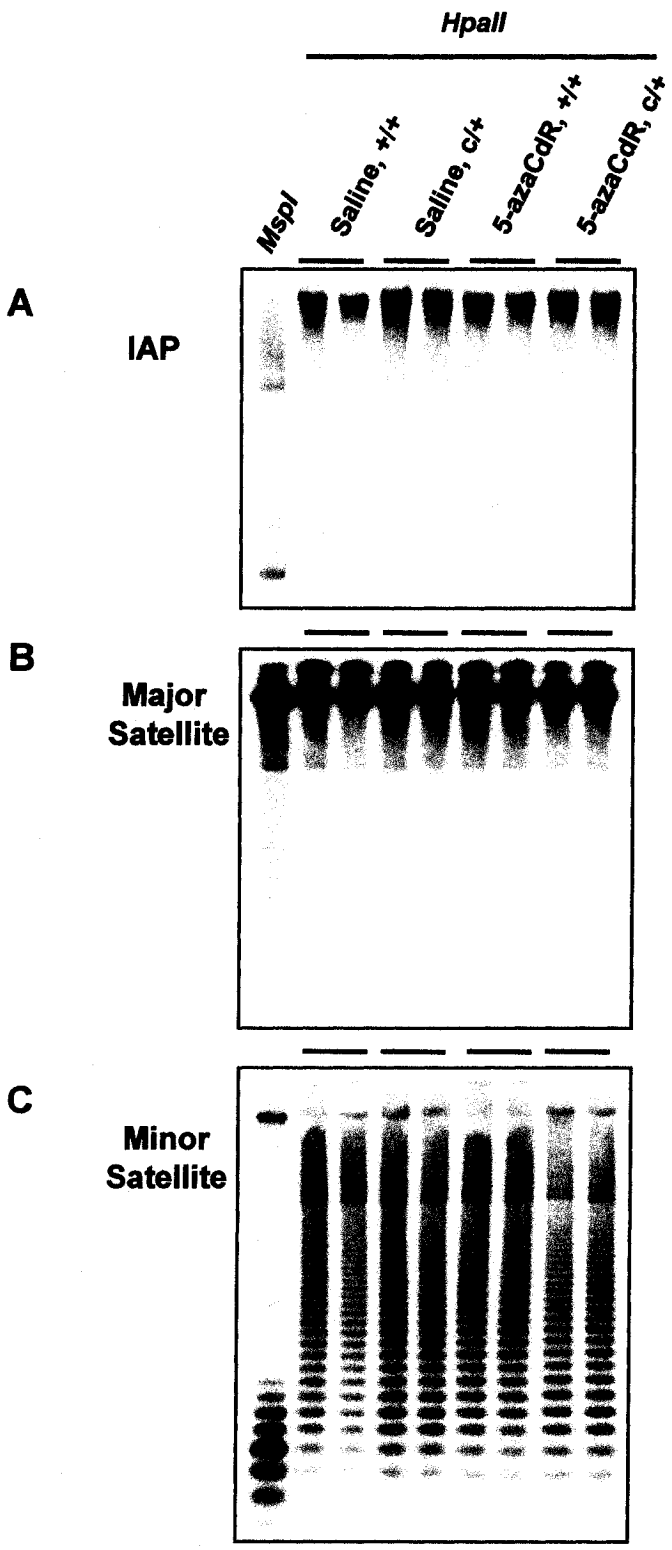

HpaII
MspI
Saline, +/+
Saline, c/+
5-azaCdR, +/+
5-azaCdR, c/+
A
IAP
B
Major Satellite
C
Minor Satellite

Figure 4.2. RLGS profiles of caudal sperm from saline and 5-azaCdR-treated *Dnmt1*$^{+/+}$ and *Dnmt1*$^{c/+}$ mice. **(A)** Representative profiles of sperm methylation from *Dnmt1*$^{+/+}$ saline-treated males and *Dnmt1*$^{c/+}$ 5-azaCdR-treated males. Open arrowheads in **(A)** represent loci hypermethylated in sperm from 5-azaCdR-treated *Dnmt1*$^{c/+}$ males compared to sperm from saline-treated *Dnmt1*$^{+/+}$ males. Filled arrowheads in **(A)** represent loci hypomethylated in sperm from 5-azaCdR-treated *Dnmt1*$^{c/+}$ males compared to sperm from saline-treated *Dnmt1*$^{+/+}$ males. **(C)** Enlargements of loci **a-c**, shown as boxes in **(A)**. Filled arrows in **(B)** represent hypomethylation of that site. Numbers across the bottom of **(C)** represent individual animals.

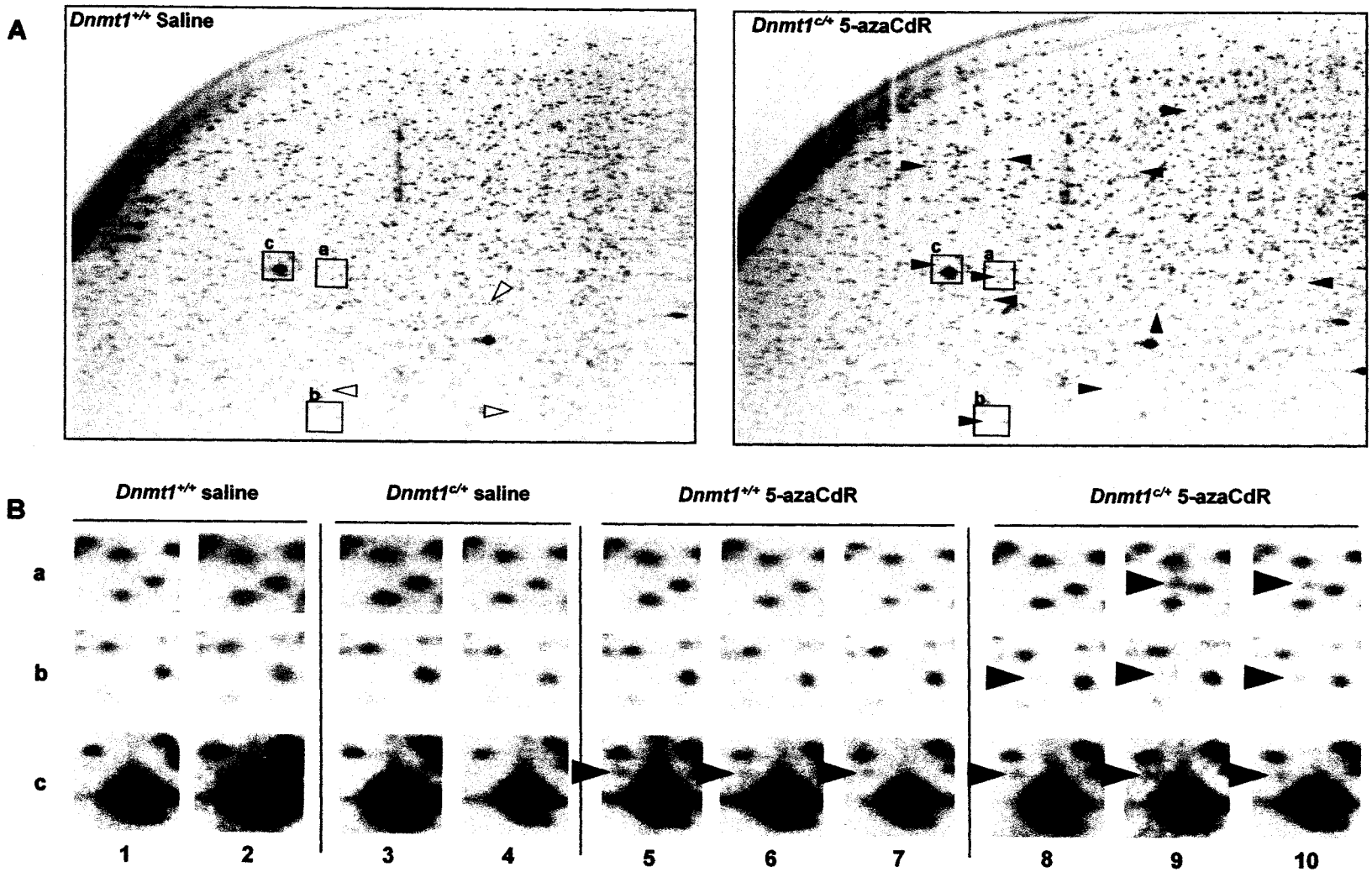

A
Dnmt1+/+ Saline
c
a
b
Dnmt1c/+ 5-azaCdR
c
a
b
B
Dnmt1+/+ saline
Dnmt1c/+ saline
Dnmt1+/+ 5-azaCdR
Dnmt1c/+ 5-azaCdR
a
b
c
1
2
3
4
5
6
7
8
9
10

Table 4.1: Quantitation of site-specific changes in sperm methylation after chronic 5-azaCdR treatment of *Dnmt1*$^{+/+}$ and *Dnmt1*$^{c/+}$ males compared to saline-treated *Dnmt1*$^{+/+}$ males.

Methylation State of *NotI* Site	Total Loci	Saline	5-azaCdR	
		Dnmt1$^{c/+}$	*Dnmt1*$^{+/+}$	*Dnmt1*$^{c/+}$
Demethylated	1294	1	5	11
Hypermethylated	1412	0	0	3

4.4.4 Sperm Motility

Sperm movement was assayed using a Hamilton-Thorne IVOS semen analyser, allowing simultaneous evaluation of several motility parameters. While generally both genotypes experienced decreases in the various motility parameters after treatment with 5-azaCdR, the reduction was less extensive in *Dnmt1*$^{c/+}$ mice. Both motility and progressive motility were significantly lowered, by 24% and 34% respectively, in sperm from 5-azaCdR-treated *Dnmt1*$^{+/+}$ males (Fig. 4.3A), but not in sperm from 5-azaCdR-treated *Dnmt1*$^{c/+}$ males (14% and 17%, respectively). For those kinetic properties which were significantly different in 5-azaCdR *Dnmt1*$^{+/+}$ sperm, the extent of change was consistently nearly twice that experienced by treated *Dnmt1*$^{c/+}$ sperm. Of the 15 CASA-evaluated parameters, 10 were significantly altered in treated *Dnmt1*$^{+/+}$ males, while only two were affected in treated *Dnmt1*$^{c/+}$ males. Although chronic exposure to 5-azaCdR did not alter STR, LIN or BCF in either group, all three sperm velocity characteristics (VCL, VSL and VAP) were significantly different in 5-azaCdR-treated *Dnmt1*$^{+/+}$ males (Table 4.2), while only one, VCL, was significantly lower in 5-azaCdR-treated *Dnmt1*$^{c/+}$ sperm; again this decrease was half of that observed for treated *Dnmt1*$^{+/+}$ males. Similarly, the proportion of rapidly moving sperm dropped substantially (29%) in treated *Dnmt1*$^{+/+}$ samples, but not in treated *Dnmt1*$^{c/+}$ sperm. Notably, in both groups the reduction in

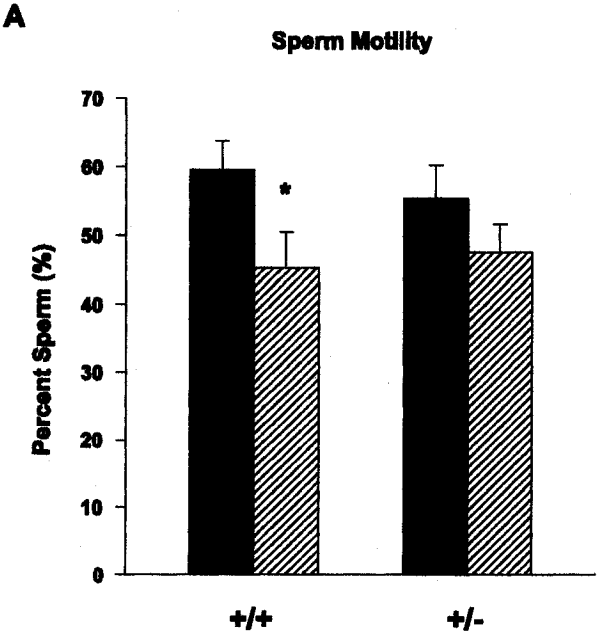
rapid sperm corresponded to an increase in static sperm, but not in medium or slow sperm. This suggests that 5-azaCdR treatment may impede the maturation of, or alter the structure of, exposed sperm such that a subpopulation of sperm is unable to move. It is possible that in addition to altered motility, the morphology of 5-azaCdR exposed sperm could also be affected. Both Area, which measures sperm head size, and Elongation, the ratio of the minor axis to major axis, were significantly different in treated *Dnmt1*$^{+/+}$ sperm compared to control values. These observations, combined with the fact that no motility variables were affected solely in 5-azaCdR-treated *Dnmt1*$^{c/+}$ sperm, support our hypothesis that *Dnmt1*$^{+/+}$ sperm are more adversely affected by chronic exposure to 5-azaCdR than *Dnmt1*$^{c/+}$ sperm, particularly in terms of their motility.

4.4.5 Fertilisation Ability is Altered in 5-AzaCdR Exposed Sperm

Both failure of sperm to fertilise oocytes and early embryo death could account for the preimplantation loss associated with chronic 5-azaCdR treatment. For example, if the kinetics of sperm movement are compromised by 5-azaCdR treatment, as suggested above, sperm may be unable to reach or penetrate the oocyte. To determine the underlying cause of elevated preimplantation loss, and thereby evaluate the ability of treated sperm to fertilise and support normal preimplantation embryo development, each male was mated to four superovulated females (total matings = 132), and embryos were collected at the one-cell stage. The presence of a vaginal plug on the morning after mating indicated successful copulation, and the copulation rate was similar for all treatment groups; only females with vaginal plugs were used for embryo collection. An average of 502 ± 76 oocytes were scored per treatment group (*Dnmt1*$^{+/+}$: saline, 329; 5-azaCdR, 580; *Dnmt1*$^{c/+}$: saline, 430; 5-azaCdR, 671); an oocyte was considered fertilised if two pronuclei were present. Whereas the incidence of fragmented oocytes/embryos was unaffected by treatment, the proportion of fertilised oocytes was dramatically reduced by 70% and 56% ($p<0.001$) after matings with treated *Dnmt1*$^{+/+}$ and *Dnmt1*$^{c/+}$ males,

Figure 4.3. Effects of chronic 5-azaCdR treatment on **(A)** sperm motility and **(B)** progressive sperm motility. Black bars represent *Dnmt1*$^{+/+}$ and *Dnmt1*$^{c/+}$ males treated with saline. Diagonal striped bars represent 5-azaCdR-treated *Dnmt1*$^{+/+}$ and *Dnmt1*$^{c/+}$ males. Bars represent means ± SEM. * $p<0.05$ vs. *Dnmt1*$^{+/+}$ control.

A


Sperm Motility
Percent Sperm (%)
70
60
50
40
30
20
10
0
*
+/+
+/-

B

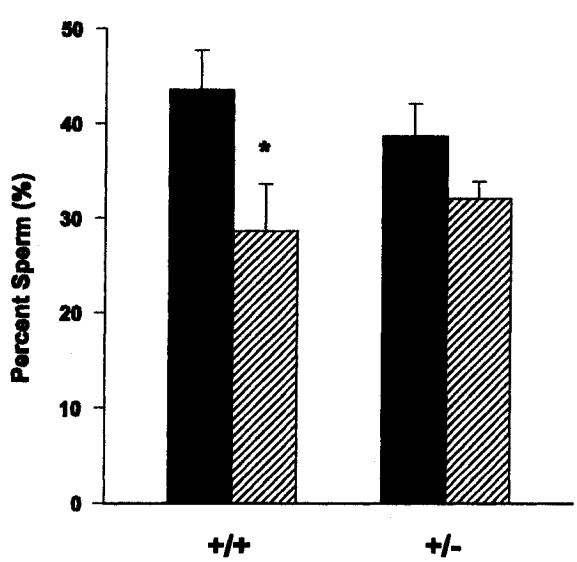

Progressive Motility
Percent Sperm (%)
50
40
30
20
10
0
*
+/+
+/-

Table 4.2: Effects of chronic 5-azaCdR treatment on *Dnmt1*$^{+/+}$ and *Dnmt1*$^{c/+}$ sperm motility parameters (as measured by CASA).

Sperm Parameter	*Dnmt1*$^{+/+}$		*Dnmt1*$^{c/+}$	
	Saline	**5-azaCdR**	**Saline**	**5-azaCdR**
VCL (μm/s) (track speed)	243.93 ± 4.91	191.54 ± 9.88*	231.04 ± 4.88	203.04 ± 3.66†
VSL (μm/s) (progressive velocity)	88.61 ± 3.13	68.17 ± 5.88*	84.74 ± 1.76	77.48 ± 2.39
VAP (μm/s) (path velocity)	125.22 ± 3.42	99.68 ± 5.40*	118.16 ± 2.51	105.61 ± 2.09$^{\phi}$
STR (%) (straightness)	69.65 ± 0.67	65.80 ± 2.78	70.82 ± 0.95	71.76 ± 1.22
LIN (%) (linearity)	37.49 ± 0.50	36.29 ± 1.62	38.04 ± 0.68	39.48 ± 0.65
Rapid Sperm (%)	52.76 ± 4.39	37.23 ± 5.09*	47.48 ± 4.73	39.04 ± 2.26
Medium Sperm (%)	6.65 ± 0.52	7.75 ± 0.92	7.76 ± 1.21	8.56 ± 2.16
Slow Sperm (%)	0.34 ± 0.12	0.30 ± 0.06	0.56 ± 0.14	0.44 ± 0.17
Static Sperm (%)	40.07 ± 4.15	54.55 ± 5.07*	44.06 ± 4.87	51.12 ± 4.13
BCF (beat cross frequency)	27.36 ± 0.58	27.30 ± 0.53	28.84 ± 0.89	29.17 ± 0.94
ALH (amplitude of lateral head movement)	13.24 ± 0.11	10.98 ± 1.29*	12.67 ± 0.17	11.28 ± 0.22†
Area	45.64 ± 3.07	35.41 ± 2.62*	43.57 ± 1.37	41.57 ± 1.40
Elongation	46.37 ± 0.97	50.65 ± 1.11*	47.04 ± 0.26	48.28 ± 0.71

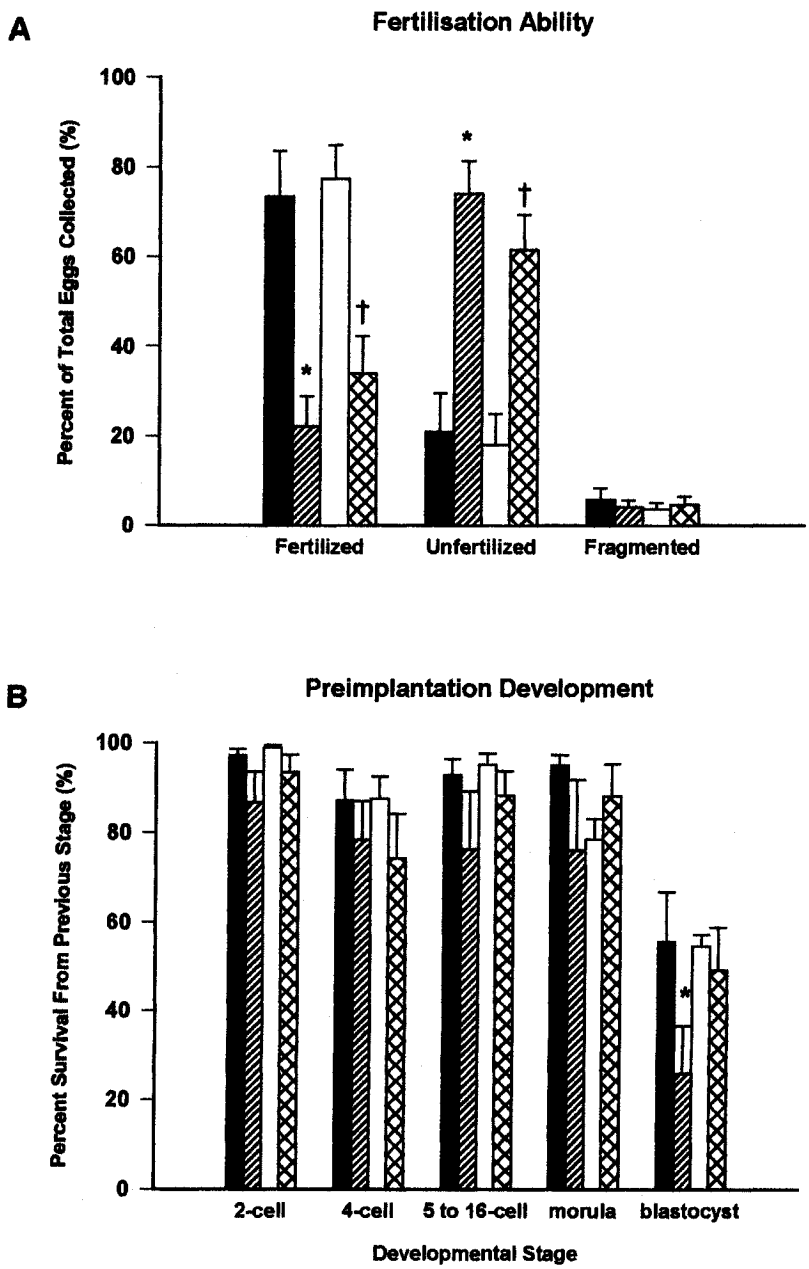
*significantly different vs. saline *Dnmt1*$^{+/+}$, $p \leq 0.05$
†significantly different vs. saline *Dnmt1*$^{c/+}$, $p \leq 0.05$
$^{\phi}p=0.059$ vs. saline *Dnmt1*$^{c/+}$

respectively (Fig. 4.4A). This decrease was reflected in a concomitant three-fold increase ($p<0.001$) in the incidence of unfertilised oocytes, but not fragmented oocytes, in both treated groups relative to their saline controls. These results suggest that for one reason or another chronic 5-azaCdR treatment renders a subset of sperm, in males of both genotypes, unable to fertilise oocytes.

4.4.6 In Vitro Embryo Development

To assess the ability of embryos sired by treated males to progress normally through preimplantation development, fertilised oocytes were placed into culture and scored daily for survival to advanced preimplantation stages. Embryo viability was calculated as the percent of embryos that survived from the previous stage. As Figure 4.4B illustrates, there was no change in the viability of embryos throughout preimplantation development, with the exception of the blastocyst stage. At this point, the embryo survival within each treatment group dropped by approximately 50%; however, the survival of embryos sired by treated $Dnmt1^{+/+}$ males dropped by 75%, which was significantly more than the $Dnmt1^{+/+}$ control group (55%) ($p<0.05$). No such decrease was observed for embryos sired by treated $Dnmt1^{c/+}$ males ($p=0.643$). It therefore appears that the elevation in preimplantation loss after matings with 5-azaCdR-treated $Dnmt1^{+/+}$ males, but not $Dnmt1^{c/+}$ males, can partly be accounted for by loss of embryos just prior to implantation.

Figure 4.4. **(A)** Effect of chronic 5-azaCdR treatment on the ability of sperm from saline and 5-azaCdR-treated *Dnmt1*$^{+/+}$ and *Dnmt1*$^{c/+}$ males to fertilise eggs. Fragmented eggs may or may not be fertilised. Percentages of fertilised, unfertilised and fragmented eggs are shown on a per male basis and represent a proportion of total eggs collected. **(B)** Viability of embryos sired by 5-azaCdR-treated males through the stages of preimplantation development. Percentages reflect the survival of embryos from the previous stage. In both **(A)** and **(B)**, black bars and white bars represent saline-treated *Dnmt1*$^{+/+}$ and *Dnmt1*$^{c/+}$ males, respectively; diagonal bars represent embryos sired by 5-azaCdR-treated *Dnmt1*$^{+/+}$ males; hatched bars represent embryos sired by 5-azaCdR-treated *Dnmt1*$^{c/+}$ males. Bars represent means ± SEM and information is presented on a per male basis. * $p<0.05$ from *Dnmt1*$^{+/+}$ control; † $p<0.05$ from *Dnmt1*$^{c/+}$ control.


A
Fertilisation Ability
Percent of Total Eggs Collected (%)
100
80
60
40
20
0
*
†
*
†
Fertilized
Unfertilized
Fragmented
B
Preimplantation Development
Percent Survival From Previous Stage (%)
100
80
60
40
20
0
*
2-cell
4-cell
5 to 16-cell
morula
blastocyst
Developmental Stage

4.5 DISCUSSION

The goal of our current study was to investigate potential reasons for the preimplantation loss observed after matings of 5-azaCdR-treated *Dnmt1*$^{+/+}$ and *Dnmt1*$^{c/+}$ males (Kelly *et al.*, 2003; Appendix I). To that end, sperm from treated males were analysed for motility, DNA methylation and the ability to support preimplantation development *in vitro*. Here we demonstrate that, as observed in our previous studies, *Dnmt1*$^{+/+}$ mice appear to be more vulnerable to the deleterious effects of 5-azaCdR than are *Dnmt1*$^{c/+}$ males. Treated *Dnmt1*$^{+/+}$ males exhibited impaired sperm motility, evidenced by significant changes in numerous motility characteristics (10 of 15 examined), as well as decreased fertilisation ability and reduced survival of sired embryos at the blastocyst stage. In contrast, *Dnmt1*$^{c/+}$ males had significant changes only in fertilisation ability and in two of the fifteen motility parameters after treatment with 5-azaCdR. However, it was the sperm of *Dnmt1*$^{c/+}$ males that displayed greater changes in DNA methylation, as demonstrated by RLGS, than did sperm of treated *Dnmt1*$^{+/+}$ males.

4.5.1 Sperm Methylation of 5-AzaCdR-Treated Mice

In agreement with early reports that genomic methylation of DNMT-deficient mice is not altered (Li *et al.*, 1992), we found that sperm methylation of saline-treated *Dnmt1*$^{c/+}$ sperm differs very little from that of wild-type males. Yet, treatment of these DNMT-deficient males allows for a considerably greater degree of hypomethylation after treatment with 5-azaCdR. By increasing inhibition of DNMT through combined genetic and pharmacological means, we observed a synergistic effect in the number of loci at which methylation was altered.

A key advantage of RLGS is that the number of altered loci is placed within the context of genomic loci that remain unchanged by treatment conditions. A total of 2706 spots are potentially visible within the analysed region of the mouse RLGS profile, of which 1412 are normally demethylated and 1294 are normally methylated (Table 4.1).

Only 11 of 1294 loci that are normally methylated exhibit demethylation after inhibition of DNMT activity, leaving greater than 99% of loci unaffected. This number (1294) is likely an underestimation of the true quantity of hypermethylated loci, because of incomplete or missing sequences in the published mouse genome, which clearly demonstrates that the number of loci affected by 5-azaCdR treatment and/or *Dnmt1* genotype is restricted to a relatively small fraction of the genome. This underestimation is particularly significant when viewed in light of the fact that the loci altered in treated $Dnmt1^{+/+}$ sperm matched roughly half the sites hypomethylated in 5-azaCdR-treated $Dnmt1^{c/+}$ males.

Of the calculated 1294 hypermethylated loci, 785 originate from the endogenous retroviral sequences *IAP*, *MusD*, and *EtnII* (Baust, 2003). These sequences are normally methylated in all adult tissues to prevent transcriptional activation (Yoder *et al.*, 1997; Walsh *et al.*, 1998). In our studies, none of the observed demethylated loci are estimated to originate from any of these repetitive elements. This is consistent with the lack of demethylation of *IAP* observed by Southern blotting. The remaining 511 normally methylated *NotI* loci are presumed to arise from single-copy sequences (Lindsay & Bird, 1987) as they are not repetitive, and they may represent transcribed sequences. In support of this, there are 62 loci hypomethylated in RLGS profiles of various mouse somatic tissues, 60 of which remain methylated under all treatment regimens tested here (data not shown; C. Oakes, unpublished data).

We can use the changes observed in the RLGS profile to estimate the effect of our treatment regimens on a genome-wide scale. The number of CpG islands within the mouse genome was recently estimated to be 15 500 (Waterston *et al.*, 2002). If we extrapolate the loci altered out of the total non-repetitive loci that could become hyper- or hypomethylated (14/1921 = 0.73% of loci are altered) to this CpG island estimate, we can predict that, with 5-azaCdR treatment of $Dnmt1^{c/+}$ mice, approximately 110 loci are

altered within the genome. Altered methylation of potentially 110 loci in sperm may result in biologically significant effects, such as altered transcriptional profiles in both the less mature male germ cells and early embryos.

The generally accepted mechanism of 5-azaCdR action is that it inhibits DNMTs during the DNA replication phase of the cell cycle and thus causes widespread hypomethylation. However, the data we have presented here indicate that the action of 5-azaCdR *in vivo* may be restricted, at least in the testis, to specific loci. Moreover, we observed hypermethylation consistently at three specific loci within treated *Dnmt1*$^{c/+}$ sperm. This was unexpected as 5-azaCdR is believed to cause only hypomethylation. However, Blanchard *et al.* (2003) also found hypermethylation at one to three loci after treatment of cancer cells with 5-azaCdR. More work needs to be done to identify a mechanism of drug action whereby 5-azaCdR can affect certain sequences and not others, in addition to causing hypermethylation along with the expected hypomethylation. The future elucidation of the sequences and genes altered in this study may provide clues to this mechanism.

4.5.2 Altered Sperm Motility

Our previous studies suggested that 5-azaCdR exposed sperm, particularly of *Dnmt1*$^{+/+}$ males, had reduced fertilising ability. In both rodents and humans, negative correlations exist between reduced sperm motility and fertility (reviewed in Turner, 2003). We therefore examined sperm motility kinetics using the CASA system, which provides a high degree of reproducibility and a wealth of data on the movement characteristics of analysed sperm. As with previous endpoints, except for DNA methylation (Kelly *et al.*, 2003, Chapter II; Appendix I), sperm motility was more extensively and significantly altered in treated *Dnmt1*$^{+/+}$ males.

In humans, progressive motility, VCL, ALH, VSL and the proportion of rapidly moving sperm are positively correlated with fertility (Parinaud *et al.*, 1996a, 1996b;

Hirano *et al.*, 2001), as are LIN and percent motile sperm (Liu *et al.*, 1991). Altered sperm motility can result from abnormal head morphology or defects in tail ultrastructure, in addition to improper maturation of the sperm (Christensen & Carrell, 2002; Chemes & Rawe, 2003; Turner, 2003; Cooper *et al.*, 2004). Morphological aberrations of the head or tail are indicative of abnormal spermiogenesis.

During epididymal passage, mammalian sperm undergo maturation and gain their forward motility, an attribute that allows them to reach and penetrate the oocyte (Orgebin-Crist, 1967; Yeung *et al.*, 1992; Yanagimachi, 1994; Tulsiani *et al.*, 1998). Of the three parameters indicative of forward sperm motion, (VSL, STR and progressive motility), two were significantly altered in the sperm of treated *Dnmt1*$^{+/+}$ males, but none were altered in *Dnmt1*$^{c/+}$ males. Significant changes in these parameters suggest epididymal maturation of *Dnmt1*$^{+/+}$ sperm may be altered, in that treated sperm may be unable to respond to molecular cues within the epididymis that are necessary for maturation. Retention of the cytoplasmic droplet has been used as an marker of defective epididymal sperm maturation (Syntin & Robaire, 2001; Henderson & Robaire, 2005) and has been correlated with decreased fertility (Keating *et al.*, 1997; Akbarsha *et al.*, 2000). In the future, examination of treated sperm for head and tail morphology as well as retention of the cytoplasmic droplet may help to clarify the underlying reasons for decreased sperm motility.

Because there were significant changes in numerous motility parameters of treated *Dnmt1*$^{+/+}$ sperm, we speculate that altered sperm motility may explain a substantial proportion of the observed decreases in fertility of these mice. The observed changes in *Dnmt1*$^{+/+}$ sperm motility are greater than in other studies that have attributed subfertility to altered sperm movement (*e.g.*, Henderson & Robaire, 2005). It should be noted, however, that altered sperm motility is not necessarily a major contributing factor to the elevated preimplantation loss observed after matings with treated *Dnmt1*$^{c/+}$ males.

4.5.3 Fertility Analyses

Preimplantation loss has been shown to be associated with paternal treatment of both *Dnmt1*$^{+/+}$ and *Dnmt1*$^{+/+}$ males with 5-azaCdR (Kelly *et al.*, 2003), but because these studies were performed *in vivo*, it was unclear whether the elevation in preimplantation loss resulted from a lack of fertilisation by sperm or from embryo death prior to implantation, or perhaps a combination of the two. To probe this further, after *in vivo* matings oocytes were flushed and evaluated for fertilisation, and embryos examined for normal growth *in vitro*. Although similar reductions in fertilisation were observed for both treated groups, we propose that the observed increase in preimplantation loss following *Dnmt1*$^{c/+}$ paternal 5-azaCdR treatment was almost solely attributable to a lack of fertilisation. In contrast, a combination of reduced fertilisation, potentially because of altered sperm motility, and elevated embryo loss just prior to implantation may explain the elevated preimplantation loss observed in matings with treated *Dnmt1*$^{+/+}$ males.

4.5.3.1 Reduced Fertilisation

A reduction in the fertilising ability of sperm could be caused by any number of factors, as fertilisation is a complex affair comprised of a complex sequence of physical and biochemical events. For successful fertilisation to occur, sperm must reach the oocyte, penetrate the surrounding cumulus cell layer, bind and penetrate the zona pellucida, and bind and fuse with the oocyte plasma membrane to induce oocyte activation and completion of meiosis (Primakoff & Myles, 2002). Defects in any one of these steps could result in arrest of the fertilisation process. For example, sluggish sperm motility may contribute to a lower proportion of fertilised oocytes, as might an inability of sperm to properly bind the oocyte.

In humans and rodents, reduced fertility is correlated with increased sperm DNA damage and abnormal sperm chromatin packaging (Yu *et al.*, 2000; Aoki & Carrell, 2003; Carrell *et al.*, 2003a; Chemes & Rawe, 2003; Codrington *et al.*, 2004). 5-AzaCdR

incorporation has been linked to alterations in chromatin structure; however, it is unclear whether these effects are mediated through DNMT-drug adduct formation or via hypomethylation (reviewed in Haaf, 1995). Changes in chromatin can lead to, or be the result of: undercondensation of chromosomes, chromosomal rearrangements and breakages, and increased sister chromatid exchange, as well as micronuclei formation (Schmid *et al.*, 1984; Feichtinger & Schmid, 1989; Davidson *et al.*, 1992; Stopper *et al.*, 1995); whatever the case, these changes compromise the integrity of the sperm DNA. Defects such as these may also perturb the timely expression of genes necessary for the structural and biochemical development of sperm, and thus, impair normal maturation of the sperm within the testis or epididymis. Likewise, defects in paternal chromatin decondensation could render the sperm DNA resistant to remodelling by the ooplasm and therefore terminate fertilisation (Agarwal & Allamaneni, 2004). It is plausible that treated sperm were able to penetrate the zona pellucida or fuse with the plasma membrane of the oocyte without fertilisation taking place; for example, in our study, we observed some eggs with sperm tails protruding, but no pronuclei formation.

4.5.3.2 Preimplantation Embryo Development

Surprisingly, loss of embryos during preimplantation development was significantly higher only in the progeny of treated *Dnmt1*$^{+/+}$ males. It is doubtful that altered sperm DNA methylation can explain this rise in embryo death, because *Dnmt1*$^{c/+}$ sperm exhibited greater changes in sperm methylation, and at the same loci as *Dnmt1*$^{+/+}$ males, but did not experience increased loss of progeny *in vitro*. The integrity of both parental genomes is paramount to normal embryo development and thus, sperm DNA damage is incompatible with both fertilisation and early embryo development (Irvine *et al.*, 2000; Morris *et al.*, 2002; Barton *et al.*, 2003; Codrington *et al.*, 2004). Should fertilisation occur, any of the earlier mentioned chromatin defects could compromise embryo growth. The decreased chromatin integrity associated with 5-azaCdR treatment

can lead to abnormal chromosome segregation (reviewed in Haaf, 1995) during mitotic and meiotic divisions resulting in aneuploidy. Diploid and aneuploid sperm are common in men with reduced fertility (Egozcue *et al.*, 2002; Templado *et al.*, 2002; Agarwal & Said, 2003; Carrell *et al.*, 2003b). Also, 5-azaCdR's cytotoxicity is increased in wild-type mice (Jüttermann *et al.*, 1995), and sites of DNMT-5-azaCdR adducts are potentially mutagenic (Jackson-Grusby *et al.*, 1997). The mutational load of *Dnmt1*$^{+/+}$ sperm DNA may therefore be higher and could result in fertilisation failure and/or embryo death.

It is possible that any of the above changes in sperm motility, fertilisation and preimplantation loss could be the result of altered DNA methylation of the paternal genome or chromatin anomalies associated with 5-azaCdR incorporation. Identification of loci with altered methylation as well as detailed examinations of sperm morphology and chromatin integrity (*e.g.*, Comet assay) should help to shed light on these effects of 5-azaCdR treatment.

4.6 ACKNOWLEDGEMENTS

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CHAPTER V

DISCUSSION

5.0 DISCUSSION

Previous studies in the rat with the cytosine analogue 5-aza underscored the importance of DNA methylation in male germ cell development. In order that more mechanistic studies of male germ cell DNA methylation might be conducted, we continued our studies in the mouse, an animal more amenable to genetic experimentation than the rat. Within this thesis, genetic (*Dnmt1* haploinsufficiency and MTHFR deficiency) and pharmacological (5-azaCdR) methods, with which to manipulate methylation in the mouse male germ line, were examined. Here, we provide evidence that pharmacological and/or genetic inhibition of components of the DNA methylation pathway detrimentally affect male germ cell development, fertility, and in some cases, progeny outcome.

Chronic administration of therapeutic doses of 5-azaCdR to male mice, such that germ cells are exposed from spermatogonia to mature sperm in the cauda epididymidis, is associated with abnormal spermatogenesis and elevated preimplantation loss. These effects of 5-azaCdR may be minimised, to some degree, by haploinsufficiency of *Dnmt1*, the maintenance methyltransferase, which suggests that the mechanism of action of 5-azaCdR on male germ cells is mediated, in some part, by DNMT. By itself, *Dnmt1* haploinsufficiency did not result in any spermatogenic defects. However, effects on the seminiferous epithelium exhibit a steep dose-response curve to 5-azaCdR, and this was reflected in pregnancy outcome; our data suggest that decreased ability of treated sperm, from males of either genotype, to fertilise contributes to the elevated preimplantation loss observed. In addition, increased loss of embryos sired by 5-azaCdR-treated $Dnmt1^{+/+}$ males occurred at the blastocyst stage. Although 5-azaCdR induced cytotoxicity was lower in $Dnmt1^{c/+}$ males, a greater degree of hypomethylation at single copy loci was observed in the sperm of these males.

Changes in gene expression after 5-azaCdR treatment or genetic ablation of

DNMT1 indicate that there are considerable differences between these models (Jackson-Grusby *et al.*, 2001; Gius *et al.*, 2004; Karpf *et al.*, 2004). However, none of these studies have compared gene expression or phenotype of cells deficient in DNMT1 versus 5-azaCdR-treated cells haploinsufficient for *Dnmt1*. Although the use of 5-azaCdR to decrease methylation may not accurately mimic genetic depletion of DNMTs, the work in this thesis is also important from a clinical point of view. Currently, 5-azaCdR is a treatment option for cancer and sickle cell anemia (Leone *et al.*, 2003; Saunthararajah & De Simone, 2004). The fact that spermatogenic effects of 5-aza and 5-azaCdR are similar in rats and mice suggests that adverse effects on male germ cells could be extrapolated to humans. Moreover, the fact that 5-azaCdR is selective for the testis at doses lower than or equal to those used clinically demands that a thorough understanding of the mechanisms underlying the effects of 5-azaCdR on germ cells be gained.

Because inhibition of one aspect of the DNA methylation pathway (DNMT activity) had such profound effects on male germ cell development, it led us to question if altering another enzyme within the methylation pathway would also affect spermatogenesis. MTHFR, a key enzyme in the folate pathway, is necessary for production of methyl groups used in methylation of numerous substrates including DNA. Our studies suggest that this enzyme, which has been implicated in heart disease and neural tube defects, is essential for normal spermatogenesis in the mouse; inhibition of MTHFR results in atrophy of seminiferous tubules and complete infertility. This lack of fertility is partially alleviated by provision of the nutritional supplement betaine throughout the prenatal and postnatal life of MTHFR-deficient males.

In these next sections, I will discuss each model separately and speculate as to potential mechanisms for the observed effects on male germ cell development and fertility, with an emphasis on follow-up studies. Finally, I will propose other approaches that could be used in the future to determine the precise role of methylation in male germ

cell development.

5.1 5-AzaCdR and the Mouse Male Reproductive System

5.1.1 The Testis as a Site of 5-AzaCdR Action

Through incorporation into DNA at sites that would normally be methylated and via covalent trapping of DNMT (rendering the enzyme inactive), 5-azaCdR causes hypomethylation of DNA (reviewed in Christman, 2002). Because the testis exhibits high levels of DNMTs and contains rapidly dividing cell populations, we believed it would be a primary target for incorporation of 5-azaCdR. Indeed, when males are treated chronically with low doses of 5-azaCdR, substantial decreases in testis weight and sperm count are observed (*Dnmt1*$^{+/+}$, Table 5.1), as well as disruption of the seminiferous epithelium (Chapter II). That we did not see changes in the seminal vesicle weights, which is a well accepted proxy for changes in androgen status, suggests that the decreases in testis weight and sperm numbers are not consequences of altered androgen production.

Within our studies, the effects of 5-azaCdR appear to be specific to the testis. At the doses used in both Chapters II and IV, systemic effects on body weight, spleen weight, epididymal weight or circulating red and white blood cells are minimal. This was surprising, as the hematopoeitic system is comprised of rapidly dividing cells; chronic treatment of rats with 5-aza decreases circulating red and white blood cells (Doerksen & Trasler, 1996), and myelosuppression is a common side-effect in clinical trials with 5-azaCdR (Leone *et al.*, 2003). Thus, it appears that, at the doses used here, 5-azaCdR is highly selective for the mouse testis. This selectivity for the testis may have clinical implications for the therapeutic use of 5-azaCdR in males of reproductive age.

5.1.2 Germ Cells Affected by 5-AzaCdR Treatment

Spermatogenesis is highly regulated, and therefore, the timing of drug administration can be used to determine which germ cell types are specifically targetted.

This method has been exploited in the paternal treatment of rats with 5-aza (Doerksen & Trasler, 1996; Doerksen *et al.*, 2000) and cyclophosphamide (Trasler *et al.*, 1985). In this thesis, three treatment durations were employed: three and seven weeks (chronic), and three days (acute). The results of all three treatment lengths suggest that the premeiotic and early meiotic cells of the testis are most sensitive to the effects of 5-azaCdR.

The abnormalities noted after three weeks treatment are consistent with cytosine analogue incorporation into the rapidly dividing cells of the testis (Appendix I). Post-treatment, considerable loss of spermatogonia, spermatocytes and round spermatids is apparent. These germ cells would have been exposed initially as spermatogonia or early meiotic cells, developmental periods when the drug can become incorporated during cell replication. Effects on the mitotic and meiotic populations can also be seen after acute treatment (Appendix II). Significant decreases in testis weight and spermatid populations occur 21 days after acute exposure, which corresponds to the targetting of mitotically and meiotically dividing germ cells during the three days of treatment. Four days after treatment initiation, increased vacuolisation and apoptosis within the premeiotic and meiotic germ cell populations are observed. It is the loss of these cells that contributed to the reduction in testicular sperm counts at 28 days. Electron microscopy could be useful in revealing ultrastructure changes that precede the loss of these germ cells.

As expected, 7 weeks of treatment, which exposes germ cells throughout their development, results in greater effects than 3 weeks of treatment (Chapter II & Appendix I). This treatment length provides a greater opportunity for 5-aza and 5-azaCdR to inhibit *de novo* and maintenance methylation, including DNA methylation after DNA repair. Seven weeks of 5-azaCdR treatment affects all germ cell populations and results in significantly reduced sperm counts. Likewise, the functional ability of sperm initially exposed to 5-azaCdR as spermatogonia is reduced as evidenced by decreased fertility rates and increased preimplantation loss.

Our results therefore suggest that the actively dividing cells of the testis, which exhibit high levels of *Dnmt1*, *Dnmt3a,* and *Dnmt3b* expression (Benoit & Trasler, 1994; Jue *et al.*, 1995; La Salle *et al.*, 2004; Watanabe *et al.*, 2004) are most vulnerable to the effects of 5-azaCdR. Others have shown a similar targetting of spermatogonial cells by 5-azaCdR (Raman & Narayan, 1995; Doerksen *et al.*, 2000). 5-AzaCdR-DNMT adduct formation and hypomethylation within these cells could cause germ cell death (as revealed by TUNEL staining) or could result in altered germ cell development. Assuming that some germ cells survive, effects may not be seen until later stages of germ cell development, manifesting as aberrations in spermiogenesis, sperm maturation, or even fertilisation. More detailed analysis of cellular morphology, through methods such as electron microscopy, would help pinpoint the timing and nature of these defects after 5-azaCdR administration. Our studies did not establish if spermatogonia or spermatocytes are more vulnerable to 5-azaCdR. A time-course study following a single high-dose treatment with 5-azaCdR, to examine seminiferous epithelium histology (including apoptosis levels) at regular intervals post-treatment, would provide information on the more immediate effects of 5-azaCdR and the progression of events thereafter. Similar studies by other groups have proved quite informative (Cai *et al.*, 1997).

Studies in the rat suggest that postmeiotic cells are resistant to 5-azaCdR (Doerksen & Trasler, 1996), likely because the drug does not have an opportunity to incorporate into the DNA of these cells; our work here is in agreement with these findings. During spermiogenesis, the chromatin undergoes extensive remodelling, becoming condensed and tightly packaged into a relatively small space, and transcription and DNA repair events halt (Oko & Clermont, 1999). However, a minimal amount of 5-azaCdR might be incorporated into postmeiotic germ cells during the unscheduled DNA synthesis that occurs during DNA repair.

Repeated exposure, through drug administration every other day allowed the

targetting of different clonal groups of germ cells. Because some clonal groups might be replicating while others are quiescent when exposed to 5-azaCdR the effects of the drug may not be uniform. Thus, in order to continually expose germ cells to 5-azaCdR while minimising toxicity, males were treated every other day. Determination of 5-azaCdR incorporation levels, by high-performance liquid chromatography (HPLC), nuclear magnetic resonance (NMR), or through the use of radiolabelled 5-azaCdR (Cihák & Vesely, 1978) could provide indications as to the extent of 5-azaCdR incorporation into the premeiotic and meiotic germ cell populations, as well as the integration of 5-azaCdR during postmeiotic repair processes. These levels could be compared to changes in DNA methylation and apoptosis.

5.2 5-AzaCdR and DNMT-Deficient Male Mice

5.2.1 Genotype-Specific Response to 5-AzaCdR

The toxicity of 5-azaCdR precludes treatment of mice with high doses of this drug. Therefore, in an effort to further challenge the DNA methylation machinery, we treated males heterozygous for a null mutation in *Dnmt1* with 5-azaCdR. We proposed that 5-azaCdR in conjunction with DNMT1 deficiency would act synergistically to further reduce methylation levels in the male germ line. Furthermore, we believed, based on other studies, that $Dnmt1^{c/+}$ males might be more resistant to the toxic effects of 5-azaCdR because of reduced adduct formation (Jüttermann *et al.*, 1994).

These hypotheses proved correct; the germ cells of $Dnmt1^{c/+}$ males treated with 5-azaCdR appeared considerably less vulnerable to the deleterious effects of 5-azaCdR, while greater changes in sperm DNA methylation were observed. As shown in Table 5.1, $Dnmt1^{+/+}$ males were more sensitive to 5-azaCdR at all male reproductive endpoints (testis weight, abnormal tubules, histology, sperm counts and sperm motility) and had less alterations in DNA methylation. These genotype-specific differences in the response of

the male reproductive system to chronic 5-azaCdR treatment indicate that the changes elicited by the drug are mediated by the presence of DNMT1.

Despite these differences in response, 5-azaCdR appears to target the same germ cell populations in both *Dnmt1*$^{+/+}$ and *Dnmt1*$^{c/+}$ males. Although *Dnmt1*$^{c/+}$ testicular histology appeared normal four days after acute treatment with 5-azaCdR, TUNEL staining revealed significant levels of apoptotic spermatogonia and spermatocytes. This is supported by the observed loss of these germ cell generations at later time points. Curiously, levels of apoptosis were similar for both genotypes, although chronic treatment results in less extensive germ cell loss in *Dnmt1*$^{c/+}$ males. Perhaps the acute treatment does not closely model the effects of chronic treatment. Alternatively, the rate of stem cell divisions might be accelerated in *Dnmt1*$^{c/+}$ males in response to germ cell depletion through apoptosis (Russell *et al.*, 1990). Studies of BrdU (5'-bromo-2'-deoxyuridine) incorporation after acute treatment with 5-azaCdR would provide information regarding the rate of spermatogonial proliferation.

Table 5.1: Summary of genotypic differences in response to chronic (7 weeks) 5-azaCdR treatment (0.1 mg/kg).

	***Dnmt1*$^{+/+}$**	***Dnmt1*$^{c/+}$**
Testis Weight	↓35%*	↓15%†
Sperm Count	↓33%*	No change
Pregnancy Rate	↓33%*	No change
Total Preimplantation Loss	↑5-fold*	↑3-fold†
Progressive Sperm Motility	↓34%*	↓17%
Fertilisation Ability	↓70%*	↓56%†
Survival to Blastocyst	↓50%*	No change
Changes in Sperm Methylation	5 loci	14 loci
* $p \leq 0.05$, vs. saline *Dnmt1*$^{+/+}$, † $p \leq 0.05$, vs. saline *Dnmt1*$^{c/+}$,	↓	↓
	Cytotoxicity & Hypomethylation	***Hypomethylation***

5.2.2 Fertility and Preimplantation Development of Progeny

Fertility analyses after chronic treatment for 7 weeks also revealed genotype-specific differences (Table 5.1). Whereas 30% of *Dnmt1*$^{+/+}$ males were infertile after treatment with 0.1 mg/kg 5-azaCdR, similarly treated *Dnmt1*$^{c/+}$ males experienced no changes in fertility. However, matings with treated males of either genotype resulted in elevated preimplantation loss.

In Chapter II, we reported that the increase in preimplantation loss was similar between treated *Dnmt1*$^{+/+}$ and *Dnmt1*$^{c/+}$ males (~three to four-fold increase). However, the decrease in full-term pregnancies in females successfully mated with treated *Dnmt1*$^{+/+}$ males (0.1 and 0.15 mg/kg 5-azaCdR) could also represent preimplantation loss. In the uteri of these females, we observed an absence of both resorption sites and non-viable embryos. Henderson and Robaire (2005) attributed the failure of full-term pregnancies in females that showed evidence of successful copulation to 100% preimplantation loss. Using this argument, preimplantation loss in the 0.1 and 0.15 mg/kg *Dnmt1*$^{+/+}$ groups can be calculated as 44% and 100%, respectively; these levels of preimplantation loss represent 5.5 and 12.5-fold increases from control values.

Preimplantation loss represents either failure of sperm to fertilise an oocyte or the loss of an embryo prior to implantation. From our *in vivo* mating and *in vitro* embryo culture experiment results, we propose that reduced fertilisation ability of a subset of treated sperm underpins elevated preimplantation loss in *Dnmt1*$^{c/+}$ males. Likewise, the majority of preimplantation loss in the treated *Dnmt1*$^{+/+}$ group may be accounted for by an impaired ability of some treated sperm to fertilise. Altered sperm motility may underlie the decreased fertilisation ability of treated *Dnmt1*$^{+/+}$ sperm and to a lesser extent, may account for some infertility in *Dnmt1*$^{c/+}$ males.

As discussed in Chapter IV, impaired ability of sperm to fertilise could arise from any number of problems, from altered sperm kinetics to a failure of sperm DNA to

decondense once the oocyte has been penetrated. *In vitro* fertilisation (IVF) studies are needed to determine the nature of this fertilisation failure. It would also be interesting to see if those sperm that are unable to fertilise eggs naturally could produce viable embryos if used to fertilise oocytes through intracytoplasmic sperm injection (ICSI). Understanding the competency of these embryos could provide some indication as to the extent of 5-azaCdR-induced damage.

In the case of *Dnmt1*$^{+/+}$ matings, increased early embryo loss was observed between the morula and blastocyst stages of development. Thus, elevated preimplantation loss in matings with treated *Dnmt1*$^{+/+}$ males can be attributed to a significant loss of embryos during preimplantation development, as well as depressed fertilisation ability of treated sperm. Because embryos were scored only until the blastocyst stage, we cannot discount the possibility that more embryo loss, in either treated group (*Dnmt1*$^{+/+}$ and *Dnmt1*$^{c/+}$), might occur around the time of implantation. The viability of surviving blastocysts, and their ability to implant, could be tested by implanting blastocysts into the uteri of pseudopregnant females (Hogan *et al.*, 1994).

While we established that there was no increase in postimplantation loss nor gross morphological defects of pups sired by paternally treated males, these observations do not preclude the possibility of effects that might not manifest until later in offspring life. For example, postnatal growth and development of pups sired by 5-azaCdR treated males was not examined; *Mthfr*$^{-/-}$ fetuses appear normal before birth, but postnatally display reduced developmental indices. Some imprinting disorders, such as Angelman syndrome, are not apparent at birth (Guerrini *et al.*, 2003), and pups of treated males could be at increased risk for cancer, behavioural disorders or reduced fertility. Paternal cyclophosphamide treatment results in progeny with altered behaviour (Adams *et al.*, 1981; Auroux & Dulioust, 1985; Auroux *et al.*, 1986), and irradiated males produce offspring that, although normal at birth, show increased predisposition to lung tumours and leukemia

(Nomura, 1982, 1991; Watanabe *et al.*, 1996; Shoji *et al.*, 1998). By allowing pups sired by treated males to reach adulthood, detection of such abnormalities may be possible.

5.3 Potential Mechanisms of 5-AzaCdR Effects on Male Germ Cells

Within this thesis, we investigated some processes that might underlie the effects of 5-azaCdR on germ cell development and function – namely, methylation of sperm DNA and sperm motility. Given the disparities in response to 5-azaCdR between the two genotypes, it would not be surprising if the actions precipitating such effects were very different. Because of 5-azaCdR's therapeutic use, much of our current knowledge of 5-azaCdR's biological consequences comes from the *in vitro* treatment of cancer cell lines, which typically have aberrant methylation dynamics, and embryonic stem (ES) cells. In addition, extrapolation from cell culture is limited, as it lacks the complexity of an *in vivo* model and therefore may not be a good predictor of events following *in vivo* germ cell exposure to 5-azaCdR. However, in the context of the known mechanisms of 5-azaCdR, possible explanations for the observed effects on male germ cells, including impaired fertility, will now be discussed. Many of these potential events are interrelated and none are mutually exclusive; it may be that multiple mechanisms are involved, any of which may be dependent or independent of the DNA hypomethylating capacity of 5-azaCdR. In order to better understand the mechanisms underlying 5-azaCdR's effects on male germ cells examination of endpoints such as gene expression, chromatin structure, and chromatin stability is required. Because altered preimplantation embryo survival likely stems back to abnormalities in male germ cell development, potential explanations for embryo loss will be addressed within this section.

5.3.1 Altered DNA Methylation

Due to its ability to deplete DNMT activity, incorporation of 5-azaCdR is reported to cause profound DNA hypomethylation after several rounds of replication (*e.g.*, Haaf,

1995; Leone *et al.*, 2003). Surprisingly, however, we observed no changes in the germ cell methylation of repeat sequences, in which the majority of m^5C is located, and found far fewer changes than expected in the methylation of single copy loci. Similar to Laird *et al.* (1995), we did see greater hypomethylation in the sperm DNA of treated *Dnmt1*$^{c/+}$ males relative to that of treated *Dnmt1*$^{+/+}$ males, but hypomethylation levels were less than expected. In our studies, repeat sequence methylation was analysed after cutting genomic DNA with *HpaII*. However, analyses of the major satellite sequence may have been more informative with the restriction enzyme *MaeII* as several groups have found changes in the methylation of this sequence only after cutting genomic DNA with *MaeII* (Dennis *et al.*, 2001; Takebayashi *et al.*, 2001; Lehnertz *et al.*, 2003).

Our RLGS data suggest that demethylation by 5-azaCdR is not a random genome-wide event, in that specific loci are consistently demethylated in members of the same treatment group; and treated *Dnmt1*$^{c/+}$ sperm exhibited changes at the same loci as *Dnmt1*$^{+/+}$ sperm (Chapter IV). Blanchard *et al.* (2003) also found consistent demethylation of specific loci in cell cultures exposed to 5-azaCdR, regardless of the dose and duration of exposure. However, why these particular loci were repeatedly affected by 5-azaCdR treatment is unknown. Of primary importance is the identification of loci with altered methylation; this may help us understand why these loci are more vulnerable to 5-azaCdR's effects than are other loci. Staining of meiotic chromosomes with the anti-methylcytosine antibody may reveal specific chromosomal locations with altered methylation. It would also be interesting to ascertain methylation of separated germ cell populations, as larger DNA methylation changes in the immature populations (relative to the more mature populations) may be indicative of repair or death of these cells prior to maturation.

When we began these experiments, we had predicted that 5-azaCdR would inhibit all catalytically active DNMTs with equal affinity. Indeed, Weisenberger *et al.* (2004)

found complete depletion of DNMT1, DNMT3a and DNMT3b after treatment of ES cells with 5-azaCdR. However, several other studies suggest that 5-azaCdR preferentially inhibits DNMT1 (Liu *et al.*, 2003; Robert *et al.*, 2003; Missiaglia *et al.*, 2005). Moreover, Robert *et al.* (2003) demonstrated that maintenance, but not *de novo*, methylation was inhibited by 5-azaCdR treatment and proposed that the selective inhibition of DNMT1 could arise from the differential nuclear localisation of the DNMT enzymes. During replication, DNMT1 associates with PCNA at the replication fork, methylating newly replicated DNA in a processive manner (Vilkaitis *et al.*, 2004); DNMT1 therefore has a high probability of encountering newly incorporated 5-azaCdR. In contrast, DNMT3a and DNMT3b are more dispersed within the cell (Bachman *et al.*, 2001; Margot *et al.*, 2001) and thus, would encounter and bind to 5-azaCdR less frequently. Furthermore, it has been suggested that DNMT3a may be able to methylate DNA without formation of a covalent intermediate (Reither *et al.*, 2003). If so, DNMT3a could avoid becoming covalently trapped by 5-azaCdR. In the premeiotic cells of the testis, expression levels of *Dnmt1*, *Dnmt3a* and *Dnmt3b* are all relatively high (La Salle *et al.*, 2004). Thus, even after selective depletion of DNMT1 by 5-azaCdR, substantial methylating activity would remain, due to active DNMT3a and DNMT3b. To identify the nature of DNMT inhibition within our system will require examination of DNMT-drug adducts (Ferguson *et al.*, 1997; Liu *et al.*, 2003) and free DNMT enzyme levels in nuclear extracts (Liu *et al.*, 2003; Robert *et al.*, 2003; Weisenberger *et al.*, 2004).

Surprisingly, in addition to hypomethylation we observed hypermethylation of three loci in the sperm of 5-azaCdR-treated *Dnmt1*$^{c/+}$ males. Other groups have also observed increased methylation at specific loci after 5-azaCdR exposure, both *in vitro* (Blanchard *et al.*, 2003) and *in vivo* (Aparicio *et al.*, 2003). The DNMTs are thought to act cooperatively (*e.g.*, Hata *et al.*, 2002; Kim *et al.*, 2002; Margot *et al.*, 2003). Ectopic methylation could therefore result from upregulation of any of the DNMT enzymes in

response to reduced enzyme activity due to covalent trapping. If DNMT3a and DNMT3b are upregulated in response to preferential DNMT1 inhibition, these enzymes may mistakenly methylate some sequences. Indeed, upregulation of DNMT1 and DNMT3a is observed in cancer cells after incubation with low doses of 5-azaCdR (Missiaglia *et al.*, 2005), and Gius *et al.* (2004) reported a three-fold increase in the expression of DNMT3B in DNMT1-deficient ES cells. *Dnmt* mRNA and protein expression, as well as DNMT enzyme activity assays would establish whether levels or activities of these enzymes change after exposure to 5-azaCdR.

While alterations in DNA methylation were not as dramatic as predicted, changes of the levels observed in RLGS are adequate to affect male germ cell development via perturbed gene expression, particularly if the extent of change throughout the genome is considered (extrapolation data, Chapter IV); possible effects on gene expression will be discussed in the next section. Again, identification of loci with altered methylation should help us to understand the processes leading to disturbed germ cell development.

While it is tempting to speculate that altered germ cell DNA methylation is the cause of the increased early embryo death after matings with treated *Dnmt1*$^{+/+}$ males, it is likely not the reason. The sperm of *Dnmt1*$^{c/+}$ males showed greater changes with respect to methylation, but embryos sired by these males did not experience higher rates of preimplantation loss. However, we cannot rule out the possibility that embryos from 5-azaCdR-treated *Dnmt1*$^{+/+}$ fathers have altered methylation. Methylation analyses via RLGS and methylcytosine staining would answer this question. It would be interesting to see if these embryos exhibit altered methylation at the same loci as their fathers' sperm.

5.3.2 Perturbations in Gene Expression

Transcriptional silencing is often associated with methylation of CpG dinucleotides in the promoter regions of genes (Bird, 2002; Jaenisch & Bird, 2003), and loss of methylation can result in ectopic gene expression (Panning & Jaenisch, 1996;

Walsh *et al.*, 1998; Jackson-Grusby *et al.*, 2001). Treatment with 5-azaCdR can also result in the re-expression of silenced genes, both *in vitro* (*e.g.*, Primeau *et al.*, 2003; Dickinson *et al.*, 2004) and *in vivo* (*e.g.*, Koshy *et al.*, 2000; Daskalakis *et al.*, 2002; Sauntharajah *et al.*, 2003); however, induction of expression may or may not be related to changes in methylation. For example, Soengas *et al.* (2001) found a dramatic increase in the expression of *Apaf-1* in melanoma cell lines, after 5-azaCdR treatment, without changes in promoter CpG methylation. As well, colon cancer cells deficient in DNMT1 experience only a 20% decrease in genomic methylation (Rhee *et al.*, 2000) but have substantial changes in gene expression (Gius *et al.*, 2004), whereas cells deficient in both DNMT1 and DNMT3B have smaller changes in gene expression than DNMT1-deficient cells (Gius *et al.*, 2004), but have a much greater loss in methylation (Rhee *et al.*, 2002). Together, these data suggest that changes in methylation may not always be related to changes in gene expression.

Spermatogenesis is a highly regulated process that requires timely and appropriate gene expression in various germ cell populations. Altered gene expression (either up- or downregulation) within any of these populations could alter the developmental programme of germ cells to form functional sperm. Even subtle changes in gene transcription can translate to dramatic differences in phenotype (Nadeau, 2003). 5-AzaCdR-induced disruption of normal gene expression could underlie the altered motility and reduced fertilisation capacity of treated sperm from $Dnmt1^{+/+}$ and $Dnmt1^{c/+}$ males. The testis is the site of 5-azaCdR action, and it is within this organ that the structures necessary for normal sperm motility develop. Changes in gene expression could lead to: structural defects in the head/tail of the sperm, resulting in altered motility; and biochemical defects that could alter the interaction between the sperm and oocyte, thus preventing fertilisation. Furthermore, expression changes could disrupt the ability of sperm to respond to maturational cues during epididymal transit.

As mentioned in Chapter IV, extrapolation of our RLGS data predicts that altered methylation may be found at approximately 100 sites throughout the genome. Although expression at not all of these loci may be governed by methylation, changes of even a few genes may be enough to disrupt the spermatogenic process. Once loci with altered methylation have been identified and their methylation status confirmed, expression analyses in earlier germ cell populations can be conducted. As well, gene arrays, which allow for the screening of expression changes in a large number of genes, will be carried out for the different germ cell populations after *in vivo* exposure to 5-azaCdR. Since modifications in methylation are not always proportional to changes in gene expression (Rhee *et al.*, 2000; Soengas *et al.*, 2001; Rhee *et al.*, 2002; Gius *et al.*, 2004), we expect much greater changes in gene expression relative to our methylation data.

5.3.3 Altered Genomic Imprinting

DNA methylation has been implicated in the establishment and maintenance of genomic imprinting (Reik *et al.*, 2001), and imprinted genes can only acquire their methylation mark upon passage through the germ line (Tucker *et al.*, 1996). 5-Aza has been shown to alter the expression of imprinted genes both *in vitro* (Eversole-Cire *et al.*, 1993; Barletta *et al.*, 1997) and *in vivo* (Efstratiadis, 1994). Men with poor semen samples exhibit perturbed sperm *H19* methylation (Marques *et al.*, 2004), but we observed no changes in *H19* methylation in sperm of male mice treated with 0.15 mg/kg 5-azaCdR (Kelly, unpublished data). However, this does not preclude changes in other paternally methylated imprinted genes, including *Rasgrf1* and *Gtlk2*. Imprinted genes have been implicated in fetal and placental development, as well as in neurological disorders and some cancers (Tycko & Morison, 2002; Lucifero *et al.*, 2004). Because there is no opportunity to correct altered imprinted gene methylation in embryos, inherited changes in imprinted gene methylation could have deleterious consequences for the offspring of males with altered sperm methylation. 5-AzaCdR can induce chromosomal aberrations

that can lead to deletions or rearrangements (reviewed in Haaf, 1995) – these defects could impair normal imprinted gene expression. Hypermethylation of normally hypomethylated paternally expressed alleles (*e.g.*, *Ins1* and *Sgce*) could have equally detrimental results for progeny. Alternatively, if 5-azaCdR insult compromises the chromatin integrity of the fertilising sperm, there may be an inability of the imprinted genes to maintain their imprints during the post-fertilisation wave of demethylation.

5.3.4 Changes in Chromatin Structure

Chromatin structure is maintained through an active interplay between methylation of DNA and histone modifications (*e.g.*, Jones *et al.*, 1998; Nan *et al.*, 1998; Fuks *et al.*, 2001; reviewed in Li, 2002). DNA methylation and methylation of lysine 9 of histone H3 (Me-H3K9) are typically associated with a condensed, transcriptionally inactive state (Maison *et al.*, 2002; Peters *et al.*, 2002). Conversely, acetylation of lysine 9 of histone H3 (Ac-H3K9) and methylation of histone H4 lysine 4 (Me-H4K4) are associated with an open chromatin configuration typical of transcriptionally active promoters. Reactivation of genes may involve formation of a more open chromatin structure, as shown by increased sensitivity to nucleases in 5-aza treated cultures (Litt *et al.*, 1997); and this open chromatin structure formation may precede changes in methylation. Takebayashi *et al.* (2001) proposed that 5-azaCdR can trap HDAC2 via covalent complex formation, which may result in hyperacetylation of heterochromatin – linked to transcriptional activity – without large changes in DNA methylation, as indicated by the continued binding of MeCP2. According to the proposed mechanism of 5-azaCdR, after its incorporation, DNA methylation should decrease with subsequent cell divisions and reach a maximum approximately 48 hours after exposure (Bender *et al.*, 1999). However, Gius *et al.* (2004) observed similar effects on gene expression after one or five days of 5-azaCdR treatment, suggesting that either active demethylation or active remodelling of chromatin played a role in gene expression changes. Similarly, 5-azaCdR

induced rapid remodelling of heterochromatin, with decreased H3K9 methylation and increased H3K4 methylation, independent of the effects on DNA methylation (Nguyen *et al.*, 2002). These studies suggest that exposure of male germ cells to 5-azaCdR may induce alterations in gene expression unrelated to changes in DNA methylation that could promote abnormal germ cell development. Perturbations in histone and chromatin remodelling could be determined by immunocytochemistry with antibodies specific to various histone modifications (*e.g.*, methylation of H3K9).

5.3.5 Induction of Chromosome Damage

Changes in chromatin structure can also render the chromatin more susceptible to damage. Treatment of cells with 5-azaCdR induces chromosomal undercondensation (Viegas-Péquignot & Dutrillaux, 1976), which results in chromosome instability (Kokalj-Vokac *et al.*, 1993), deleterious effects such as increased sister chromatid exchange (Albanesi *et al.*, 1999), and increased gene amplification due to unequal breakage during crossing over (reviewed in Haaf, 1995). However, it is unknown whether demethylation precedes or follows chromosome decondensation (reviewed in Haaf, 1995). Based on more recent findings, it is not unreasonable to assume that changes in chromatin condensation could occur independent of changes in methylation. Deficiency in DNMT1 results in increased rates of chromosomal rearrangement and gene loss by mitotic recombination (Chen *et al.*, 1998), although this could be the result of limited interaction with other proteins rather than decreased methylation.

Pairing of undercondensed regions can lead to recombination, breakages, deletions, and rearrangements (Schmid *et al.*, 1983; Haaf *et al.*, 1986; reviewed in Haaf, 1995), many of which result in unbalanced translocations in the next generation (Kokalj-Vokac *et al.*, 1993); 5-aza is also known to alter centromere separation (Rodriguez *et al.*, 2001). Such chromosomal damage could alter gene expression in developing germ cells or may contribute to production of sperm with increased levels of DNA fragmentation,

which has been associated with reduced fertility (Carrell *et al.*, 2003a). To test for DNA damage in treated sperm, the single-cell gel/Comet assay could be used. Chromosomal aberrations could also result in the production of aneuploid germ cells, some of which may undergo cell cycle arrest or apoptosis at a meiotic check point to prevent production of aneuploid gametes (Roeder & Bailis, 2000). In humans and mice, male infertility is often associated with increased rates of aneuploidy (Oppedisano *et al.*, 2002; Templado *et al.*, 2002; Argwal & Said, 2003; Carrell *et al.*, 2003b, 2004). Aneuploidy of haploid germ cells could be evaluated using fluorescence *in situ* hybridisation or other immunocytochemical means (*e.g.*, CREST antiserum, whose punctuate staining of kinetochores reflects the number of chromosomes).

5.3.6 Altered Repair Mechanisms

DNA methylation has been proposed to play a role in the mismatch repair (MMR) pathway, perhaps as a means of strand discrimination via CpG hemimethylation (Hare & Taylor, 1985; Robertson & Jones, 1997), although others have contested this idea (Drummond & Bellacosa, 2001). Recently, DNMT1 was identified in a screen for MMR genes (Guo *et al.*, 2004) and DNMT1 deficiency was linked to microsatellite instability (Guo *et al.*, 2004; Kim *et al.*, 2004; Wang & Shen, 2004). Kim *et al.* (2004) found that this microsatellite instability was not associated with local changes in methylation. Covalent trapping of DNMT1 could therefore lead to elevated levels of microsatellite instability because of reduced MMR efficiency. Changes such as these could alter the survival of affected germ cells or, more importantly, could result in increased rates of tumorigenesis in offspring of treated males.

5.3.7 Germ Cell Apoptosis

Following acute treatment with 5-azaCdR, significant increases in apoptosis were observed in the seminiferous tubules of both *Dnmt1*$^{+/+}$ and *Dnmt1*$^{c/+}$males. Low levels of apoptosis are normal within the testis (Russell *et al.*, 1990); the number of apoptotic germ

cells can increase in response to testicular insult (Hsueh *et al.*, 1996; Cai *et al.*, 1997; Blanco-Rodriguez & Martinez-Garcia, 1998; Doerksen *et al.*, 2000). 5-AzaCdR may cause cells to undergo apoptosis (Yamada *et al.*, 1996; Ferguson *et al.*, 1997; Wang *et al.*, 1998), perhaps due to induction of apoptosis genes via promoter demethylation. Similar results have been observed in *Dnmt1*$^{-/-}$ mice, in which levels of apoptosis are increased (Li *et al.*, 1992). However, activation of apoptosis can occur without concomitant changes in promoter methylation (Soengas *et al.*, 2001). Similarly, Karpf *et al.* (2001) found that *p53* induction after 5-azaCdR treatment did not correspond to increased *p53* transcripts, suggesting that activation of the p53 pathway occurred independently of DNA hypomethylation. It is unknown, however, whether 5-azaCdR induces apoptosis in germ cells (*e.g.*, via changes in gene expression) or if apoptosis is a method by which the testis rids itself of 5-azaCdR-damaged germ cells. As mentioned earlier, there exist checkpoints during spermatogenesis, where abnormal germ cells undergo cell-cycle arrest or are induced to undergo apoptosis (Sassone-Corsi, 1997; Roeder & Bailis, 2000). 5-AzaCdR-induced chromosomal damage may be recognised at such a checkpoint and subsequent induction of apoptosis could occur. Germ cell apoptosis in *Dnmt1*$^{c/+}$ males may be related primarily to aberrations caused by hypomethylation, whereas the increased germ cell apoptosis observed in *Dnmt1*$^{+/+}$ males may be equally due to hypomethylation and direct 5-azaCdR-induced chromosomal damage.

5.4 MTHFR Deficiency

MTHFR is necessary for the production of the methyl groups required for methylation of DNA. In the mouse, MTHFR activity is highest in the testis relative to other adult organs (Chen *et al.*, 2001), suggesting a role for this enzyme in spermatogenesis. In Chapter III, we found that mice nullizygous for *Mthfr* have small testes, histological evidence of abnormal germ cell development, and are infertile. These

results provide evidence that MTHFR is necessary for spermatogenesis. The MTHFR model is complex and it is difficult to study spermatogenesis within MTHFR-deficient males because of the considerable systemic effects elicited by deficiency in MTHFR, particularly because survival of *Mthfr*$^{-/-}$ mice is severely reduced. It would therefore be ideal to create a germ cell-specific knockout of this enzyme in order to study its role in spermatogenesis without confounding systemic effects (which would eliminate difficulties in obtaining large enough populations of MTHFR-deficient males); this has been done successfully for *Dnmt3a* and *Dnmt3b* (Kaneda *et al.*, 2004).

5.4.1 Localisation and Timing of the MTHFR Defect

Spermatogenesis in *Mthfr*$^{-/-}$ males fails early in postnatal development; germ cell numbers at 18.5 dpc were normal. At 6 dpp, germ cell numbers were dramatically decreased and levels of apoptosis were four-fold higher in the testis of *Mthfr*$^{-/-}$ males. We suggested that the observed decrease in germ cells was the result of apoptosis of gonocytes that fail to replicate postnatally or that do not relocalise to the basement membrane and are therefore improperly aligned within the developing seminiferous tubule. To define the timing of this defect (when germ cell loss occurs) will require examination of *Mthfr*$^{-/-}$ testes on each day between 18.5 dpc and 6 dpp. At each time point, histology should be carefully analysed, with germ cells quantified using GCNA staining and levels of germ cell proliferation and apoptosis conducted via BrdU (*e.g.*, Nagano *et al.*, 2000) and TUNEL labelling, respectively.

In order to understand the role of MTHFR in spermatogenesis, it is imperative that MTHFR expression in the testis be characterised. Determination of levels of *Mthfr* mRNA and protein expression (via quantitative RT-PCR, Western blotting and immunocytochemistry) through development and within the various germ cell populations of the adult testis should help to define the point at which MTHFR-deficiency impacts spermatogenesis. Moreover, it is unclear whether the testicular defect that leads

to male infertility resides within the testicular somatic cells or within the germ cells themselves. To aid in this discrimination, germ cell transplant studies will be conducted whereby spermatogonial stem cells from *Mthfr*$^{-/-}$ males will be transplanted into germ-cell deficient mice and *Mthfr*$^{-/-}$ males will be the recipients of stem cells from normal males.

5.4.2 Nutritional Amelioration of the MTHFR Defect

Betaine, an alternative methyl donor, is used clinically to treat severe MTHFR deficiency (Haworth *et al.*, 1993; Al Tawari *et al.*, 2002; Bönig *et al.*, 2003) and has been found to improve *Mthfr*$^{-/-}$ pup survival, as well as liver and brain defects associated with MTHFR deficiency (Schwahn *et al.*, 2004). In this thesis, we demonstrated that provision of betaine to *Mthfr*$^{-/-}$ males throughout gestation and postnatal life results in improved male reproductive indices, including testicular histology, sperm counts and fertility. We postulated that alterations in the transmethylation pathway may underlie the adverse effects of MTHFR-deficiency and that betaine supplementation helped to maintain methyl production necessary for the transmethylation reactions. These reactions include methylation of DNA, which we have shown to be important for normal male fertility (Doerksen *et al.*, 2000; Kelly *et al.*, 2003; Chapter IV). Currently, we are examining the methylation of sperm from animals studied in Chapter III, via RLGS. Because so few sperm were observed in the testes of *Mthfr*$^{-/-}$ males it may be necessary to collect germ cells through laser capture microdissection, an extremely time-consuming method. If possible, it would be ideal to compare the germ cell methylation levels of untreated *Mthfr*$^{-/-}$ males and those provided with betaine. Moreover, it would be interesting to compare the methylation of sperm from fertile and infertile *Mthfr*$^{-/-}$ males given supplemental betaine, to show conclusively that methyl group production via MTHFR is necessary for DNA methylation of male germ cells.

MTHFR deficiency decreases the pool of methyl groups available for the

transmethylation reactions. It would therefore be interesting to combine the MTHFR model with 5-azaCdR treatment. However, prior to initiation of such studies, it would be ideal to create a model heterozygous for both DNMT1 and MTHFR deficiency in order to minimise cytotoxicity due to DNMT-drug adducts.

5.4.3 Clinical Implications

There is currently a debate as to whether MTHFR has a role in male infertility in humans. Bezold *et al.* (2001) found that in infertile men, the incidence of 677C→T homozygosity was double the rate of this genotype in fertile men. However, other studies have not found such a link (Ebisch *et al.*, 2003; Stuppia *et al.*, 2003). Our data provide support for the link between MTHFR and normal testicular functioning and show that provision of a methyl donor can partially ameliorate the infertility associated with MTHFR-deficiency. The thermolabile enzyme associated with the 677C→T polymorphism is stabilised through increased folate intake (Jacques *et al.*, 1996). It may therefore be necessary to stratify/categorise infertile and fertile men by folate intake/plasma levels when such comparisons are done.

5.5 Alternative Ways to Modify DNA Methylation

Since this project began five years ago, there has been an explosion of information regarding the intricacies of chromatin structure and transcriptional regulation. Our view of chromatin remodellers as static participants has dramatically shifted. It is now apparent that by altering one piece within the chromatin remodelling puzzle, many other pieces are affected – all the pieces are interconnected. For example, DNMT1 was believed to play a role in methylating DNA; if this gene were knocked out, its effects on other systems could be related back to the hypomethylation resulting from DNMT1's absence. DNMT1 is now known to interact with a plethora of other proteins involved in chromatin remodelling, replication, and mismatch repair. Therefore, inhibition of

DNMT1 or any of the catalytically active DNMTs not only affects methylation of DNA, but may also alter repair pathways, histone modifications and more; these changes may all occur independently of hypomethylation. This complexity makes the creation of a model of hypomethylated male germ cell DNA extremely difficult. How does one tease apart those effects that are due to hypomethylation and those that are due to DNMT interactions with proteins in other pathways? It also seems that each cell type reacts differently to similar situations; reduction of DNMT1 in colon cancer cells elicits different effects than in ES cells (*e.g.*, Li *et al.*, 1992; Rhee *et al.*, 2000; Weisenberger *et al.*, 2004). What this means is that while information from other cell types is useful, each system itself must be studied in great detail. It also indicates that while cell culture is informative on a level where numerous aspects can be controlled, it does not mimic the intricacies of an *in vivo* model. More effort should be put into taking *in vitro* information and testing it *in vivo*. However, our MTHFR model clearly illustrates the problems associated with an *in vivo* model. The folate pathway is universally required and deficiency in any of the enzymes within it brings about numerous systemic effects. For a more thorough understanding of MTHFR's role in spermatogenesis and its affects on germ cell methylation, a germ cell-specific knockout is necessary.

Over the past several years there have been great leaps in our understanding of DNA methylation. The methyltransferase family has grown exponentially and new members have been characterised. As well, techniques such as Cre-mediated deletion of genes in specific tissues are mainstream and have been used to generate germ cell-specific knockouts of DNMT3a and DNMT3b (Kaneda *et al.*, 2004). Throughout this discussion I have made suggestions as to future experiments within the models we have utilised in this thesis. However, there are a few other approaches which could be used alone, or in combination with the methods applied within this thesis, to lower methylation within the male germ line.

5.5.1 Antisense Technology

In vivo administration of antisense oligonucleotides (ODN), directed against any or all of the DNMTs, to male mice (via injection into the testis) could be used to decrease DNA methylation during spermatogenesis (Ramchandani *et al.*, 1997; Milutinovic *et al.*, 2004). Given that the expression of the various DNMTs differs during spermatogenesis (Fig. 1.4), it would be interesting to administer antisense specific to these DNMTs during their windows of peak expression. Alternatively, RNAi or siRNA directed to any of the DNMTs could be used instead of antisense (Suzuki *et al.*, 2004; Ting *et al.*, 2004). It might also be informative to use antisense or RNAi against DNMT3a or DNMT3b in *Dnmt1*$^{c/+}$ males. To help better elucidate the molecular underpinnings of DNMT1 deficiency in male germ cells antisense technology could also be used to reduce DNA methylation in cultures of testicular germ cells (Jeong *et al.*, 2003). This approach is also of clinical interest; currently, Phase I clinical trials testing the anticancer therapeutic value of the DNMT1 antisense inhibitor, MG98 are underway (Stewart *et al.*, 2003).

5.5.2 Targetting of Folate Pathway Enzymes

One function of the folate pathway is to produce the methyl groups required for transmethylation reactions in the cell. Our studies here demonstrated that inhibition of a key folate pathway enzyme (MTHFR) has severe consequences for sperm production. Although several folate pathway enzymes have been deleted in mice using gene-targetting approaches, few studies have examined effects on methylation or male germ cell development. Of primary interest would be methionine synthase (MTR), which is expressed in the human testis (Li *et al.*, 1996), and the reduced folate carrier 1 (RFC1). Inhibition of RFC1 causes embryonic lethality before 9.5 dpc (Zhao *et al.*, 2001), but high dose folic acid supplements enable live births of nullizygotes; however, these mice soon exhibit growth retardation, abnormal seminiferous tubules, and die within the first two weeks of life (Zhao *et al.*, 2001). One obstacle to the investigation of these enzymes and

their roles in reproduction is the obviating systemic effects that deficiency in any one of these enzymes causes. As with MTHFR mice, tissue-specific knockouts of these genes would be necessary to determine their role, if any, in spermatogenesis and methylation of germ cell DNA. As well, subtle interactions between dietary folate and heterozygotes might be studied to elucidate potential roles of these enzymes in germ cell development.

5.5.3 Diet and Nutrition

Deficiency or supplementation of some nutrients may alter DNA methylation (Van den Veyver, 2002). Through dietary manipulation of methyl group donors, methylation can be altered. Rats fed a methyl-deficient diet experienced DNA hypomethylation, which was reversible within a matter of weeks once rats were returned to a methyl-adequate diet (Wainfan *et al.,* 1989; Christman *et al.*, 1993). However, the oncogenes *c-myc* and *c-fos* remained unmethylated, indicating that long-term methyl deficiency may result in permanent phenotypic changes (Christman *et al.*, 1993). In our studies, diets supplemented with betaine improved spermatogenesis considerably. It would be interesting to otherwise supplement the diets of $Mthfr^{-/-}$ males to see if similar results would be achieved with other nutrients. Conversely, nutritional deficiencies may underlie some forms of male infertility. In the female, the link between folate and reproduction is well-established (reviewed in Nelen, 2001), but the role of this nutrient in male fertility is not known. Would male $Mthfr^{+/-}$ mice fed methyl or folate-deficient diets experience reduced fertility? Can such diets further reduce methylation in 5-azaCdR treated $Dnmt1^{+/+}$ and $Dnmt1^{c/+}$ germ cells? Recent studies in the mouse have indicated that the nutritional status of the parents may affect the phenotype of offspring through changes in methylation (Waterland & Jirtle, 2003).

5.5.4 Other Epigenetic Modifications

As mentioned earlier, there exists a tight link between DNA methylation and histone modifications (Fuks *et al.*, 2000, 2003a, 2003b; Rountree *et al.*, 2000; Ballestar &

Esteller, 2002; Lehnertz *et al.*, 2003; Espada *et al.*, 2004). 5-AzaCdR is a powerful inhibitor of DNA methylation (Jones & Taylor, 1980) and acts synergistically with HDAC inhibitors such as trichostatin A (TSA) (Cameron *et al.*, 1999). In addition, 5-azaCdR appears capable of interrupting the silencing feedback loop between DNA methylation and histone methylation, thereby restoring gene expression, whereas HDAC inhibitors do not have this capacity (Nguyen *et al.*, 2002; reviewed in Issa, 2003; Kondo *et al.*, 2003). Clinical trials are now underway using combinations of histone deacetylases and 5-azaCdR as potential cancer therapy (reviewed in Claus & Lübbert, 2003; Leone *et al.*, 2003).

Effects of 5-azaCdR on histone modifications are not limited to genes silenced through promoter-associated methylation (Kondo *et al.*, 2003). LSH (lymphoid-specific helicase) and ATR-X (from α-thalassemia/mental retardation syndrome, X-linked) are members of the SNF2/helicase family of proteins that remodel chromatin, and are postulated to modulate DNA methylation in mammalian cells (Hendrich & Bickmore, 2001; Li, 2002). Mice deficient in LSH die soon after birth and exhibit demethylation of a number of different repetitive elements, including satellite, *LINE-1*, and *IAP* sequences as well as some single-copy sequences, which suggests a role for this enzyme in the maintenance of DNA methylation patterns during development (Geiman *et al.*, 1998; Dennis *et al.*, 2001). ATR-X is localised to pericentromeric chromatin in both human and mouse cells (Gibbons *et al.*, 2000; Berube *et al.*, 2002). Patients with ATR-X syndrome have hypomethylated rDNA genes and hypermethylated Y-chromosome-specific repeats (Gibbons *et al.*, 2000), suggesting an interaction between chromatin remodelling and DNA methylation. However, the precise role of ATR-X in chromatin function is unknown, as are the roles that LSH and ATR-X play in spermatogenesis. As well, ES cells deficient in Suv29h histone methyltransferases have altered methylation at pericentriomeric satellite sequences (Lehnertz *et al.*, 2003). Characterisation of testicular

phenotypes in these models would provide information on the intricacies of the DNA methylation pathway and its interrelationship with histone modifications. These models may also be combined with 5-azaCdR or any of the drugs mentioned in the following section.

5.5.5 Other Pharmacological Agents

In the past few years, several alternative hypomethylating drugs have gained recognition, including zebularine, valproate and procainamide. Zebularine is a ribonucleoside cytosine analogue that inhibits DNA methylation through the same mechanism as 5-aza and 5-azaCdR (Hurd *et al.*, 1999); similar to 5-azaCdR, zebularine preferentially sequesters DNMT1 (Cheng *et al.*, 2003, 2004). Although zebularine is not as potent a hypomethylating agent as 5-azaCdR, it is considerably more stable in aqueous solution, has a much longer half-life (Barchi *et al.*, 1992; Kelley *et al.*, 1986), and can be administered by oral gavage (Cheng *et al.*, 2003). Valproate is a histone deacetylase inhibitor used to treat epilepsy; this drug can demethylate DNA and induce gene expression independently of DNA replication (in both proliferating and non-proliferating cells) via its promotion of histone acetylation (Alonso-Aperte *et al.*, 1999; Phiel *et al.*, 2001; Manev & Uz, 2002; Detich *et al.*, 2003; Zhang *et al.*, 2004). Chronic exposure of male rats to valproate results in decreased testicular weight, testicular atrophy, and spermatogenic arrest at high doses (Cohn *et al.*, 1982; Walker *et al.*, 1990; Yerby & McCoy, 1999; Nishimura *et al.*, 2000; Sveberg *et al.*, 2001), but the impact of this treatment on germ cell methylation has not been studied. It would be interesting to exploit the ability of valproate to diminish DNA methylation in non-proliferating cells. Combination treatment with 5-azaCdR and valproate may allow induction of hypomethylation in both the actively dividing and post-meiotic cells in the testis. Another drug with which to inhibit DNA methylation is the antiarrhythmic drug, procainamide (Cornacchia *et al.*, 1988; Hibino *et al.*, 1998); it is a competitive DNMT

inhibitor that binds to GC-rich DNA and prevents DNMT-DNA interaction (Thomas & Messner, 1986; Zacharias & Koopman, 1990). Procainamide can induce hypomethylation and gene expression both *in vitro* and *in vivo* (Yang *et al.*, 1997; Segura-Pacheco *et al.*, 2003; Villar-Garea *et al.*, 2003; Alikhani-Koopaei *et al.*, 2004). The differing mechanism of DNA methylation inhibition, relative to 5-azaCdR, makes this drug an attractive one with which to compare effects of 5-azaCdR. Moreover, procainamide might also be used in wild-type (*Dnmt1*$^{+/+}$) mice with limited toxicity, as compared to 5-azaCdR. Treatment of germ cell culture with 5-azaCdR and/or any of these drugs may help to elucidate the molecular events that result from disruption of DNA methylation.

5.6 Summary

The work presented in this thesis has contributed to our knowledge and understanding of mechanisms by which alterations in DNA methylation can influence male germ cell development and function. Our studies suggest that the DNMTs work together in maintaining methylation within the male germ line. The studies within this thesis also have clinical relevance. We have demonstrated that at a therapeutically relevant dose 5-azaCdR treatment results in altered methylation at potentially 30 to 100 genomic loci; these alterations may impact on the appropriate gene expression required for normal spermatogenesis. Our results indicate that altered sperm development and function could result after treatment of reproductive-age males with 5-azaCdR. Even if sperm were incapable of natural fertilisation, the advent and increased use of artificial reproductive technologies, such as intracytoplasmic sperm injection (ICSI), means 5-azaCdR damaged sperm could be used to fertilise eggs. If these sperm carry DNA damage incurred through drug-induced hypomethylation or other aberrations, progeny outcome could be altered deleteriously, perhaps in ways that may not be apparent until

offspring are mature.

Furthermore, our results suggest that mutations in MTHFR, and perhaps other folate pathway enzymes, may contribute to some forms of male infertility. We found that infertility caused by complete inhibition of MTHFR can be ameliorated with the addition of a simple nutrient, the alternate methyl donor betaine. This dietary alleviation of infertility may hold potential therapeutic value for the treatment of some male infertility. Here, we have shown that genetic or pharmacological disruption of critical enzymes within the DNA methylation pathway, including non-methylating enzymes, results in spermatogenic disturbances in male mice. There are numerous directions in which the studies described here can be taken, all of which would contribute to our knowledge of the DNA methylation pathway and its role in male germ cell development.

5.7 Original Contributions

1. Doses of 5-aza used to treat male rats elicited greater effects on male mice than on rats, suggesting that the testes of mice are more sensitive to the effects of this agent.

2. In the male mouse, doses of 5-aza and 5-azaCdR of approximately the same potency have similar effects on the male reproductive system.

3. Chronic exposure of wild type (*Dnmt1*$^{+/+}$) male mice to low doses of the hypomethylating drug 5-azaCdR for seven weeks (exposing germ cells throughout spermatogenesis) resulted in dose-dependent decreases in testis weight, increased seminiferous epithelial abnormalities, and decreased sperm counts. These males also experienced dose-dependent decreases in fertility.

4. Chronic exposure of *Dnmt1*$^{c/+}$ male mice, haploinsufficient for the primary methyltransferase DNMT1, to low doses of 5-azaCdR for seven weeks (throughout spermatogenesis) resulted in decreased testis weight, but no significant increase in histological abnormalities of the testis, nor reductions in sperm count or fertility, as indicated by full-term pregnancies. These results indicate that *Dnmt1*$^{c/+}$ males are less susceptible to the deleterious effects of 5-azaCdR.

5. As determined by TLC, overall genomic methylation of CCGG sites decreased in a dose-dependent manner, but was significant only after treatment of *Dnmt1*$^{+/+}$ males for seven weeks with the highest dose (0.15 mg/kg) of 5-azaCdR. Decreases in CCGG methylation were similar for in *Dnmt1*$^{+/+}$ and *Dnmt1*$^{c/+}$ males treated at the same dose level.

6. Despite differences in testis weights, sperm counts and histological abnormalities between *Dnmt1*$^{+/+}$ or *Dnmt1*$^{c/+}$ males treated chronically with low doses of 5-azaCdR, matings with both groups resulted in elevated preimplantation loss.

7. Elevated preimplantation loss after matings with *Dnmt1*$^{c/+}$ males appeared to be the result of impaired fertilisation ability, not loss of embryos, whereas for

Dnmt1$^{+/+}$ males preimplantation loss was likely a combination of impaired fertilisation and increased loss of embryos at the blastocyst stage.

8. *Dnmt1*$^{+/+}$ and *Dnmt1*$^{c/+}$ males treated chronically with 5-azaCdR exhibited altered sperm motility, although significant changes were observed only in *Dnmt1*$^{+/+}$ males. Altered sperm motility could play a role in decreased fertilisation ability.

9. As determined by RLGS, chronic treatment of male *Dnmt1*$^{c/+}$ mice with 5-aza-CdR resulted in greater changes in sperm DNA methylation, at single copy loci, than did treatment of *Dnmt1*$^{+/+}$ males. DNA methylation of the repeat *IAP*, major satellite, and minor satellite sequences was not altered.

10. More severe male reproductive effects were observed in *Dnmt1*$^{+/+}$ males treated with 5-azaCdR than in treated *Dnmt1*$^{c/+}$ males. This suggests that testicular effects of 5-azaCdR are mediated by DNMT1.

11. Effects elicited by acute treatment (three days) of *Dnmt1*$^{+/+}$ or *Dnmt1*$^{c/+}$ males with 0.3 mg/kg 5-azaCdR appeared, from testicular histology, to be reversible.

12. The seminiferous epithelium of *Dnmt1*$^{+/+}$ males responded more quickly to acute treatment with 0.3 mg/kg 5-azaCdR than did that of *Dnmt1*$^{c/+}$ males. Within one week of acute treatment with 5-azaCdR, testicular histological abnormalities were observed in *Dnmt1*$^{+/+}$ males; abnormalities were not seen in *Dnmt1*$^{c/+}$ males until two weeks after treatment. Similarly, for 5-azaCdR-treated *Dnmt1*$^{+/+}$ and *Dnmt1*$^{c/+}$ males, testis weight significantly decreased at 21 and 28 days post-treatment, respectively.

13. Despite differences in testicular histology, similar increases in germ cell apoptosis were observed after acute treatment with 5-azaCdR in *Dnmt1*$^{+/+}$ and *Dnmt1*$^{c/+}$ males. The majority of apoptotic germ cells were the mitotically and meiotically dividing germ cells in stages I-IV and V-VII.

14. Male mice deficient in MTHFR (*Mthfr*$^{-/-}$) were completely infertile and exhibited gross spermatogenic abnormalities, which suggests that MTHFR is necessary for

spermatogenesis. With respect to the testis, *Mthfr*$^{+/-}$ males did not show an intermediate phenotype.

15. MTHFR deficiency results in loss of germ cells soon after birth as evident by decreased germ cell numbers and elevated apoptosis, indicating that MTHFR may be developmentally regulated in the mouse testis.

16. Provision of supplemental betaine to *Mthfr*$^{-/-}$ males, both prenatally and postnatally, resulted in significantly improved testicular histology, sperm counts and fertility. Although substantial, these improvements were not to the level of wild-type.

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APPENDIX III

Copyright Permissions & Compliance Forms

McGill University
Animal Use Protocol – Research

Guidelines for completing the form are available at www.mcgill.ca/fgsr/rgo/animal/

Protocol #: 3595
Investigator #: 685
Approval End Date: June 30, 2005
Facility Committee: MCH

☐ Pilot ☐ New Application ☒ Renewal of Protocol # 3595

Title (must match the title of the funding source application): Mechanisms of regulation of gene expression in the mammalian germ line.

C level

1. Investigator Data:

Principal Investigator:	Jacquetta Trasler, M.D., Ph.D.	Office #:	412-4400, x25235
Department:	Pediatrics	Fax#:	412-4331
Address:	Montreal Children's Hospital Research Institute	Email:	jacquetta.trasler@mcgill.ca

2. Emergency Contacts: Two people must be designated to handle emergencies.

Name:	Jacquetta Trasler	Work #:	412-4400, x25235	Emergency #:	631-9123
Name:	Josee Martel	Work #:	412-4400, x23291	Emergency #:	450-689-9730

3. Funding Source:

ACTION	✓	DATE
	✓	
☐ NOT APPROVED		

External ☒
Source (s): CIHR
Peer Reviewed: ☒ YES ☐ NO**
Status : ☒ Awarded ☐ Pending
Funding period: 2000-2005

Internal ☐
Source (s):
Peer Reviewed: ☐ YES
Status: ☐ Awarded ☐ Pending
Funding period:

** All projects that have not been peer reviewed for scientific merit by the funding source require 2 Peer Review Forms to be completed . e.g. Projects funded from industrial sources. Peer Review Forms are available at www.mcgill.ca/fgsr/rgo/animal/

Proposed Start Date of Animal Use (d/m/y): or ongoing ☒

Expected Date of Completion of Animal Use (d/m/y): or ongoing ☒

Investigator's Statement: The information in this application is exact and complete. I assure that all care and use of animals in this proposal will be in accordance with the guidelines and policies of the Canadian Council on Animal Care and those of McGill University. I shall request the Animal Care Committee's approval prior to any deviations from this protocol as approved. I understand that this approval is valid for one year and must be approved on an annual basis.

Principal Investigator: [signature] Date: June 25/2004

Approval Signatures:

Chair, Facility Animal Care Committee:	[signature]	Date: July 6, 2004
University Veterinarian:	[signature]	Date: July 6, 2004
Chair, Ethics Subcommittee(as per UACC policy):	—	Date: —
Approved Period for Animal Use	Beginning: July 1, 2004	Ending: June 30, 2005

☐ This protocol has been approved with the modifications noted in Section 13.

08 SEP 2004

Centre universitaire de santé McGill
McGill University Health Centre

Internal Permit #: 7-2006-04

Permit Holder: Jacqueta Trasler **Office:** PT.232

Department: Pediatric Human Genetics **Telephone:** 25235

(A) Location

Room	Classification
PT.233*	Basic
PT.234*	Basic

Room indentified by an asterix (*) must have a door warning sign

(B) Authorized Activity

Isotope	Max Manipulated (MBq)	Max Purchase (MBq)
P32	3.7	18.5

(C) Authorised Users

Last Name	First Name	P32	P33	S35	H3	C14	Fe59	I125	Ca45	Co57	Cr51	Na22	Rb86
Chan	Donovan	☑	☐	☐	☑	☐	☐	☐	☐	☐	☐	☐	☐
Deng	Liyuan	☑	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Kelly	Tamara	☑	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Lasalle	Sophie	☑	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Lucifero	Diana	☑	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Martel	Josée	☑	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Neaga	Oana-Raija	☑	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Oakes	Christopher	☑	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Sun	Wei David	☑	☐	☐	☑	☐	☐	☐	☐	☐	☐	☐	☐
Toppings	Mark	☑	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Trasler	Jacqueta	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

(D) Conditions/Notes

The persons listed in section (C) are authorised to use the designated radioisotopes. The radioisotopes and their respective activities listed in section (B) can only be used in the laboratories listed in section (A) in accordance with the conditions listed in section (D). Importation, storage, manipulation and disposition of radioactive material must be performed in conformity with our CNSC licence, with Federal regulations and with the MUHC Radiation Safety Policies and Procedures. A copy of the CNSC consolidated licence is available from the Radiation Protection Service (ext. 43866).

Approved by: _____
Radiation Safety Manager (43866)

Date issued: Wednesday, November 03, 2004

Expiration date: December 31, 2006

McGill University
Animal Use Protocol – Research

Guidelines for completing the form are available at www.mcgill.ca/fgsr/rgo/animal/

Protocol #: 3595
Investigator #: 685
Approval End Date: June 30, 2005
Facility Committee: MCH

☐ Pilot ☐ New Application ☒ Renewal of Protocol # 3595

Title (must match the title of the funding source application): Mechanisms of regulation of gene expression in the mammalian germ line.

C level

1. Investigator Data:

Principal Investigator:	Jacquetta Trasler, M.D., Ph.D.	Office #:	412-4400, x25235
Department:	Pediatrics	Fax#:	412-4331
Address:	Montreal Children's Hospital Research Institute	Email:	jacquetta.trasler@mcgill.ca

2. Emergency Contacts: Two people must be designated to handle emergencies.

Name:	Jacquetta Trasler	Work #:	412-4400, x25235	Emergency #:	631-9123
Name:	Josee Martel	Work #:	412-4400, x23291	Emergency #:	450-689-9730

3. Funding Source:

ACTION	✓	DATE
	✓	Sept 1/04
	☐ NOT APPROVED	

External ☒
Source (s): CIHR
Peer Reviewed: ☒ YES ☐ NO**
Status : ☒ Awarded ☐ Pending
Funding period: 2000-2005

Internal ☐
Source (s):
Peer Reviewed: ☐ YES ☐ NO
Status: ☐ Awarded ☐ Pending
Funding period:

** All projects that have not been peer reviewed for scientific merit by the funding source require 2 Peer Review Forms to be completed . e.g. Projects funded from industrial sources. Peer Review Forms are available at www.mcgill.ca/fgsr/rgo/animal/

Proposed Start Date of Animal Use (d/m/y):	or ongoing ☒
Expected Date of Completion of Animal Use (d/m/y):	or ongoing ☒

Investigator's Statement: The information in this application is exact and complete. I assure that all care and use of animals in this proposal will be in accordance with the guidelines and policies of the Canadian Council on Animal Care and those of McGill University. I shall request the Animal Care Committee's approval prior to any deviations from this protocol as approved. I understand that this approval is valid for one year and must be approved on an annual basis.

Principal Investigator:	Date: June 25/2004

Approval Signatures:

Chair, Facility Animal Care Committee:		Date: July 6, 2004
University Veterinarian:		Date: July 6, 2004
Chair, Ethics Subcommittee(as per UACC policy):		Date: —
Approved Period for Animal Use	Beginning: July 1, 2004	Ending: June 30, 2005

☐ This protocol has been approved with the modifications noted in Section 13.

08 SEP 2004

November 23, 2004

Tamara,

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Tamara Kelly
tamara.kelly@mail.mcgill.ca

Tamara Kelly
4140 Av. de Melrose
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H4A 2S4

Tamara Kelly
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McGill University Health Centre
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Please let me know if you have any questions.

Sincerely,

Alison Heimburger
Communications and Publications Coordinator
American Society of Andrology, *Journal of Andrology*
alison@wjweiser.com

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Tamara Kelly
McGill University
tamara.kelly@mail.mcgill.ca

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Tamara LJ Kelly
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CANADA

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