THE EFFECTS OF ADVANCED PATERNAL AGE ON GERM CELL CHROMATIN

By

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This thesis is dedicated to my parents.

To my father, Dan Fice, for encouraging me to climb every mountain.

And to my mother, Shirley Fice, for making sure I've gotten to the top safely.

ABSTRACT

Delayed parenthood is a current trend in society, capturing abundant attention from the public. This trend has been correlated with genetic disorders and detrimental outcomes in the offspring of aged mothers and fathers. Though the consequences of maternal aging are well understood, paternal aging is only now being studied. As many of the disorders observed are multigene disorders, we propose that their source is a disruption of sperm chromatin integrity. We hypothesize that due to aging, there is multilevel genome disorganization. The objective of this thesis is to explore the impact of advanced paternal age on fertility, by examining telomere dynamics, gene expression, and DNA methylation in germ cells, using the Brown Norway rat model. This study is designed to capture the impact of age on germ cells earlier during the spermatogenic process. These objectives were accomplished by pursuing three aims: measuring germ cell telomere length across spermatogenesis with advanced age, examining differential gene expression, and determining changes in DNA methylation with aging in post-meiotic round spermatids and mature spermatozoa. Young (4 mos) and aged (18 mos) Brown Norway rats were compared, resembling young (20-30 yrs) and elderly (45+ yrs) humans. First, we measured telomere length in pachytene spermatocytes (diploid), round spermatids (haploid), and sperm from the caput (immature) and cauda (mature) epididymidis from young and aged rats. Telomere length significantly decreases ($p<0.05$) with aging in our rat model. In immature sperm, telomere length decreased from 115.6 kb (+/- 14) to 93.3kb (+/- 12). In mature sperm, it decreased 142.4 kb (+/- 33) to 105.3 (+/- 5). Given differential telomere lengths, we hypothesize that there will be a disruption of chromatin positioning and interactions that could potentially alter the gene expression profile in sperm from aged rodents. For aim two, we used mRNA sequencing to examine the mRNA transcriptome in round spermatids. Through differential expression analysis, Ingenuity Pathway Analysis, and Gene Ontology enrichment, we observed 220 differentially expressed genes due to aging in round spermatids. There are 211 upregulated genes, and 9 downregulated genes with a log2FC > |1|. Based on pathway analysis, these genes sort to pathways involved in: immune response, oxidative stress, cellular protrusions, and reproduction. Once sorted in greater detail, 157 out of the 220 altered transcripts are relevant in the context of spermatogeneic processes. These transcripts are highly involved in: sperm motility, capacitation, the interaction between germ cells and Sertoli cells, fertility, and epigenetics. This suggests genetic bases for many of the changes observed with advanced paternal age at the round spermatid level. Due to alterations in gene expression associated with aging, for aim three we examined DNA methylation in round spermatids, and mature spermatozoa using whole genome bisulfite sequencing. The results suggest a strong trend in hypermethylation in both the round spermatids and sperm with aging, with the majority of the differential methylation falling between |10-20%| difference in methylation. The hypermethylation is spread throughout the genome, with the majority

of the hypermethylation in intergenic regions such as SINE/LINE regions. LINE-1 DMRs had differential methylation levels between 10-20%. Interestingly, the effect of aging can also be noted in the DNA methylation process from round spermatid to spermatozoa, as there are many more DMRs (hyper and hypomethylated) when examining the process of spermatids to spermatozoa in an aged rat. Taken together, these results provide new insight into the mechanisms leading to disrupted fertility and potential genetic alterations in the offspring of older men.

RÉSUMÉ

La parentalité retardée est une tendance dans la société, captant une abondante attention de la part du public. Cette tendance a été corrélée avec des troubles génétiques et des effets néfastes sur la progéniture de mères et de pères âgés. Si les conséquences du vieillissement maternel sur la progéniture sont bien connues, ce n'est que récemment que les effets du vieillissement paternel commencent à être étudiés. Comme les troubles observés sont souvent multigéniques, nous proposons que leur source est une perturbation de l'intégrité de la chromatine des spermatozoïdes. Nous émettons l'hypothèse qu'en raison du processus de vieillissement, il existe une désorganisation du génome à plusieurs niveaux, y compris les télomères, la méthylation de l'ADN et l'expression des gènes. L'objectif de cette thèse est d'explorer l'impact de l'âge paternel avancé sur la fertilité, en examinant la dynamique des télomères, l'expression des gènes et la méthylation de l'ADN dans les cellules germinales, en utilisant le modèle du rat Brown Norway, Les objectifs de cette thèse sont: mesurer la longueur des télomères des cellules germinales au cours de la spermatogenèse, examiner l'expression génique différentielle et déterminer les changements au niveau de la méthylation de l'ADN des spermatides rondes et des spermatozoïdes matures lors du vieillissement. Une comparaison entre des rats Brown Norway jeunes (4 mo) et âgés (18 mo) a été fait, simulant des populations humaines jeunes (20-30 ans) et âgées (45 ans+). Nous avons premièrement mesuré la longueur des télomères dans les spermatocytes pachytènes, les spermatides rondes et les spermatozoïdes de la tête de l'épididyme et de la queue de l'épididyme obtenus de rats jeunes et âgés. Nous avons observé une diminution significative de la longueur des télomères ($p<0,05$) avec le vieillissement. La longueur des télomères a diminué de 115,6 kb (+/- 14) à 93,3 kb (+/- 12) dans les spermatozoïdes immatures et de 142,4 kb (+/- 32) à 105,3 (+/- 5) dans les spermatozoïdes matures. Nous émettons donc l'hypothèse qu'avec l'âge, il y a une perturbation de l'emplacement et des interactions de la chromatine qui a le potentiel de modifier le profil d'expression génique dans le sperme des rats. Pour interroger cette hypothèse, nous avons conçu comme deuxième objectif le séquençage de l'ARNm pour examiner le transcriptome dans les spermatides rondes post-méiotiques. En nous servant d'outils d'analyse génique différentielle, d'analyse de l'enrichissement des voies biologiques et d'ontologie génétique, nous avons observé l'expression différentielle de 220 gènes. L'expression de 211 gènes a augmentée tandis que l'expression de 9 gènes a diminué par un changement de $\log2 > |1|$ en conséquence du vieillissement des spermatides rondes. D'après l'analyse de l'enrichissement des voies biologiques, ces gènes jouent un rôle dans la réponse immunitaire, le stress oxydatif, les protrusions cellulaires et la reproduction. L'analyse plus approfondit et plus en détail révèle que 157 des 220 gènes altérés sont impliqués dans des processus importants pour la spermatogenèse, entre autres, la motilité des spermatozoïdes, la capacitation, l'interaction entre les cellules germinales et les cellules de Sertoli, la fertilité et l'épigénétique. Ainsi, un bon nombre des changements observés avec l'âge paternel avancé au

niveau des spermatides rondes seraient associés à des changements génétiques. En raison des altérations de l'expression des gènes observées associées au vieillissement, comme troisième objectif, nous avons examiné la méthylation de l'ADN dans les spermatides rondes et les spermatozoïdes matures en appliquant le séquençage génome entier au bisulfite. Les résultats suggèrent une forte tendance à l'hyperméthylation dans les spermatides rondes et les spermatozoïdes avec l'âge, avec la majorité des différences de méthylation se trouve entre |10-20%|. L'ADN hyperméthylée est répartie sur tout le génome et se situe, en grande partie, dans les régions intergéniques telles que les régions SINE/LINE. Les DMR LINE-1 avaient des niveaux de méthylation différentiels entre 10 et 20 %. De plus, le nombre augmenté de régions avec méthylation différentielle (hyper- et hypométhylé) entre les spermatides rondes et les spermatozoïdes âgés suggère que le procès de méthylation de l'ADN lors de la transformation de la spermatide ronde en spermatozoïde mature semble également être affecté par l'âge chez le rat. Ensembles, ces résultats apportent des nouveaux renseignements au sujet des mécanismes qui mènent à une fertilité perturbée chez les hommes plus âgés et les potentielles conséquences génétiques chez leur progéniture.

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List of Abbreviations

3C	Chromatin conformation capture
5-mC	5-methyl cytosine
5-hmC	5-hydroxymethyl cytosine
8-oxo-dG	8-oxo-2'-deoxyguanosine
ADHD	Attention deficit hyperactivity disorder
AKT	Protein kinase B
ALT	Alternative lengthening of telomeres
APA	Advanced paternal age
ASRM	American Society for Reproductive Medicine
ATAC-seq	Assay for transposase accessible chromatin sequencing
BER	Base excision repair
BN	Brown Norway
BORIS	Brother of the Regulator of Imprinted Sites
BP	base pairs
BRDT	testis specific bromo-domain containing protein
BSA	Bovine serum albumin
BTB	Blood-Testis Barrier
CAT	Catalase
CHG	Nucleotide base cytosine separated by one nucleotide from guanine
CHH	Nucleotide base cytosine on its own
CO2	Carbon dioxide
CpG	cytosine-phosphate-guanine
CpG	Nucleotide base cytosine paired with guanine

CTCF	CCCTC-binding factor
CTCFL	CCTC-binding factor like
CYP11A1	cytochrome P-450 cholesterol side-chain cleavage enzyme
CYP17A1	17-hydroxylase/C17-20-lyase
DDR	DNA damage response
DEG	differentially expressed gene
DMR	differentially methylated region
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferases
DSB	Double strand breaks
DTT	dithiothreitol
ERK	Extracellular signal regulated kinase
FACs	Flow-cytometry analysis
FISH	Fluorescence in situ hybridization
FLCs	Fetal Leydig cells
FPKM	Fragments Per Kilobase of transcript per Million reads mapped
FSH	Follicle stimulating hormone
GnRH	Gonadotropin releasing hormone
GO	Gene ontology
GSK3	Glycogen synthase kinase 3
HAT	histone acetyl transferases
HDAC	histone deactylases
Hi-C	Chromatin conformation capture method examining all chromatin interactions
HP1	Heterochromatin protein 1

HPG axis	Hypothalamic-pituitary-gonadal axis
HR	homologous recombination
HSD17B	17ß-Hydroxysteroid dehydrogenase
HSD3B	3-hydroxysteroid dehydrogenases
ICSI	intracytoplasmic sperm injection
IPA	Ingenuity Pathway Analysis
KEGG	Kyoto Encyclopedia of Genes and Genomes
LH	Luteinizing Hormone
LHR	luteinizing hormone receptor
lncRNA	long noncoding RNA
MARS	matrix association regions
MAT	methionine adenosyltransferase
MMR	Mismatch repair
mRNA	messenger RNA
MTHFR	Methylenetetrahydrofolate reducatase
ncRNA	noncoding RNA
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
NIA	National Institute on Ageing
PCA	Principle component analysis
piRNA	Piwi-interacting RNA
PS	Pachytene spermatocyte
PTM	post translational modification
qPCR	Quantitative polymerase chain reaction
RA	retinoic acid

RAR	retinoic acid receptor
RNA	Ribonucleic Acid
RNA-Seq	RNA-Sequencing
ROS	Reactive oxygen species
rRNA	ribosomal RNA
RS	Round spermatid
rTL	Relative telomere length
sAC	Soluble adenylyl cyclase
SAM	s-adenosyl-methionine
SC	Sertoli cell
scRNA	Small conditional RNA
SD	Sprague-Dawley
SGK	Serum glucocorticoid kinase
sncRNA	small noncoding RNA
SOD1	Superoxide dismutase
SOGC	Society of Obstetricians and Gynecologists of Canada
sRNA	small non protein coding regulatory RNA
SSC	Spermatogonial stem cell
StAR	steroidogenic acute regulatory protein
TADs	topologically associated domains
TERC	Telomerase RNA component
TERRA	Telomeric repeat containing RNA
TERT	Telomerase reverse transcriptase
TET	Ten-eleven translocation
THF	tetrahydrofolate

TP	transition protein
TPSO	translocator protein
TRF1/2	Telomere binding proteins 1/2
tRF	Transfer RNA fragments
tRNA	Transfer RNA
TSS	Transcription start site
TTS	Transcription termination site
UTR	Untranslated region
WGBS	Whole genome bisulfite sequencing
WHO	World Health Organization

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Format of the Thesis

This thesis is a manuscript based thesis, conforming to section I.C of the "Guidelines for Thesis Preparation: of the Faculty of Graduate Studies and Research of McGill University." The manuscripts are presented in the order in which they were published or submitted for publication. We retain the right to the manuscripts in this thesis according to the copyright agreements of the respective publishers, provided that this thesis is not published commercially or used for commercial purposes. Chapter one is an introduction to advanced paternal age, male fertility and germ cell chromatin, with background on the theories of aging, animal models to study aging, spermatogenesis, DNA damage, chromatin compaction, and epigenetics. The chapter ends with the rationale for the studies in this thesis and the objectives of the thesis. Chapters 2 and 3 are published manuscripts, and Chapter 4 is in preparation with collaborators. Connecting texts are included between chapters to ensure continuity. Chapter 5 is a general discussion of the findings, and recommendations for future work. References are included at the end of each chapter. In Chapter 4, Dr. Donovan Chan aided in DNA methylation bioinformatics analysis. The candidate completed all of the experiments and analysis.

Contribution of Authors

All experiments presented in this thesis were performed by the candidate. With the exception of bioinformatics for RNA-sequencing done by Novogene Corporation, and initial bioinformatics for whole-genome bisulfite sequencing, which were done by Novogene Corporation and Dr. Donovan Chan.

Land Acknowledgement

McGill University is located in Tiohtià:ke/Montréal, on the unceded lands of Indigenous peoples. The Kanien'kehá:ka Nation is recognized as the stewards and custodians of the lands and waters on which McGill was founded. These lands have long served as a site of meeting and exchange amongst Indigenous peoples, including the Haudenosaunee and Anishinabeg nations. I acknowledge and thank the diverse Indigenous peoples whose presence marks this territory on which I have completed the works in this thesis.

Considerations of Gender and Sex

This thesis focuses on reproductive aging in humans/rodents with XY chromosomes. The author recognizes that not all XY individuals identify as men, have the biologically male reproductive organs, or produce testosterone and spermatozoa. However, in agreement with the field of paternal aging, gendered language is present in this text when discussing the subject. Paternal in this instance would refer to all individuals that produce spermatozoa.

CHAPTER 1

INTRODUCTION

1. Male Reproduction

The male reproductive tract is composed of the brain and the male reproductive organs: the testes, epididymides, vas deferens, ejaculatory ducts, prostate gland, seminal vesicles, bulbourethral glands, urethra, and the penis (Fig. 1-1). Sperm are continuously produced in the seminiferous tubules of the testis through the process of spermatogenesis (Okafor et al., 2022). The seminiferous tubules have a basement membrane comprised of collagen and laminin. These tubules are supported by cells within the testicular interstitium, including Leydig cells and vascular, immune, and peritubular cells. The peritubular cells lie along the basement membrane of the seminiferous tubules, providing support and contractile motion. The contractile motion aids in spermiation and the movement of spermatozoa through the seminiferous tubule lumen and into the epididymis. Leydig cells are accompanied by their undifferentiated mesenchymal progenitor cells and are the site of testosterone biosynthesis. The vascular cells include endothelial, smooth muscle, and perivascular cell types. Under normal conditions, the interstitium is also home to testicular macrophages. Testicular macrophages have been shown to regulate Leydig cell function and Sertoli cell action and support spermatogenesis (Fujisawa, 2006).

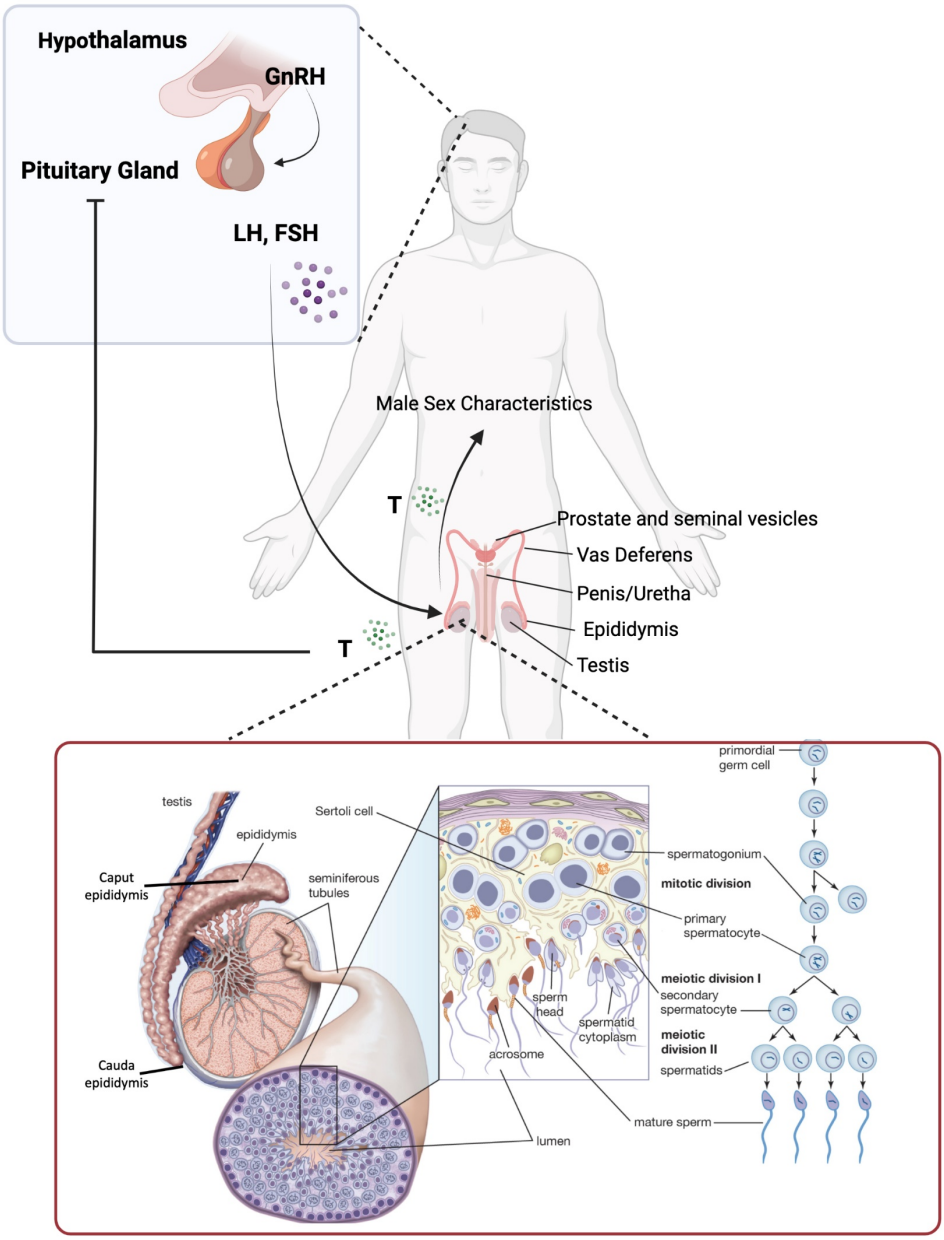

Figure 1-1: Overview of male reproduction. Beginning in the brain with the HPG axis. GnRH from the hypothalamus stimulates the secretion of LH and FSH from the pituitary gland. FSH acts on the Sertoli cells as a regulator of spermatogenesis. LH acts on the Leydig cells of the testis to produce testosterone. Testosterone goes on to produce male sex characteristics and regulate spermatogenesis. Spermatogenesis occurs within the testis, with all cell types shown herein. Created with BioRender, and modified from Encyclopedia Britannica.

1.1 Somatic cells of the testis

A limited number of somatic cells within the testis support the development of spermatozoa. The germ cells develop within the seminiferous tubules but are supported by various cells throughout this process.

1.1.1 Leydig Cells

The Leydig cells are the site of testosterone biosynthesis, providing the high concentration of intratesticular testosterone necessary for spermatogenesis. The adult Leydig cells (ALCs) are located within the interstitial space and are accompanied by their undifferentiated progenitors. ALCs develop from stem cells in the post-natal mammal, going through four distinct phases of development from stem Leydig cells (SLCs), progenitor Leydig cells (PLCs), immature Leydig cells (ILCs), and finally to ALCs (Chen et al., 2010).

In rodents, the first steps of Leydig cell differentiation take place during the first weeks of post-natal life in XY individuals. However, the full ADCs are not differentiated and testosterone-producing until puberty (Chen et al., 2010). Adult Leydig cells have relatively slow, or non-existent, turnover during the lifespan (Teerds et al., 1989). There is much debate within the field of andrology as to whether PLCs or ILCs can differentiate into adult Leydig cells after toxicant insult, such as with ethane 1,2-dimethanesulphonate (EDS). Indeed, after treatment with EDS, there is a depletion in ALCs and, subsequently, a replacement with a new population of Leydig cells (Kerr et al., 1985; Molenaar et al., 1985; Jackson et al., 1986; Morris et al., 1986). The cellular origin of the new population of LCs is still being researched.

1.1.2 Sertoli Cells

Sertoli cells were first described by Enrico Sertoli in 1865 and named after him in 1888. Sertoli cells are the somatic nurse cells or support cells within the seminiferous tubules. Sertoli

cells extend from the basement membrane of the seminiferous epithelium up toward the lumen, with branch-like projections that form crypts for germ cell support (França et al., 2016). Sertoli cells are constantly remodeling throughout spermatogenesis in order to allow for the progression of germ cells from the basement membrane to the lumen. As the germ cells develop, they become more deeply embedded within the Sertoli cell and are finally released through spermiation (França et al., 2016). Sertoli cells also provide signaling support to the germ cells through cytokines, glycoproteins, and metal transport proteins such as transferrin and ceruloplasmin (França et al., 2016). The metal transport proteins were among the first studied and showed that metal ions could be taken up at the basement membrane of the seminiferous epithelium by Sertoli cells and transferred to developing germ cells (Leichtmann-Bardoogo et al., 2012).

Binding with one another, Sertoli cells form the blood-testis barrier (BTB) through tight junctions. The BTB allows for the testis-immune privilege, as a balance must be maintained where the body is protected from external pathogens and the germ cells are protected from the body's innate immune system (Washburn et al., 2022). Though tight junctions form a clear BTB, the immune system of the testis requires immune modulation by the Sertoli cells. They secrete and respond to many immunoregulatory factors and induce regulatory immune cells from the interstitial space (Washburn et al., 2022). They also act to phagocytize apoptotic germ cells and the residual bodies of developing germ cells (Nakanishi and Shiratsuchi, 2004).

1.2 Hormonal Control of Male Reproduction

Hormonal control of male reproduction is through the hypothalamic-pituitary-gonadal axis (HPG axis) in XY individuals. The hypothalamus in the brain releases gonadotropin-releasing hormone (GnRH) under the regulation of sex hormones, neurotransmitters, and

environmental cues or stressors (Genazzani et al., 2000; Bova et al., 2014; Spergel, 2019). GnRH then stimulates the secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary gland into circulation. At the level of the pituitary gland, many modifying molecules, such as neuropeptide Y, are able to regulate GnRH action by binding antagonistically to the GnRH receptor (Parker et al., 1991). FSH stimulates the Sertoli cells of the testis to produce many proteins including androgen-binding protein and inhibin (Kerr et al., 1992). Inhibin can also act as a negative regulator of the HPG axis through its action on the pituitary gland. LH acts on the Leydig cells of the testis to stimulate testosterone production (Fig. 1-2). Testosterone production in the testis is critical, as testosterone is the primary driver of spermatogenesis and must exist at high concentrations within the testis. Testosterone acts as a negative regulator at the hypothalamus and pituitary gland level (Okafor et al., 2022).

In the synthesis of testosterone, LH acts on the luteinizing hormone receptor (LHR) and among many functions, stimulates adenylyl cyclase to produce cyclic-AMP (cAMP) that then activates cAMP-dependent kinase PKA (Stojkov et al., 2013). PKA activates the conversion of cholesterol into its more bioactive forms; cholesterol is transported into the mitochondria the steroidogenic acute regulatory protein (StAR) and the translocator protein (TPSO). This functions as the rate-limiting step of steroidogenesis. Once inside the Leydig cell mitochondria, cholesterol is converted by cytochrome P-450 cholesterol side-chain cleavage enzyme (CYP11A1) into pregnenolone. In the endoplasmic reticulum, the remaining steps of testosterone biosynthesis take place, with the conversion of pregnenolone by 3-hydroxysteroid dehydrogenases (HSD3B) to progesterone. Then, 17-hydroxylase/C17-20-lyase (CYP17A1) catalyzes progesterone to androstenedione. Finally, androstenedione is converted into

testosterone by 17ß-Hydroxysteroid dehydrogenase (HSD17B) (Haider, 2007; Henrich and DeFalco, 2020).

Testosterone then diffuses through the Leydig cell membrane and into the blood circulation to have effects on a wide array of tissues in the body and into the seminiferous epithelium to drive spermatogenesis.

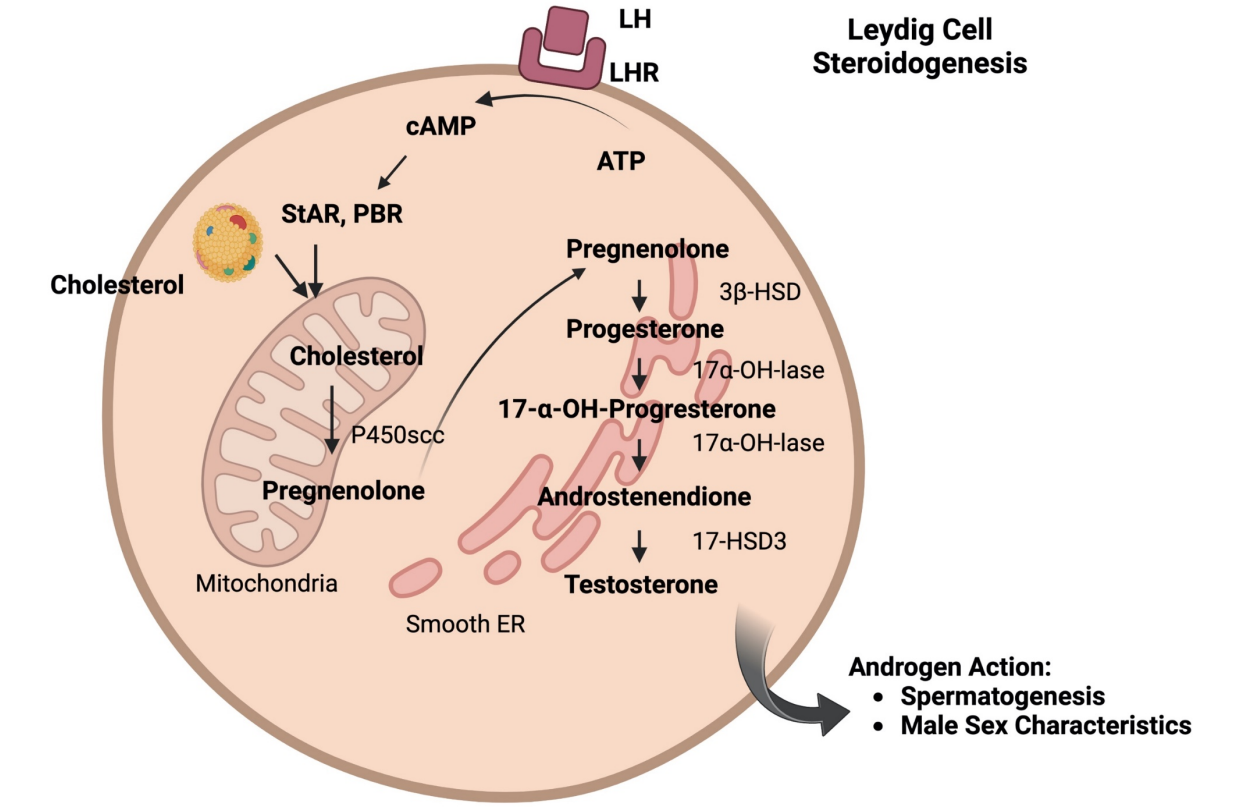

Figure 1-2: Steroidogenesis within the Leydig cell. Briefly, LH binds to the LHR, which triggers the production of cAMP and the production of StAR, and PBR. StAR and PBR bring cholesterol into the mitochondria to begin testosterone production. P450scc converts cholesterol to pregnenolone that is then converted by 3beta-HSD into progesterone. 17-alpha-OH-lase converts progesterone to androstenedione in two steps through a 17-alpha-OH-progresterone intermediate. Androstenedione is then converted to testosterone by 17-HSD-3. Testosterone then is released from the Leydig cell to act on the somatic cells to determine male sex characteristics or on the Sertoli cells to regulate spermatogenesis. Created with BioRender.

1.3 Spermatogenesis

Spermatogenesis is the process by which spermatozoa are continuously produced within the seminiferous tubules of the testis from the onset of puberty and during adulthood. The germline is established and maintained by the spermatogonia stem cells (SSCs), capable of self-renewal and differentiation. The SSCs sit at the basement membrane of the seminiferous epithelium and either undergo mitosis to maintain the stem cell pool or differentiate to form spermatozoa (Diao et al., 2022). The differentiating SSC undergoes a further mitotic division to form primary spermatocytes. The primary spermatocytes undergo meiosis I to form secondary spermatocytes. The spermatocytes are the active meiotic cell type during spermatogenesis, and in this stage, abundant DNA damage is introduced and repaired. Secondary spermatocytes undergo meiosis II to form round spermatids (Griswold, 2016). The haploid round spermatids are the last transcriptionally active cell type. Round spermatids undergo a DNA reorganization process during spermiogenesis, where the histone-bound DNA is gradually replaced by a cysteine-rich protein called protamine (O'Donnell, 2014). It is during this process as well that spermatids shed their cytoplasm through residual bodies and develop the acrosome and a tail. The acrosome caps the anterior sperm nucleus and is an organelle containing many hydrolytic enzymes to aid sperm-egg binding and penetration (Berruti and Paiardi, 2011). The germ cells are referred to as elongating spermatids throughout spermiogenesis, noted from their structure. Once the developing germ cells have gained a tail and the complete acrosome, they are poised at the edge of the seminiferous epithelium for spermiation. Spermiation is the release of spermatozoa into the seminiferous tubule lumen to transit the spermatozoa to the caput of the epididymis (Griswold, 2016). This transit is controlled by the contractile motion generated by the peritubular cells. Spermatogenesis and epididymal transit require ~74 days in humans and ~56 days in rats (Griswold, 2016).

1.4 Cycle of the seminiferous epithelium

The production of spermatozoa through spermatogenesis is a cyclical process and occurs in a wave. Yves Clermont was the first to observe and characterize the patterns he saw in the seminiferous epithelium. He consistently observed that the same types of germ cells could be found in the one histological section of a seminiferous tubule (Leblond and Clermont, 1952). He recognized this pattern and came up with a method whereby researchers can now sort spermiogenesis into stages (Fig. 1-3). The staging is done by examining nucleus size, cell shape, and acrosome development. In the rat, there are fourteen stages (I-XIV), each with distinct cell compositions (Clermont and Leblond, 1953). Interestingly, Clermont also noted that in rodents, when one section of a seminiferous tubule begins a new round of spermatogenesis, the segment next to it will begin shortly after that, and this continues across a whole seminiferous tubule. This process is quite complex in humans, and there are many stages within one tubule side by side. Instead of progressing through the tubule like a wave, it happens more like a winding corkscrew (Clermont, 1963).

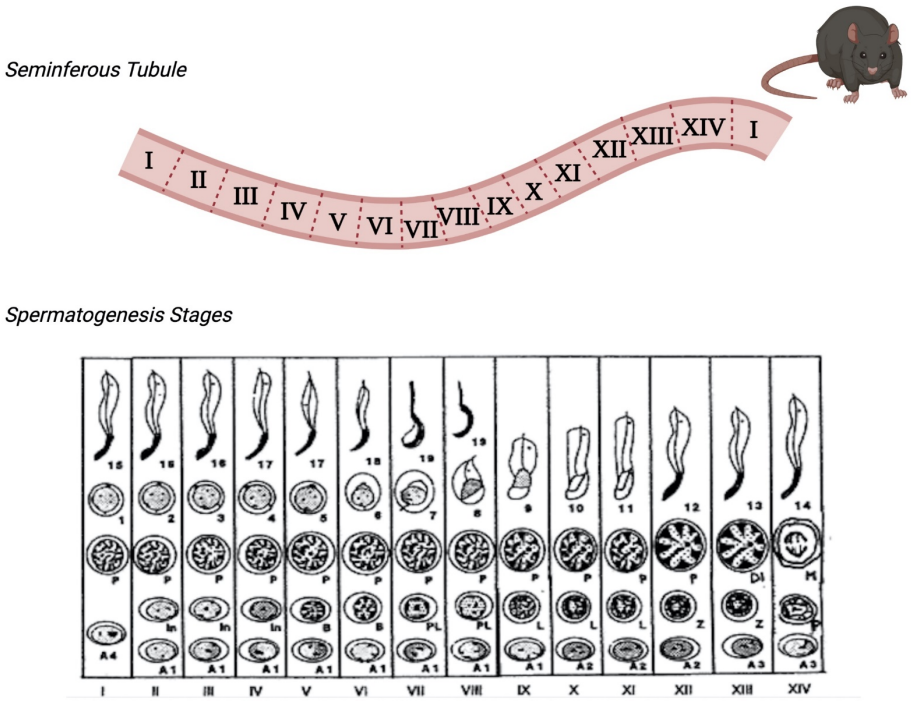

Figure 1-3: Cycling of the seminiferous epithelium. The seminiferous tubule is divided into various stages (I-XIV) representing the wave-like nature., and the spermatogenesis stages in the rat shown below. Created with BioRender. Modified from Homma-Takeda et al., 2001 (License # 5517741386597).

1.5 Epididymal Sperm Maturation

The epididymis is a small tubular organ that is adjacent the testis within the scrotum. It consists of four main segments: the initial segment, caput, corpus, and cauda. As the names suggest, these components begin where the efferent ducts merge and form the initial segment. Spermatozoa continue to move through the caput (head) of the epididymis, transit through the corpus (body) of the epididymis, and are finally stored in the cauda (tail) of the epididymis. As spermatozoa transit the epididymis, they gain functional motility, fertilizing capabilities, and undergo the final steps of chromatin compaction. The process of chromatin compaction is

complete in the epididymis, as the environment presents oxidizing conditions that allow for thiol groups contained within protamine molecules to form disulfide bonds (Calvin and Bedford, 1971; Bedford and Calvin, 1974; Saowaros and Panyim, 1979). The protamine disulfide bonds are additionally stabilized by zinc molecules in the epididymis (Björndahl and Kvist, 2009). Thiol oxidation also occurs in proteins during transit through the epididymis, leading potentially to stabilization especially in the flagella (Mercado et al., 1976; Gervasi and Visconti, 2017). Following spermiation, the cytoplasmic droplet also migrates during epididymal transit from the sperm neck to the annulus (Cooper, 2011). The cytoplasmic droplet may function in providing energy, ion balance or other molecules necessary for sperm function (as reviewed by: Gervasi and Visconti, 2017). The sperm membrane also undergoes remodelling, with the addition, removal and modification of the sugars and lipids that constitute the sperm plasma membrane (Gervasi and Visconti, 2017). These changes to the membrane predominantly aid in the rearrangements necessary for sperm-egg fusion. Finally, sperm gain the ability to be progressively motile during transit through the epididymis. The mechanism of this is still unclear, but it is presently hypothesized that this is due to various signaling cascades during epididymal transit, specifically protein kinase A phosphorylation, and serine/threonine phosphatases such as the testis specific ser/thr phosphatase PPP1CC2 (Sasaki et al., 1990; Vijayaraghaven et al., 1996). This process depends on other signaling molecules and pathways, including: glycogen synthase kinase 3 (GSK3), cyclic AMP (cAMP) activation by soluble adenylyl cyclase (sAC), or potentially Akt (protein kinase B) and SGK (serum glucocorticoid kinase) (Esposito et al., 2004; Hess et al., 2005; Somanath et al., 2004; Vadnais et al., 2013). More recent analyses are proposing entirely separate pathways for sperm maturation through pathways such as Wnt signaling (Koch et al., 2015).

The sperm proteome and RNA payload also change in composition during epididymal transit. Yanagimachi et al. (1985) first described so-called epididymosomes, small vesicles resting on the sperm membrane within the epididymis. It was hypothesized then that these vesicles provided proteins to the sperm that aided in their maturation (Sullivan and Saez, 2013; James et al., 2020). These proteins largely include glycosylphosphatidylinositol (GPI)-anchored proteins and other sperm surface binding proteins, that aid in proper formation of the sperm plasma membrane and fertilizing ability (Sullivan et al., 2007). Other proteins that are acquired during epididymal transit are those in the polyol pathway; they metabolize sugars necessary for metabolism and energy (Sullivan et al., 2007). Studies have shown that during transit through the epididymis, many surface proteins also undergo post-translational modifications that are gained or lost (Cooper, 1998). Other acquired proteins function in sperm capacitation, the acrosome reaction, sperm-egg binding, fertilization, and sperm motility (as reviewed by Gervasi and Visconti, 2017).

As a result of the varying roles of the epididymis in sperm maturation and final steps of chromatin compaction, research on the contribution of the epididymis to epigenetic regulation is being done. Specifically, Nixon et al. (2015) have studied the contribution of the epididymis to sperm small non-coding regulatory RNAs (sRNAs). The hypothesis is that the epididymosomes are the likely vesicle for transporting these molecules. Thus, ongoing studies are examining epididymosome content and contributions to spermatozoa (Trigg et al., 2019).

After leaving the epididymis, sperm undergo the final process required for fertilization, i.e., capacitation; this normally occurs in the female reproductive tract but can also be induced experimentally. Capacitation was first described by Chang (1951) and Austin (1951), each independently drawing the same conclusions. More recently capacitation has been further

divided into fast and slow events, regulated by similar molecules HCO3-, sAC and cAMP (Salicioni et al., 2007). Flagellar movement is a fast event, and the ability to carry out the acrosome reaction via an agonist and tyrosine phosphorylation are the late events. Briefly, capacitation is characterized by removal of cholesterol from the plasma membrane; this is followed by an influx of calcium ions through the HCO3- stimulation and membrane ion channels such as CatSper (Ren and Xia, 2010); secondary messengers are then activated including sAC and finally cAMP is produced (Osheroff et al., 1999) (reviewed by Jin and Yang, 2017). These changes are associated with an increase in cellular pH. Additionally, the extracellular signal regulated kinase (ERK) pathway is activated by extracellular ligands and intracellular ROS. Both ERK activation, and sAC signaling events lead to phosphorylation events, namely tyrosine phosphorylation (Visconti and Kopf, 1998). The process of capacitation is also under the regulation of other proteins and molecules, various hormones including estrogen and progesterone (Ded et al., 2013), and free radicals such as superoxide (Griveau et al., 1994; de Lamirande and Gagnon, 1993; Herrero et al., 1999).

1.6 Controls of spermatogenesis

Spermatogenesis involves many levels of regulation, including endocrine, autocrine, and paracrine signaling. Primarily, testosterone is known to be a driver of spermatogenesis. The Sertoli cell is theorized to be the main target of testosterone responsible for driving spermatogenesis. During the spermatogenic cycle, levels of the androgen receptor (AR) in Sertoli cells rise and fall, suggesting that they control the cyclicity of the seminiferous epithelium (Bremner et al., 1994). The AR is a nuclear receptor, often facilitating the expression or inhibition of genes required for Sertoli cell function or spermatogenesis support. Without testosterone, most germ cells do not complete meiosis. However, the action of testosterone in

meiotic regulation is likely indirect (Walker, 2011). To determine the potential indirect action of testosterone, studies have examined Sertoli cell-specific AR knockouts and found that in these animals, there is a disruption in the BTB and an overall dysfunction of Sertoli cell signaling (Wang et al., 2006). Additionally, FSH signaling in Sertoli cells acts independently and together with testosterone support spermatogenesis. Without FSH, the ratio of Sertoli cells to germ cells decreases, representing their decreased ability to support numerous germ cells. There is partial fertility in a rodent model with constitutive FSH-receptor activity and a knockout of the LH receptor (minimal testosterone production). This suggests the complementariness of FSH and testosterone action (Oduwole et al., 2018).

Recently, retinoic acid (RA) has been studied as an additional regulator of spermatogenesis (reviewed by Tipping and Griswold, 2022; Gewiss et al., 2021; Teletin et al., 2017; Griswold, 2016). The process of RA regulation of spermatogenesis is both germ cell intrinsic and extrinsic through Sertoli cell action (Lin et al., 2008). Though there are retinoic acid receptors (RARs) expressed in germ cells, RA mediates its effects on spermatogenic regulation mainly through the Sertoli cell RARs (reviewed by Tipping and Griswold, 2022). The action is primarily in the initiation of spermatogenesis, as Sertoli cells synthesize RA to support the entry of SSCs into meiosis (Raverdeau et al., 2008). Sertoli cell produced retinol acts on the SSCs to activate gene expression, such as Mafb, necessary for the transition into differentiating cells (Raverdeau et al., 2008) When the germ cells progress to spermatocytes, they are able to synthesize their own RA and thus maintain spermatogenic cycling and the differentiation of spermatogonia. Sertoli cell synthesized RA remains necessary for spermiation and release from the seminiferous epithelium (Raverdeau et al., 2008). These findings have been confirmed by studies using RAR knockout mice; specifically RAR-alpha deficient mice are sterile and do not

undergo normal spermatogenesis (Vernet et al., 2006; Chung et al., 2009; Teletin et al., 2019). Knockout of retinoid x receptor (RXR) beta, either at the level of the whole organism or the Sertoli cell alone, result in a loss of spermiation, suggesting that Sertoli cell RA action is necessary for spermiation (Vernet et al., 2008).

2. Sperm Chromatin

2.1 Spermiogenesis and chromatin compaction

Somatic cells can take advantage of a beads-on-a-string configuration bound by histones to form nucleosomes. While this permits sufficient compaction of DNA in a normal cell nucleus, the spermatozoa require much tighter compaction to allow for their small size and streamlined shape for optimal motility. Before meiosis, germ cells benefit from nucleosome packaging and undergo dramatic reconfiguration only after the round spermatid stage through spermiogenesis. The transition of histone-bound DNA to protamine-bound DNA involves many changes to the histone dynamics within the cell, primarily the hyperacetylation of histone variant 4 (H4) (Pivot-Pajot et al., 2003). The histone acetylation process is controlled by histone acetyltransferases (HATs) and histone deacetylases (HDACs). Hyperacetylation of H4 at lysines 5 and 8 serves as a signal to recruit testis-specific bromodomain-containing protein (BRDT) (Meistrich et al., 2003). If this initial step in chromatin reorganization is disrupted, there is resulting infertility suggesting that it is a critical step in the process. BRDT directs the removal of histones and the replacement with arginine and lysine-rich transition proteins (TPs). There are two transition proteins, and as a result, they likely have some functional redundancy between the pair (Yu et al., 2000; Zhao et al., 2001). The transition proteins are transiently present on the spermatid DNA and are replaced by cysteine-rich, basic protamines (Sonnack et al., 2002).

Protamines were initially discovered in 1874 by Miescher and are small arginine-rich proteins (Olivia, 2006). There are two reported protamines, with protamine one expressed in all mammals and protamine two expressed in some species, including humans and mice. During epididymal transit, arginine residues on the cysteine groups undergo oxidization to form disulfide bonds. The disulfide bonds allow the DNA to form a tighter toroidal configuration (Fig. 1-4) similar to a slinky or a stack of rings (Oliva, 2006). This protamine-bound configuration is much tighter than a nucleosomal configuration, not only allowing for a smaller nucleus but also for the protection of the DNA and a transcriptionally inactive state to be maintained (Oliva and Dixon, 1991).

Approximately 1-2% of the sperm genome in rodents and up to 8-15% of the human sperm genome remain bound to histones (Balhorn, 2007). There is debate about what the retained histone regions may confer for the early embryo. However, the histone-bound regions include telomeric DNA and regions read by the embryo immediately after fertilization. The histone-bound regions may also aid in DNA anchoring to the sperm nuclear matrix (Kramer and Krawetz, 1996).

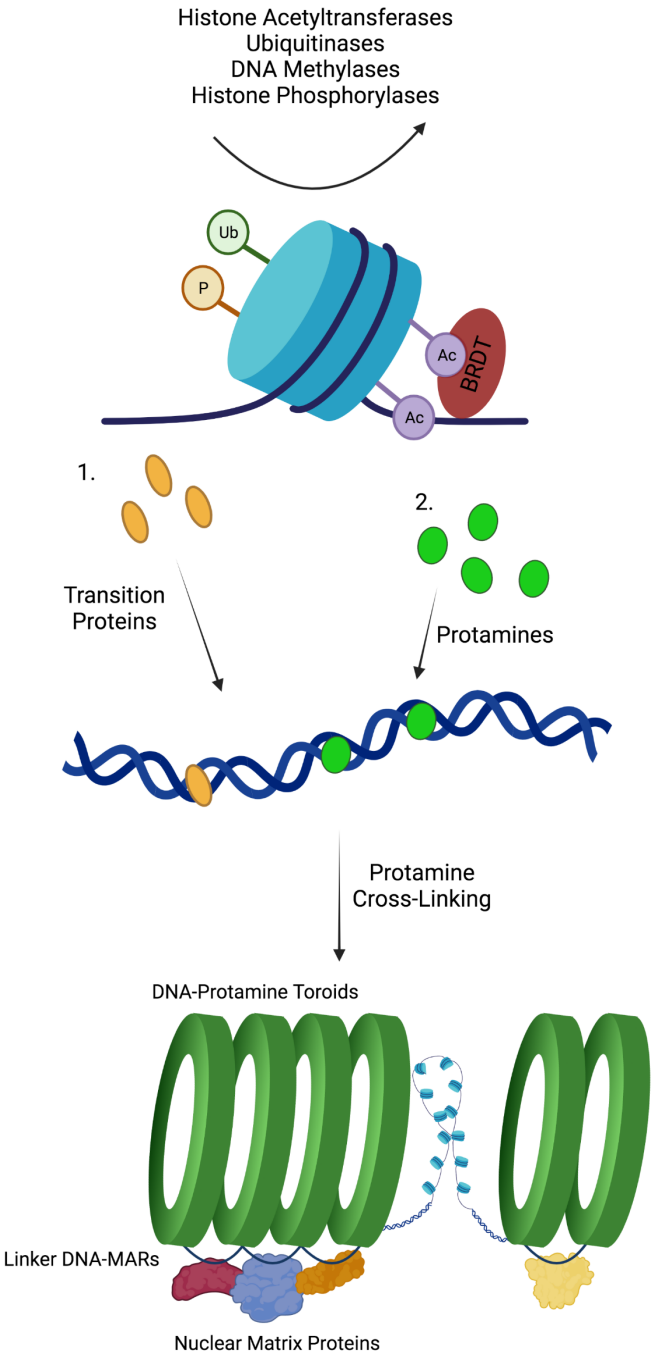

Figure 1-4: Germ cell chromatin rearrangement during spermiogenesis. Beginning with nucleosome-bound DNA, gradually replaced with transition proteins and protamines. DNA-protamine toroids, nucleosome-bound DNA, and linker regions bound to the nuclear matrix.

2.2 The Sperm Nuclear Matrix

The nuclear matrix is a group of proteins that provide a transient structure for chromatin anchoring within the nucleus, comparable in some ways to the cellular cytoskeleton. The nuclear matrix, though, has a dynamic nature to accommodate the genetic activity (Balhorn et al., 1988). The core nuclear matrix was initially discovered in cancer cells using electron microscopy, where it was observed that a small group of proteins remained bound to DNA after high salt extraction (Balhorn et al., 1988). In somatic cells, these core nuclear matrix proteins bind to linker regions between nucleosomes at specific sites to ultimately form matrix-associated regions (MARs). The site specificity has been studied to determine the functionality of MARs. It has been found that these regions are often topoisomerase II sites, AT-rich sequences involved in DNA replication, or origins of replication (Wilson and Coverley, 2013; Ward, 2018).

Sperm DNA is packaged with a combination of protamines and histones. The DNA linker regions bind to the nuclear matrix and form sperm MARs (Belokopytova, et al., 1993). Coffey and Ward extensively studied the relationship between protamine DNA packaging and the sperm nuclear matrix, ultimately determining that the protein-DNA configuration provided by the nuclear matrix in sperm is sufficient and necessary for fertilization and development (Ward and Coffey, 1991). The functional necessity of the sperm nuclear matrix configuration has been determined using intracytoplasmic sperm injection (ICSI). In these studies, researchers injected various combinations of DNA and nuclear matrix components (Shaman et al., 2007; Gawecka et al., 2013; Kramer and Krawetz, 1996). Successful fertilization and pronuclear formation were achieved with control sperm and the nuclear matrix with DNA fragments at MARs. When either isolated DNA or isolated DNA with a reconstituted matrix was injected, fertilization and pronuclear formation were unsuccessful. Similarly, paternal pronuclear formation is delayed

after fertilization if the nuclear matrix is damaged (Gawecka et al., 2013). As a result of these studies, it has been established that the sperm nuclear matrix is essential for fertilization.

The sperm MARs are maintained after fertilization, potentially priming the DNA for accessibility to specific genes required immediately after fertilization. This includes developmentally relevant genes and those the early embryo requires to reorganize the paternal genome (Kramer and Krawetz, 1996). An issue presents itself with primed developmentally relevant genes, as sperm MARs are more susceptible to DNA damage during epididymal transit (Ribas-Maynou et al., 2014). Accompanying these findings is the hypothesis that the matrix provides a template for recognition in the early embryo and guides protamine replacement by maternal histones while potentially priming sites for transcription by anchoring them to MARs (Kramer and Krawetz, 1996).

2.3 Sperm Chromatin Organization

Though we begin to understand sperm chromatin organization through the lens of the nuclear matrix, additional studies have been done to examine how various DNA elements and chromosomes are spatially arranged within the nucleus. Starting in the early 1990s, studies were done using fluorescence in situ hybridization (FISH) to examine the location of chromosomes relative to one another and the positioning of centromeres and telomeres in the sperm nucleus (Haaf and Ward, 1995). In porcine sperm, it was determined that each chromosome has distinct locations, forming distinct chromosome territories that may be functionally relevant in the developing embryo (Foster et al., 2005; Zalensky et al., 1995). Examining specific chromosomal elements, Zalensky et al. found that centromeres cluster towards the center of the sperm nucleus form a chromocenter, while telomeres extend outwardly toward the periphery. The outward extension of telomeric repeats allows for telomeric dimers or tetramers, which link the

chromosomes together in a hairpin configuration (Zalensky et al., 1995). This fits the general hypothesis that the nucleus has a gene-rich center and a gene-poor periphery (Zink et al., 2001). The dispersal of telomeres towards the periphery also fits with somatic cell nuclear organization, in which the telomeres form a 'bouquet' within the nucleus (Scherthan, 2001). A newly proposed model suggests multiple chromocenters and interspersed telomeric dimers throughout the nucleus (Ioannou et al., 2017).

High-resolution of chromatin organization in somatic cells has allowed us to further define regions of the nucleus. These formerly observed chromosome territories can be broken downinto topologically associated domains (TADs), compartments, and chromatin loops (Fraser et al., 2015). A chromatin loop contains all functional elements necessary for the transcription of a gene, including promoters and enhancers. In male germ cells, chromatin loops have been studied with immunohistochemistry techniques probing for proteins identified in somatic cells. A highly conserved zinc finger protein, CCTC-binding factor (CTCF), has been one of the main protein complexes associated with the formation of chromatin loops. CTCF binds to DNA with cohesion as a co-factor, thus determining functional genomic units. In the testis, a specific paralog of CTCF is called Brother of the Regulator of Imprinted Sites or CCTC-binding factor-like (BORIS/CTCFL) (Sleutels et al., 2012). BORIS is a likely regulator of imprinted gene DNA methylation in the germline and thus acts as a gene regulator. BORIS also regulates the expression of Stra8 in the testis, a retinoic acid signaling gene and meiotic gatekeeper (Sleutels et al., 2012; Ma et al., 2018).

The chromatin conformation capture techniques are the leading edge of determining the chromatin configuration within the sperm nucleus. These methods allow for the relationship between genomic elements to be predicted based on the interaction frequency observed in the

data by combining DNA cross-linking with next-generation sequencing. Recently, these chromatin conformation capture techniques have been applied to the sperm genome through Hi-C. Hi-C is a chromatin conformation capture technique that allows for the assessment of all interactions within the genome. Sperm chromatin shows many long-range contacts within the DNA sequence. This is consistent with tight packaging as areas of the genome that would typically be far apart in space are able to localize more frequently (Dekker et al., 2013; Dostie and Bickmore, 2012). These methods have also been applied across spermatogenesis; it was found that during the early stages of spermatogenesis, chromatin organization is highly variable, with no compartmental organization. The germ cells gain strong compartmentalization by the end of spermatogenesis in the round spermatid stage. While the chromatin is undergoing rapid packaging changes from histone to protamine, it is also being reorganized within the nucleus (Battulin et al., 2015; Jung et al., 2017; Ke et al., 2017).

The accessibility of sperm chromatin during spermatogenesis is also of interest, given the tight configuration of chromatin after spermiogenesis. As such, the assay for transposase-accessible chromatin (ATAC) sequencing has been used to assess the chromatin accessibility of the developing germ cells. During the process of spermatogenesis, there appears to be a ~35% reduction in open chromatin (ATAC peaks) from pachytene spermatocytes to round spermatids (Maezawa et al., 2018). This reduction in accessible chromatin is consistent with what has been established regarding the repackaging of chromatin during spermiogenesis and what was observed with Hi-C methodologies. The use of knockout models can further elucidate potential regulators of these processes, such as the knockout for *Scml2,* which was observed as a potential contributor to the closing of open chromatin. Additionally, examining the sequence of open chromatin identified through ATAC-sequencing will allow us to understand what the open

regions of chromatin are and if they hold functional significance as previously hypothesized. Indeed, studies have shown that in the human male germline, using single-cell ATAC-sequencing, upwards of 100,000 open regions were promoters, gene bodies, or CpG islands (Wu et al., 2022).

2.4 Epigenetic changes during spermatogenesis

Epigenetics is the process by which the expression of genes is altered without alteration to the DNA sequence. There are three major mechanisms by which epigenetic information is passed from one cell generation to the next: DNA methylation, histone modification, and non-coding RNAs. Presently, these are the most well-studied epigenetic mechanisms in male germ cells. Many studies are actively investigating the effects of the environment (toxicants, lifestyle factors, age) on the sperm epigenome and the implications this may have for fertility or offspring development (Braun, 2001; Balhorn, 1982; Kitamura et al., 2015; Wilson and Jones, 1983; Dad et al., 2012; DeBaun et al., 2003; Gosden et al., 2003; Maher, 2005; Lazaraviciute et al., 2014).

2.4.1 DNA methylation

DNA methylation is a common epigenetic mechanism that regulates gene expression and imprinting through the methylation of the 5-carbon cytosine residues (5-mC) at cytosine-phosphate-guanine (CpG) dinucleotides. DNA methylation regulates gene expression, with hypermethylation of promoters leading to gene silencing by blocking access to transcriptional machinery and hypomethylation increasing gene expression through facilitating access of transcriptional machinery and RNA polymerase (Lazaraviciute et al., 2014; Jones, 2012). In germ cells, alterations in DNA methylation can result in abnormal spermatogenesis and subsequently impaired fertility.

DNA methylation is a process intimately linked to folate metabolism through one-carbon metabolism, summarized in Figure 1-5 (Smith et al., 2013). Briefly, folate is brought in through diet or supplementation. It is metabolized by reductase enzymes in the liver but ultimately enters the folate cycle as tetrahydrofolate (THF). Serine or glycine acts as a carbon donor to form 5,10-methyl THF. Methylenetetrahydrofolate reductase (MTHFR) converts this to strictly 5-methyl THF. 5-methyl THF acts as a carbon donor to recycle homocysteine to methionine as part of the DNA methylation cycle. Methionine is then converted by methionine adenosyltransferase (MAT) to s-adenosyl-methionine (SAM). SAM then facilitates DNA methylation by acting as a methyl donor for DNA methyltransferases (Smith et al., 2013; Clare et al., 2019). Interestingly, MTHFR is expressed at a fivefold higher level in the testis than in other tissues. The Trasler lab has shown the necessity for MTHFR for sperm development and normal spermatogenesis in both the parent (F1) and in their offspring (F2) fed a normal diet (Chen et al., 2001; Karahan et al., 2021). The Kimmin's lab has also shown that folate-deficient mice have altered sperm DNA methylation in regions associated with cancer and chronic human illness (Lambrot et al., 2013).

DNA methylation is maintained and removed by a variety of catalytic enzymes such as: DNA methyltransferase 1,2 and 3 (DNMT1/3A/3B), and ten-eleven translocation (TET) dioxygenase activity by TET3 (as reviewed by Janssen and Lorincz, 2021). DNMT1 is known as a maintenance DNA methyltransferase, active predominantly during DNA replication to ensure the replacement of 5mC at methylated sites through binding partner UHRF1 (Bostick et al., 2007). DNMT3A/B have been found to catalyze de novo DNA methylation. The access of DNMT3A/B to DNA for de novo methylation is heavily dependent on histone methylation (Li et al., 2011). Repressive histone marks (i.e; H3K9me3, H3K36me2/3) providing access and stabilization to DNMT3A/B and activating histone marks (H3K4me3) blocking access to the

DNMT3A/B complex (as reviewed by: Li et al., 2011; Saksouk et al., 2015; Nicetto and Zarat, 2019). This interaction is due to elements of the DNMT3A/B complex, specifically the ADD motif, as without this motif DNMT is able to methylate regions with activating histone marks (Otani et al., 2009; Zhang et al., 2010). TET enzymes are one of the most well studied mechanisms for DNA demethylation, where they hydroxylate 5mC to 5hmC, that is recognized by base excision repair machinery for replacement. This process is error prone and leads to frequent mutations (Santos et al., 2013; Shi et al., 2017).

There are two main reprogramming events that lead to the proper methylation of sperm DNA (Monk et al., 1987; Kafri et al., 1992; Reik et al., 2001; reviewed by Smallwood and Kelsey, 2022). After fertilization at the 2-cell stage, both maternal and paternal chromatin are demethylated rapidly, likely by Tet proteins (Mayer et al., 2000; Oswald et al., 2000; Gu et al., 2011), with imprinted genomic regions remaining. At the blastocyst stage, methylation is redeposited onto the DNA. The second DNA methylation erasure is during embryonic development when the primordial germ cell DNA is demethylated. In primordial male germ cells, the DNA methylation is replaced in the prospermatogonia during mitotic arrest before birth of the male and after birth prior to puberty for the female (Hajkova et al., 2002). During this phase of epigenetic reprogramming the DNA methylation at imprinted loci is also lost; however, it is somehow re-established (Seisenberger et al., 2012; Kremsky and Corces, 2020). There are additional regions coding for active or mobile insert elements that may be mutagenic that appear to remain silenced during this process, though this mechanism is still being actively researched (Hackett et al., 2013).

Many groups are now investigating the significance of altered DNA methylation in sperm. Several studies show intergenerational (F0 to F1) and transgenerational (F0 to F1, F2, F3, etc.) epigenetic inheritance mediated by paternal DNA methylation (Chastain and Sarkar, 2017; de Castro Barbosa et al., 2016; Dunn and Bale, 2011; Illum et al., 2018; Lambrot et al., 2013; King and Skinner, 2020). Radford and colleagues (Radford et al., 2014)) have been one of many groups to explore the transmission of metabolic disorders through the sperm DNA methylome. When F0 dams were malnourished, the F1 males had differentially methylated regions (DMRs) in their sperm, and the F2 offspring displayed altered metabolic functions. This study did not directly prove a mechanism for this inheritance, but the results suggest a strong link between DNA methylation and intergenerational phenotypic inheritance.

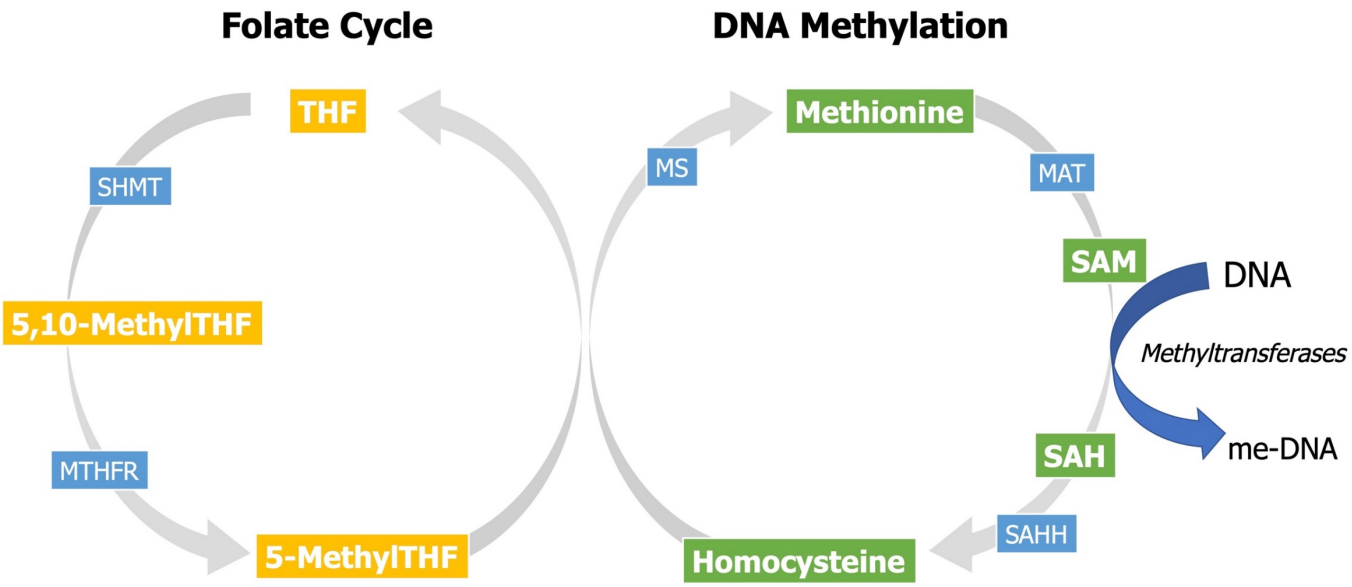

Figure 1-5: Simplified One Carbon Metabolism. Folate-metabolism and DNA methylation cycles.

2.4.2 Histone variants and histone post-translational modifications

Until spermiogenesis, the male germ cell DNA is bound by histones, similar to somatic cell DNA. During spermatogenesis, the regulation of histones has functional significance during meiosis. Histones come in five main variants, H2A, H2B, H3, and H4, with H1 being the linker histone between nucleosomes (Talbert and Henikoff, 2021). Histones form heterodimers, binding selectively to one another to package the DNA. Within each of the five main variants, there are numerous additional paralogs. In addition to the array of histone variants, there are numerous post-translational modifications (PTMs), with at least 18 PTMs having been identified. They include acetylation, B-N-acetyl-glucosaminylation, ADP ribosylation, butyrylation, citrullination/deimination, crotonylation, formylation, glutarylation, hydroxylation, 2-hydroxyisobutyrylation, malonylation, mono-, di-, and tri-methylation, phosphorylation, proline isomerization, propionylation, succinylation, sumoylation, and ubiquitination (Huant et al., 2015;

Zhao and Garcia, 2015). These changes alter the physiochemical properties of nucleosomes, modify the 3D conformation of germ cell chromatin, determine heterochromatic and euchromatic states, and play a role in the progression through the various steps of meiosis. In pachytene spermatocytes, the paired chromosomes must undergo homologous recombination. Here, the cells must also undergo active gene transcription in the autosomal chromosomes, which have been highly condensed since leptonema. The opening of the autosomal chromosomes is due to the incorporation of different histone variants and PTMs, including the replacement of H3 by H3.3, TH2A, TH2B, H1T, H2A ubiquitination, and H3K9 acetylation (Page et al., 2012). This process is delayed in the sex chromosomes that are still inactivated, forming the XY/sex body. The formation of the sex body in germ cells is known as meiotic sex chromosome inactivation and involves numerous histone repressive marks such as H3K9me2/3 (Histone 3, Lysine 9, di / tri-methylation) and the macroH2A variant (Fernandez-Capetillo et al., 2003; Turner et al., 2004).

Histone methylation plays a role in the regulation of gene expression of genes necessary for spermiogenesis. Methylation of H3K9/36 (denoted as H3K9me2/3) is necessary to allow for the expression of transition protein and protamine genes. Okada et al. (2007) have shown that H3K9me2 regulates the expression of transition protein and protamine genes via JHDM2A.

The process of spermiogenesis and DNA repackaging is reliant on histone variants and PTMs. The main histone modification during this process is the hyperacetylation of H4 (Wang et al., 2019). Hyperacetylation of H4K5/8/12/16 is essential for the remodeling of chromatin and incorporation of transition proteins, as it recruits BRDT (Wang et al., 2019; Ketchum et al., 2018). The role of hyperacetylation is further supported by studies examining gene expression of the histone acetyltransferases responsible for the acetylation of H4K, Kat2b, and Kat8. The

expression of these genes is high at the onset of spermiogenesis but declines gradually (Li et al., 2021). Histone ubiquitination of H2A and H2B allows for the acetylation of H4K16 through the recruitment of males absent on the first (MOF) acetyltransferase complex (Ketchum et al., 2018; Chen et al., 1998).

Though the germline undergoes drastic repackaging to form protamine-bound toroidal DNA, some histones are retained in sperm DNA packaging. Various groups report different findings that seem to be species specific, with human sperm retaining as much as 15% (Gatewood et al., 1987) of the histones or as little as 4% (Hammoud et al., 2010). Rodent sperm on the other hand retains less, with as little as 1-10% in the mouse (Brykczynska et al., 2010). Sperm histone modifications are an active area of research within the field of fertility, focusing on how histone modifications may lead to transgenerational epigenetic inheritance. The functionality of retained nucleosomes remains elusive; they are found in promoter, enhancer, and super-enhancer regions often associated with CTCF binding in earlier germ cell stages and in the embryo (Jung et al., 2017). There are a few proposed mechanistic explanations for histone retention, including CTCF binding, inherent histone modifications, and non-coding RNAs (Hoghoughi et al., 2020; Jung et al., 2019; Zhang et al., 2017). The retained histones have been associated with regions that are developmentally relevant in the embryo (Hammoud et al., 2009). Further advances have been made to examine the genic regions that retain nucleosomes in sperm. Yamaguchi et al., (2018) found that these regions often overlapped with regions that were developmentally relevant, or involved in the process of spermatogenesis.

The retained sperm histones are a modulator of epigenetic and transgenerational inheritance (Torres-Flores and Hernández-Hernández, 2020). Using ChIP-Seq methodologies, it is possible to examine specific histones and their modifications in sperm, such as repressive or activating

marks. Exposure studies to the pesticides vinclozolin and dichlorodiphenyltrichloroethane have shown that sperm H3 methylation sites are altered with exposure, and this is maintained in the F3 sperm (Ben Maamar et al., 2018). High-fat diet and paternal obesity also alter H3 methylation, specifically levels of an H3K4 methylation (Terashima et al., 2015; Pepin et al., 2022). The enrichment of histone marks, either activating or repressive, has been associated with regions implicated in spermatogenesis and embryonic development (Teraskima et al., 2015; Siklenka et al., 2015; Teperek et al., 2016; Pepin et al., 2022).

2.4.3 Noncoding RNAs

As RNA sequencing methods are rapidly becoming more accessible, the number of studied noncoding RNAs (ncRNA) is increasing. The ncRNAs have two classifications, the small ncRNAs (sncRNA), and the long ncRNAs (lncRNA) (Saxe and Lin, 2011; Joshi and Rajender, 2020). The identification of ncRNAs, their roles, and the mechanisms by which they act has been a major area of research in germ cell biology. In male germ cells, ncRNAs often have functions in the regulation of gene expression, chromatin conformation, and genome stabilization. Entirely new categories of RNAs are being discovered in germ cells, including the Piwi interacting RNAs (piRNAs).

Germ cells become transcriptionally inactive after spermiogenesis and thus must transcribe all relevant RNAs prior to spermiogenesis. Many of these transcripts are translationally repressed by RNA-binding proteins and are stored in germ cell granules such as the chromatoid body (Lehtiniemi and Kotaja, 2017). Many of these RNAs are lost when spermatids shed their cytoplasm through the residual body before spermiation. After spermiation,

only ~50 fg of RNA remains in each spermatozoon, in contrast to ~12 pg of RNA/cell in somatic or earlier germ cells (Concha et al., 1993; Krawetz, 2005; Schuster et al., 2016).

Noncoding RNAs are among the regulators of the highly dynamic and stage-specific germ cell transcriptome (Ball et al., 2016; Chen et al., 2017; Jan et al., 2017; Laiho et al., 2013; Smorag et al., 2012). Primarily micro RNAs (miRNAs) are one class of ncRNAs that regulate gene expression by binding with some complementarity to mRNA and marking them for degradation, thereby suppressing specific gene expression (O'Brien et al., 2018). miRNAs have positive and negative effects, solely dependent on the mRNA they are regulating (Voorhoeve and Agami, 2007). Two miRNAs, miR-146 and miR-221, have been characterized to inhibit spermatogonial stem cell differentiation (Chen et al., 2017; Kotaja, 2014). These miRNAs target the c-Kit mRNA, which codes for a tyrosine kinase receptor essential for initiating spermatogonial differentiation, leading to c-Kit suppression (Smorag et al., 2012; Huszar and Payne, 2013). During the pachytene spermatocyte stage, piRNAs, ncRNAs 24-31 nucleotides long, which associate with the PIWI proteins, are involved in rapid mRNA decay (Gou et al., 2014). During spermiogenesis, two clusters of microRNAs from the same family, miR-449 and miR-34b/c clusters play a role in the condensation of sperm chromatin and formation of the flagellum. The male double knockout for these microRNAs is sterile due to the production of abnormal, immotile, and decondensed spermatozoa (Wu et al., 2014; Yuan et al., 2015).

As the germ cell epigenome is going through a rapid rearrangement during germ cell development, there are additional protective elements germ cells must employ in order to protect the DNA from transposable element insertion. In germ cells, piRNA along with PIWI binding proteins have been observed to target transposable elements to enhance the degradation of the transposon mRNA and prevent genomic insertion. The most prominent of these ncRNAs are the

pi-RNAs (Hirakata and Siomi, 2016; Iwasaki et al., 2015; Russel et al., 2017; Sarkar et al., 2017). The transfer RNA-derived small RNAs or tRFs have also been noted as inhibitors of transposable elements in a mouse stem cell line (Shorn et al., 2017).

Despite the removal of most RNAs through spermiation, mature spermatozoa have a vast array of ncRNAs, including long ncRNAs, fragmented ribosomal RNAs, tRFs, microRNAs, pi-RNAs, and mitochondrial RNAs. In lower quantities, the following RNAs have also been identified: repetitive elements, quiescent RNAs, intronic retained elements, snoRNAs, and YRNAs (Schuster et al., 2016; Jodar et al., 2013; Krawetz et al., 2011; Nixon et al., 2019). There is a class of microRNAs (cluster 17-92) that are present in testicular sperm, absent in sperm from the caput of the epididymis, but present after epididymal transit when the sperm are in the cauda. This suggests that the epididymis is contributing ncRNAs to the sperm through epididymal transit, with convincing evidence suggesting these RNA payloads are delivered by epididymosomes (Sharma et al., 2018).

Beyond the role of ncRNAs in genetic regulation during spermatogenesis and epididymal transit, research is now focusing on the role of sperm ncRNAs in fertilization and post-fertilization embryo development. Some studies suggest that ncRNAs may play a role in intergenerational epigenetic transmission (Chen et al., 2016; Fullston et al., 2013). In male runners, for example, the sperm miRNA payload was significantly different from a group of control men. The difference in miRNA transcriptome was correlated with offspring who had decreased anxiety and stress responses (Short et al., 2017). Similarly, the ncRNAs in sperm from infertile men were significantly different from those of fertile controls. This suggests that sperm ncRNAs may be a strong biomarker for male fertility parameters (Burl et al., 2018). When Sun et al. (2021) examined oligozoospermic men compared to normozoospermic controls, they found

differentially expressed ncRNAs involved in gene expression regulation, endoplasmic reticulum trafficking, and apoptosis.

2.4.4 Telomeres

Telomeres are the repeat regions at the end of chromosomes in all cells throughout the body. Telomeric repeats are made up of the nucleotide sequence TTAGGG (T: thymine, A: adenine, G: guanine). They provide stability to the genome and act as anchoring points for DNA within the nucleus. During meiosis, they help to pair homologous chromosomes, form the synapsis, and aid in homologous recombination (Scherthan, 2007). Telomeric DNA, with double-stranded G rich repeats, end in a 3' overhang, this overhang can fold back onto itself and invade the double-stranded telomeric DNA. This folding back creates what is known as a T-loop, and is thought to have protective effects in evading cellular DNA damage responses and chromosome fusion events (Griffith et al., 1999). Telomeres are bound by a multiprotein complex known as the Shelterin complex, or telosome. This complex contains proteins PTOP/POT1/TPP1, or the telomere binding factors TRF1, TRF2 and their interacting protein RAP1 and TIN2 (Chan and Blackburn, 2002; Liu et al., 2004). These proteins have functions in protecting and stabilizing the telomeres. Specifically, POT1 and TRF2 help protect the chromosome ends from DNA damage repair machinery and chromosome ligation via non-homologous end-joining (Celli and Lange, 2005; Hockemeyer et al., 2006). This ultimately aids the cell in avoiding DNA damage responses such as apoptosis and cell cycle arrest (Chan and Blackburn, 2002). Telomeres are maintained as heterochromatic regions through histone methylation, specifically H3K9me and H4K20me (as reviewed by: Blasco, 2007). There are low levels of histone acetylation as well, at H3 and H4 (Bonetti et al., 2007). The heterochromatic regions, though, are not stable and often spread into the neighboring regions of DNA, silencing

nearby genetic information through what is called telomere position effects (Blasco, 2007; Wang et al., 2018). In addition to methylated histones, telomeres are packaged by a H3K9me3 binding protein heterochromatin protein 1 (HP1) (Koering et al., 2002; Garcia-Cao et al., 2004; Gonzalo et al., 2006).

The subtelomere contains similar histone modifications, and heterochromain proteins (HP1); however, the subtelomere contains heterochromatic DNA methylation and does not contain the Shelterin complex of proteins. DNA methylation within the subtelomere may have functionality in regulating telomere length, as decreases in DNA methylation within the subtelomere has been associated with elongated telomeres (Gonzalo et al., 2006).

Telomeres have also been proposed as an epigenetic mechanism of their own, for which there is some debate. Telomere length is determined in each cell of the developing embryo, and determine the replicative potential of each cell within the body, thereby determining the phenotype without changing the DNA sequence (Blasco, 2007). Thus, telomeres present an interesting potential epigenetic function, as they are both controlled by epigenetic mechanisms and may inherently contribute to epigenetics.

In germ cells, telomeres are actively maintained by the enzyme telomerase, which is absent, or expressed at very low levels in most somatic cell types. Telomerase is a ribonucleoprotein with a telomerase RNA component (TERC) and telomerase reverse transcriptase (TERT) (Ozturk, 2015). The RNA component facilitates the addition of complementary DNA by the reverse transcriptase, to prevent telomere degradation. The activity of telomerase in germ cells has been studied, with conflicting evidence to suggest that it is most

active in spermatogonial stem cells and early germ cells, but declines after the spermatid stage (Achi et al., 2000; Eisenhauer et al., 1997; Ravindranath et al., 1997)

Sperm telomeres have been studied in relation to the offspring, and studies have shown that the sperm telomere length is correlated with the offspring telomere length. Sperm length has also been studied in the context of fertility, suggesting that decreased telomere length leads to poor fertility outcomes during assisted reproductive therapies (Gentiluomo et al., 2021). In clinical settings, telomere length is being proposed as a marker for sperm quality. It is relatively easy to measure using qPCR and may provide insight into the fertility or sperm quality and DNA integrity. There are no set limits for optimal telomere length, though, and it is likely that telomeres that are either too short or too long could pose issues for sperm quality.

2.4.5 Cooperating epigenetic mechanisms

Though each of these epigenetic mechanisms presents a strong case for its contribution to germ cell epigenetic regulation, none act in a vacuum, but rather act in concert with one another, this further complicates our understanding of epigenetics and the specific contributions of each mechanism. The current body of literature focuses on individual epigenetic mechanisms; however, the future of this field relies on understanding how the mechanism act together to regulate gene expression and phenotypic observation.

Lambrot et al. (2013) have made an effort to link histone modifications and DNA methylation. They have shown that areas with H3K4me3 in human sperm correspond to areas of DNA hypomethylation and are found in gene-rich CpG regions, like promoters. Another study demonstrated the interplay between ncRNA, DNA methylation, and histone modifications (Beck et al., 2021). When males were exposed to a toxicant in utero, the sperm from the F1, F2, and F3

progeny had ncRNAs overlapping differentially methylated regions (DMRs). The DMRs had significant overlap with histone retention sites and altered histone modifications. Together, these studies are beginning to indicate that epigenetic mechanisms work together in a cooperative manner.

2.5 DNA damage and DNA damage repair during spermatogenesis

The highly replicative and dynamic nature of the male germline endangers the DNA to many types of lesions. These include single and double-strand breaks (DSB), interstrand or intrastrand cross-links, base mismatches, and modifications. In order to repair the array of damage that may occur during spermatogenesis, germ cells have evolved a wide range of DNA damage repair pathways, referred to as the DNA damage response (DDR) (Jackson and Bartek, 2009). The process of DDR involves sensors of damage, mediators of sensor signaling, transducers, and effector classes of proteins. The major repair pathways in germ cells include base-excision repair (BER), nucleotide-excision repair (NER), mismatch repair (MMR), homologous recombination (HR), and nonhomologous end joining (NHEJ) (Bailly and Gartner, 2013).

Each germ cell type uses different repair pathways. Spermatogonial stem cells primarily use NHEJ and HR to repair DSBs that occur during mitosis. MMR-related genes are expressed in spermatogonia, and the spermatocytes, suggesting that more than one repair pathway may be active in spermatogonia (Richardson et al., 2000). In spermatocytes, the main repair pathway is HR, with the necessary components Rad51 and Dmc1. There is less NER in these cells, as these cells can be eliminated by apoptosis rather than repaired (Xu et al., 2005). Post-meiotic spermatids do not typically undergo complete DNA damage repair; however, there is some evidence to suggest that these cells may use NHEJ (Ahmed et al., 2007), for example, that they

contain Ku70, a necessary factor in NHEJ (Ahmed et al., 2013). As NHEJ is a template free repair, it is hypothesized that this is a major source of the male DNA *de novo* mutation bias (Grégoire et al., 2018). During spermiogenesis, when the spermatids have begun to elongate, there is an overall downregulation of the DNA repair-associated transcripts, with some studies reporting that there is likely no active repair in these stages (van Loon et al., 1993; Olsen et al., 2003). The process of spermiogenesis may require transient double-stranded DNA breaks, and their repair with NHEJ, to facilitate the repackaging with protamines, again allowing for *de novo* mutations at this stage (Leduc et al., 2008). Once spermiogenesis is complete, the protamine insertion and chromatin compaction protect the DNA. When spermatozoa are mature, it is unlikely that any DNA damage response elements are active as there are few proteins and RNAs in sperm. Additionally, the chromatin is tightly compacted; thus, the repair machinery is unlikely to make contact with the DNA.

3. Advanced Paternal Age and Reproductive Aging

In industrialized countries, including Canada, the age at which individuals choose to start a family has been steadily increasing since the 1970s. For men, this has resulted in about a 3 - year increase in the age at which they become first-time fathers regardless of ethnicity (Khandwala et al., 2017). The societal factors contributing to delayed parenthood are vast, ranging from goals of higher education, career demands, and financial stability. In addition to individuals waiting longer to have children, we also are faced with an aging population of men in Canada who are able to father children until much later in life, especially with the advent of assisted reproductive technologies.

The effects of advanced paternal age (APA) are less well-known than those of the well-studied maternal age. As women reach a finite age at which they are no longer able to reproduce

and go through menopause, there are substantiated results to show their oocytes decline in quality (Pellestor et al., 2003). The decline in oocyte quality can primarily be attributed to abnormal chromosome segregation and results in reduced fertility and increased risk of poor pregnancy outcomes. It is increasingly common knowledge that women over 35 are at risk of children with trisomy 21 (Yoon et al., 1996; Crawford and Steiner, 2015)Though the effects on men's fertility are less striking, they still pose a hazard to the offspring through subtle alterations to spermatozoa (sperm) (Crow, 1997).

The first noted disruption in male reproduction with aging is through the hypothalamic-pituitary-gonadal (HPG) axis, as there is a significant decrease in testosterone production with age, often referred to as andropause, or more appropriately, as late onset hypogonadism (LOH) (Singh, 2013; Kaufman et al., 2019). The source of LOH has been studied by examining Leydig cell function and characterizing the remaining sex hormones (GnRH, LH, testosterone). Using the Brown Norway (BN) rat model of reproductive aging, we now know that with age, the Leydig cells have a decreased ability to respond to stimulation by LH, causing an overall decrease in testosterone production, consistent with what is seen in humans (Midzak et al., 2008). The observed decrease in testosterone has potential effects on male reproductive endpoints.

Though spermatogenesis often continues with APA, several fertility parameters and testicular histology are perturbed (Dong et al., 2022). Testicular histology shows age-related disruptions, including loss of testicular cells (somatic and germ cells), reduced vasculature, thickening of the seminiferous tubule basement membrane, and reduced tubule epithelium. The abnormal testis environment is met with abnormal sperm motility and morphology. Taken

together, disruptions in the testis translate to a decrease in an overall semen volume equivalent to approximately 0.22 mL for every five years of age (Beguería et al., 2014).

There is correlative epidemiological evidence that show alterations to a couple's fertility when the man is of advanced paternal age, even when adjusted for maternal age (Sharma et al., 2015; Bray et al., 2006). These studies found that in men over 40 years of age, there was an increased time to pregnancy (TTP) and increased incidence of preterm births, stillbirths, low birth weight progeny, and preeclampsia. Beyond pregnancy outcomes, there have been reported increases in neurological disorders in the offspring of older men. These disorders include attention deficit hyperactivity disorder (ADHD), autism, and schizophrenia (Dong et al., 2022; Frans et al., 2013; Grether et al., 2009; Malaspina et al., 2015; D'Onofrio et al., 2014). There is an increasing number of studies that are exploring causality between these disorders and the effects of aging on spermatozoa.

Following the early studies displaying paternal age effects, the American Society for Reproductive Medicine set a limit of 40 years for sperm donors. The Society of Obstetrics and Gynecologists of Canada (SOGC) has also used these findings to set an age threshold for advanced paternal age at 40 (Liu and Case, 2011). Though sperm donation is prohibited over 40, and major societies recognize this as APA, there remains no universally accepted age threshold for men's fertility.

3.1 Theories of Aging

The theories of aging represent a set of hypotheses that reflect the alterations seen with aging throughout various systems. There are two main classes of aging theories, 1) programmed aging theories and 2) damage aging theories. Programmed aging theories include programmed

longevity, telomere theory, endocrine theory, and immunological theory. For example, Hayflick proposed that all cells have a limited number of times they will divide before cell senescence will occur (Hayflick, 1980). Damage aging theories include wear and tear theory, rate of living theory, cross-linking theory, free radical theory, and DNA damage theory (Jin, 2010). Within the germ cells, all theories of aging may be applicable and likely interact with one another to result in the major alterations observed in sperm with advanced paternal age. Although it is important to identify what are the various theories and biologically relevant pathways that contribute to the overall process of germ cell aging, this renders the study of aging a particularly challenging endeavor.

3.2 Effects of aging on the somatic cells of the testis

3.2.1 Leydig Cells

The Leydig cells are the site of testosterone biosynthesis in the testis, converting cholesterol to testosterone once activated by LH. Due to aging, there is a decrease in the Leydig cell's ability to respond to stimulation by LH, suggesting a likely disruption in the cyclic-AMP signaling cascade (Chen et al., 2002).

3.2.2 Sertoli Cells

The Sertoli cells are somatic support cells within the seminiferous tubules, providing physical and signaling support to developing germ cells. As previously mentioned, they are responsible for a host of functions ensuring the initiation and successful completion of spermatogenesis. Beyond supporting spermatogenesis, Sertoli cells function to establish and maintain the BTB; however, with aging, there is evidence that the BTB integrity is disrupted and becomes more permeable to toxicant insult (Levy et al., 1999). There is an apparent decrease in

Sertoli cell number with aging, and a decrease in cell quality, with apparent cell degeneration (Jiang et al., 2014). There is also a host of dysregulated genes in Sertoli cells from aged mice (Dong et al., 2022), including those involved in gap junction maintenance, ERα-NRF2, and growth factor signaling such as through Insl3 (Shiba et al., 2021; Zhao et al., 2020; Crespo et al., 2021). From a recent study of whole testis single-cell RNA sequencing in men, it is evident that the transcriptome of Sertoli cells from aged men is distinct from that of young men, with major differences in cellular metabolism genes (Nie et al., 2022). Similar results were observed at the RNA level when examining specific transcripts in Sertoli cells for cell communication, where Syed and Hecht (2001, 2002) proposed that there was a potential disruption of signaling between germ and Sertoli cells. The decrease in the Sertoli cell number is accompanied by a decrease in the number of germ cells that one Sertoli cell can support with aging (Jiang et al., 2014). Upon examination of testis sections from aged rats, it became clear to Levy et al. (1999) that there were gaps where germ cells should be embedded within the Sertoli cells. These findings together suggest that there is a disruption in Sertoli cell biology due to aging.

3.3 Effects of aging on germ cells

One of the first observations made about the effects of advanced age in rodents was that there was a general disruption in the overall organization of the germ and somatic cell type in the seminiferous epithelium (Wang et al., 1993). This immediately suggested that there were additional effects due to aging worth studying. It has also been reported that within the germline, there is an accumulation of mutations due to aging (Walter et al., 1998). Since then, studies have reported that with aging, there is no change in the number of spermatogonial stem cells (SSCs) that comprise the stem cell pool giving rise to the germline; however, there is a decrease in their ability to form germ cell colonies and their longevity is reduced (Paul et al., 2013). In mice,

despite potentially fewer or less functional SSCs due to age, those that remain at one year of age are capable of generating a germ cell colony, indicative of their ability to contribute to spermatogenesis; when the mice reach two years of age, though, their replicative potential and the colony size they form decreases (Zhang et al., 2006). Thess studies suggest that as the stem cell pool ages, the remaining cells become less functional. Concordant with the decrease in SSCs, there are also fewer germ cells of the remaining cell types. The spermatocytes that remain tend to show more apoptosis than the spermatocytes of young mice (Barnes et al., 1998). During meiosis in mice, there is an observed disruption in chromosome pairing, synapsis, and homologous recombination, though cells with these defects appear to be removed through cycle checkpoints (Vrooman et al., 2014). In post-meiotic spermatids from men, abnormalities in some rodents appear in the: acrosome, nuclear membrane, excessive cytoplasmic droplets, irregular nucleus shape, and intranuclear inclusions (Paniagua et al., 1987). In humans, multinucleated round spermatids have also been observed, suggesting a potential disruption in membrane integrity or complete meiosis (Nistal et al., 1986). Similar observations have been made in hamster spermatids, where they, too, had abnormalities in the acrosome and nuclear membrane. In addition, in hamsters, the developing flagella were curved (Calvo et al., 1995). The effects seen at the round spermatid stage likely contribute to the effects of age on abnormal sperm morphology and motility. Sperm progressive motility is two-fold lower in men aged 50 or more relative to men aged 40-50 (Pino et al., 2020). It is reported that the overall fertilizing ability of human sperm decreases by 0.3% per year for paternal age, likely due to issues with motility or capacitation (Diao et al., 2013; Bartolacci et al., 2018). As previously mentioned, germ cell DNA integrity decreases with age, and this remains consistent with mature spermatozoa. With aging,

the DNA fragmentation index increases significantly in sperm (Belloc et al., 2014; Evenson et al., 2020).

3.4 Epigenetic changes during spermatogenesis due to aging

Aging has been shown to affect the chromatin of germ cells through most major mechanisms of epigenetic regulation. This includes DNA methylation, histone modifications, noncoding RNAs, and telomere length. It is important to note here, that all modifications act together in the process of genetic regulation, epigenetic inheritance, and chromatin structure.

3.4.1 DNA methylation

Aging and aging-associated diseases have been linked to alterations in DNA methylation in somatic cells (Issa, 2000; Wilson et al., 1987; Ono et al., 1989). It is logical that with overlapping factors, such as altered folate metabolism with aging, the germ cells will also be affected by the aging process. Pioneering studies in this area compared embryos generated from the germ cells of aged rats with rats treated with a demethylating agent, 5-azacytidine, and found that the results were similar. Suggesting a likely DNA methylation perturbation in the sperm from aged rats (Oakes et al., 2003). In mouse sperm, global DNA methylation and hydroxymethylation are not significantly altered due to age (Kobayashi et al., 2016; Milekic et al., 2015). Kobayashi et al. (2016) note, though, that there is differential methylation at some promoters within the mouse genome. The hypomethylated promoters they observed were enriched for gene regions involved in spermatogenesis and meiosis. When studying the mouse sperm DNA methylome, the CpG shores surrounding transcription start sites or splice junctions are hypomethylated in the sperm of older individuals (Cao et al., 2020; Milekic et al., 2015). Milekic et al. (2015) have reported that sperm DNA hypomethylation at CpGs near transcription start sites and hypermethylation of splice junctions was maintained in brain sample DNA from

the offspring of older fathers, however, to a lesser extent. The offspring of older fathers also displayed subsequent alterations in the expression of 17 transcripts in the brain and altered behavior (Milekic et al., 2015).

In many somatic cell types, there is a drive to develop an epigenetic clock – whereby one can predict the biological age of a person by their DNA methylation levels. In somatic cells, there is hypomethylation associated with aging, that allows for these predictions. In spermatozoa from men of different ages, data indicate that the DNA methylation and hydroxyl-methylation levels increase by 1.76% and 5% per year, respectively (Jenkins et al., 2013). The correlation of age and sperm DNA methylation alterations is so strong with aging that models can be used to accurately predict age based on sperm DNA methylation. When analyzing differential methylation of 2.65 million CpG sites from aged men, many sites are hypermethylated regions (62%), with a smaller proportion of hypomethylated regions (38%) (Cao et al., 2020). The nature of the DMRs is also of importance, with most hypermethylation found in distal gene regions and hypomethylation found proximal to gene transcription start sites. This suggests that though there may be more hypermethylation overall, areas relevant to gene transcription may be more likely to have increased expression due to the hypomethylation observed. Given the issues that have been observed with advanced age in the offspring, it is interesting that the DMRs were found to cluster near gene regions that were related to metabolic aging and neurodevelopment. Some have proposed that altered methylation in regions specific to development may be biased due to the more open nature of chromatin at developmentally relevant genes (Ashapkin et al., 2022).

3.4.2. Histone modifications

Very few studies have begun to examine the effects of aging on sperm histone methylation (as reviewed by: Ashapkin et al., 2022). Firstly, a mouse model for aging has shown that in the

spermatogonial stem cells, alterations in H3K27me3 at sites required for the expression of differentiation genes during SSC differentiation (Liao et al., 2021); additionally, they reported alterations in H3K27me3 at Wnt and TGF-B signaling genes. Two additional studies examining sperm histone methylation have been done in mouse models as well. Xie et al., (2018) found that when examining histone repressive (H3K27me3) and activating marks (H3K4me3), there were disruptions in genes for longevity and spermatogenesis. In addition, 90% of the altered histone modifications were found within one region on chromosome 5 that has been associated with differential DNA methylation as a result of age in hematopoietic stem cells (Taiwo et al., 2013). It has been observed that, with aging, repressive histone marks, H3K9me3 and H3K27me2/3, have a disrupted balance with decreased H3K9me3 and increase H3K27me2/3 (Tatehana et al., 2020). It was also reported that the activating mark H3K4me2 was decreased in sperm from aged mice (Tatehana et al., 2020). Studies of histone modifications in sperm remain technically challenging to undertake, as this requires expertise in sperm ChIP-seq. Consequently, there are limited studies on altered histone modifications in sperm due to aging.

3.4.3 Noncoding RNAs

There are few studies on the effects of aging on testicular ncRNAs. Emerging data suggest that advanced paternal age dysregulates ncRNAs in rat sperm (Suvorov et al., 2020). Mainly, the proportions of ncRNAs shift with aging. Specifically, rRNA and lncRNA decrease while tRFs, piRNA, and miRNA increase. The targets of the miRNA and piRNA have been reported to be developmental and metabolism-related genes, consistent with other epigenetic reports and what is known about the effects of paternal age on the offspring. miR-125a-5p has been reported to be significantly upregulated in sperm from aged mice and is negatively associated with DNA integrity and embryo development (Liang et al., 2021). The same group

found that another miRNA, miR-574, is upregulated in sperm from aged mice and is associated with inhibited ATP production through impairing mitochondrial function (Ma et al., 2020). Offspring exposed to tRFs from aged sperm during zygotic development had altered gene expression for neurodevelopment genes and related anxious behaviors (Guo et al., 2021). These data support the hypothesis that ncRNAs are contributing to epigenetic inheritance as a consequence of an altered sperm epigenome.

3.4.4 Telomeres

It is likely that epigenetic regulators are contributing to the organization and maintenance of telomeres with aging. In somatic cells, the dynamics of telomere length during aging are well documented, with a consistent decrease in telomere length in most cell types. This shortening is due to the loss of telomeric repeats with each mitotic division (Shammas, 2012). In male germ cells, though, no such clear trend has been established. The developing sperm contain active telomerase until spermiogenesis, suggesting the potential for germ cell telomere maintenance or lengthening (Bekaert et al., 2004). The results thus far on telomere length have been conflicting and have spanned methods and species. In mice, there exists evidence to suggest that with aging, sperm telomere length decreases, similar to somatic cells (de Frutos et al., 2016). When similar studies were conducted in humans, the evidence was contrary to somatic cells and mice sperm, with an increase in sperm telomere length with age (Kimura et al., 2008; Laurentino et al., 2020). It remains possible that the effect of age on sperm telomere length is species specific. A likely hypothesis to explain this disparity is that the lifetime activity of telomerase is much longer in a man, given the drastic lifespan discrepancies between species. Essentially, this suggests that telomerase has more time in the germ cells of aged men to add telomeric repeats than it would in a rodent model.

There are now studies to suggest that oxidative stress may also contribute to a loss of telomere integrity with aging. Telomeres and their associated proteins are more susceptible to DNA damage by oxidative stress (Moazamian et al., 2022).

3.5 Germ cell gene expression with aging

Studies linking direct germ cell gene expression changes to aging are rare and either use whole testis RNA or examine specific genes, such as DNA damage repair in spermatocytes (Han et al., 2021; Paul et al., 2011). Using microarray analysis, studies done on mice spermatogonia revealed that *Icam1*, and *Selp* transcripts were affected by aging (8 months); these two transcripts are considered as specific aging markers, as they are also affected during aging of hematopoietic stem cells (Kokkinaki et al., 2010). One of the first studies to examine individual germ cell types was done recently in humans. Nie et al. (2022) performed single-cell RNA sequencing on whole testis samples from young and aged men and used bioinformatics to identify cell types based on how closely the cell types were related. They found that in germ cells, there were few changes in SSC RNAs and that no significant differential gene expression was present in early germ cells, with changed only emerging at the elongated spermatid stage (Nie et al., 2022). In the elongated spermatid stage, they report upregulated genes related to protein targeting and downregulated genes related to peptide chain elongation and oxidative phosphorylation (Nie et al., 2022). These results conflict with rodent-based literature for differential gene expression with advanced aging, as significant differential expression had been observed in SSCs, pachytene spermatocytes, and round spermatids (Kokkinaki et al., 2016; Paul et al., 2011).

3.6 DNA damage and repair with aging in male germ cells

In men that are over 35, DNA damage is three-fold higher in spermatozoa when compared to men under 35 (Singh et al., 2003). Comet assays for DNA damage reveal that with

advancing age, there is a higher rate of sperm with DNA fragmentation (Schmid et al., 2007). With age, germ cells accumulate DNA damage, such as 8-oxodG, due to the poor removal of DNA adducts and oxidized purines (Paul et al., 2011). There is evidence that some sperm retain these DNA adducts after spermatogenesis, as the developing germ cells maintain them without undergoing apoptosis (Ahmed et al., 2007; Zubkova et al., 2005). Studies in aging animals have examined the DNA damage repair pathways in various cell types. As mentioned previously, after meiosis, most germ cells do not undergo DNA damage repair and thus do not have abundant repair machinery. In aged rodent spermatocytes, that normally are capable of repair, genes involved in the BER pathway are downregulated (Paul et al., 2011).

The increased DNA damage is associated with decreased conception and increased rates of miscarriage. Mutations can be repaired in the zygote after fertilization; sperm from aged and infertile men may contribute to preimplantation loss prior to the opportunity for repair (Zenzes et al., 1999).

This phenomenon has many possible sources, including the accumulation of DNA mutations due to continuous cell divisions of spermatogonial stem cells, accumulation of exposures to toxicants over the lifetime, decreased ability to check and correct chromosome segregation during mitosis/meiosis, increased oxidative stress, and decreased ability to repair DNA damage (Aitken and Baker, 2013). The increase in oxidative stress caused by reactive oxygen species (ROS) due to aging has been well studied in the Robaire lab, where they found that germ cells from aged mice and rats have a decrease in the enzymes required to neutralize oxidative stress and related increases in oxidative stress-related DNA damage. Mice that were modified to have an over-expression of catalase (*Cat*) had sperm that showed reduced oxidative damage during aging when compared to wild type mice (Selvaratnam and Robaire, 2016b). In

instances where the neutralizing genes were knocked out, the DNA damage and oxidative stress phenotypes seen with aging were exacerbated (Selvaratnam and Robaire, 2016a).

3.7 Animal Models of Reproductive Aging

Though obtaining semen samples from men is of relative ease, it remains unfeasible to study developing germ cells at a large scale. In some instances, human testis samples can be obtained; however, these do not allow for genetic or pharmacological intervention, and studies using these tissues can be quite restricted by their availability. As a result, to study the process of aging during spermatogenesis, rodent models have been widely used.

3.7.1 The Brown Norway Rat

The primary model used to study reproductive aging since the early 1990s is the Brown Norway (BN) rat (*Rattus norvegicus*). As of 2004, the Brown Norway rat has a fully sequenced genome. Presently the genome version is at rn_6 (Gibbs et al., 2004). This model has been suggested by the National Institute on Ageing as the optimal rodent model for aging studies. Specifically, Brown Norway rats are an excellent model as they have age-related endocrine alterations similar to those seen in humans. BN rats experience a decrease in testosterone, with no decrease in serum LH and slight increases in FSH (Gruenewald et al., 1994). With aging, other rat models tend to develop primary tumors in the pituitary, testis, adrenal and/or liver altering serum hormones and thus making it a challenge to ascertain the origin of age-related effects in the testis (Taylor and Mowat, 2020). These rats also generally live comfortably into old age, approximately 30-36 months, without developing additional age-related pathologies such as obesity or cancers.

Using this model, many groups have reported the effects of advanced paternal age on the testis and the epididymis, as well as on spermatogenesis and on spermatozoa. Wang et al. (1993) reported that with aging, there is an overall disruption in the testis histology and spermatogenesis. Wright et al. (1993) observed that there were Leydig cell alterations due to aging in the BN rat that were similar to the pathology noted in men. Zirkin's group has continued to use the BN rat to advance our understanding of the effects of aging on steroidogenesis in the Leydig cell. Levy et al. (1999) reported that with aging, there were segment-specific changes in the epididymis of the BN rat and that the blood-epididymis barrier was disrupted with aging. When examining the effects on sperm, the Robaire lab has shown that due to aging in the BN rat, there is an overall decrease in fertility and poor outcomes in the progeny (Serre and Robaire, 1998). Other reported changes from this include: sperm structural and motility changes (Syntin and Robaire, 2001), fewer spermatogonial stem cells (Paul et al., 2013), altered antioxidant enzymes (Zubkova and Robaire, 2004; Zubkova and Robaire, 2006; Weir and Robaire, 2007), increased oxidative stress DNA damage (Zubkova and Robaire, 2005; Selvaratnam et al., 2015), and differential expression of DNA repair pathway genes in spermatocytes (Paul et al., 2011).

4. Rationale and formulation for the project

Correlative epidemiological studies demonstrate a link between advanced paternal age and potentially detrimental effects on their offspring. The effects of aging on fertility have been well established, with a decrease in fertility and decreased sperm quality. The purpose of the studies presented in this thesis is to evaluate the genetic basis for the changes observed with advanced paternal age through overall chromatin organization, gene expression, and epigenetic regulation.

The objectives of this thesis are to:

1. Measure telomere length in male germ cell of young and aged Brown Norway rats
2. Evaluate the effects of aging on gene expression in post-meiotic round spermatids
3. Examine the effects of aging in post-meiotic round spermatids and mature spermatozoa on DNA methylation

For these studies, adult wild-type Brown Norway rats were aged to either 4-6 months of age or 18-20 months, representing human populations of 20-30 or 45+ years of age, respectively. As previously mentioned, Brown Norway rats are the standard model used in the study of aging, with many age-related effects similar to those observed in humans. We then collected various germ cell types from the testes using the STA-PUT method to isolate germ cell types and spermatozoa from the epididymides. For objective 1, we aimed to observe telomere length changes across spermatogenesis, as this had not previously been characterized. We used all pachytene spermatocytes as a pre-meiotic germ cell type, round spermatids as post-meiotic germ cells, and sperm from the caput and cauda of the epididymis to capture the effect of epididymal transit. Here, we used qPCR for telomeric repeat measurements. For objective two, we focused on round spermatids as they are the last transcriptionally active germ cell type and would be transcribing RNAs required for spermiation and sperm functionality. We used Novogene Corporation to do paired-end mRNA sequencing. For objective three, we examined round spermatids as they are the last germ cell stage prior to chromatin repackaging with protamine during spermiogenesis, and mature spermatozoa as the marks in sperm would be potentially transmitted to the offspring. We were interested here not only in the direct effect of age on each cell type, but also on the effect of age on the transition between cell stages. We used Novogene Corporation again for whole genome bisulfite sequencing. The three objectives, together, elucidate the effect of aging on epigenetic regulation and gene expression in Brown Norway rats.

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CHAPTER 2

TELOMERE DYNAMICS THROUGHOUT SPERMATOGENESIS

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Abstract

Telomeres are repeat regions of DNA that cap either end of each chromosome, thereby providing stability and protection from the degradation of gene-rich regions. Each cell replication causes the loss of telomeric repeats due to incomplete DNA replication, though it is well-established that progressive telomere shortening is evaded in male germ cells by the maintenance of active telomerase. However, germ cell telomeres are still susceptible to disruption or insult by oxidative stress, toxicant exposure, and aging. Our aim was to examine the relative telomere length (rTL) in an outbred Sprague Dawley (SD) and an inbred Brown Norway (BN) rat model for paternal aging. No significant differences were found when comparing pachytene spermatocytes (PS), round spermatids (RS), and sperm obtained from the caput and cauda of the epididymis of young and aged SD rats; this is likely due to the high variance observed among individuals. A significant age-dependent decrease in rTL was observed from 115.6 (±6.5) to 93.3 (±6.3) in caput sperm and from 142.4 (±14.6) to 105.3 (±2.5) in cauda sperm from BN rats. Additionally, an increase in rTL during epididymal maturation was observed in both strains, most strikingly from 115.6 (±6.5) to 142 (±14.6) in young BN rats. These results confirm the decrease in rTL in rodents, but only when an inbred strain is used, and represent the first demonstration that rTL changes as sperm transit through the epididymis.

Introduction

The male germline is biologically unique in many ways, ranging from cellular structures to chromatin packaging and enzymatic activity. Telomeres are no exception to this statement, with telomere dynamics in male germ cells being distinctly different from those of somatic cells.

Telomeres are 5′-TTAGGG-3′ repeat sequences that cap the ends of chromosomes to give the genome protection and stability from progressive shortening after DNA replication caused by the incomplete replication of the 5′ end by DNA polymerase [1]. The progressive shortening of telomeres due to the end-replication problem can be mitigated by the enzyme telomerase; it maintains telomeres by the addition of new repeats. Containing both a protein (TERT) and an RNA template (TERC), the enzyme functions as a reverse transcriptase synthesizing a single strand of telomeric DNA complementary to TERC onto the 3′ overhang. This newly synthesized telomeric DNA strand is then used for lagging strand synthesis by DNA replication machinery [1]. Alternative lengthening of telomeres (ALT) by homologous recombination is another mechanism by which telomeres and subtelomeric regions are able to retain their length in the absence of active telomerase [2].

The existing literature presents data on the length and spatial arrangement of telomeres, and the activity of telomerase within germ cell nuclei as spermatogenesis progresses [3]. Beginning in spermatogonia, telomerase is most active and telomere length is hypothesized to be shorter relative to fully mature spermatozoa [4]. The telomeres at this stage are randomly positioned; however, during mitosis, they align to either pole of the cell in preparation for cytokinesis. In spermatocytes, telomerase levels are high and telomeres follow a similar alignment once meiotic events are initiated. Round spermatids have similarly high levels of telomerase at the onset of spermiogenesis; however, these levels decrease as the cells become transcriptionally inactive during chromatin compaction [4]. At this stage, fluorescence in situ hybridization (FISH) experiments have also shown that telomeres spread randomly throughout the cell nucleus. Interestingly though, FISH experiments often display a reduced number of telomeres due to their apparent dimerization. This has been shown as the number of telomeres present at the final

stages of spermiogenesis is half of the expected number, suggesting that they are co-localizing [5–7]. Some hypotheses have been put forth about the nature of this interaction, and through FISH experimental staining for p and q arms of chromosomes 3 and 6, it appears that the telomeres of each chromosome bind to each other in a loop-like fashion [7]. Throughout spermiogenesis and epididymal transit, germ cells undergo dramatic chromatin repackaging. There is a gradual replacement of most histone-bound nucleosomes first with transition proteins and then with protamines, to form a tight toroidal conformation [8]. This repackaging event does not completely void the cell of histones, and approximately 10–15% of histones are retained in human sperm [9–11], while in rodents, only 1–2% of histones are retained [11,12]. Fully mature sperm maintain dimeric telomeres, as shown in round spermatids, though they contrast with earlier germ cells as little to no telomerase activity has been observed [13]. Telomere length in spermatozoa is longer than in somatic cells and has been measured at approximately 6–20 kb in humans [14–18]. Spermatozoa also appear to have a specific organization of telomeres, with the telomeric regions of chromatin found toward the nuclear periphery or bound to the nuclear membrane. This observation has been shown for many species, including humans, rodents, primates, and bovine [14,19]. It has been postulated that the combination of histone-bound telomeres and their arrangement at the nuclear periphery serves a functional role after fertilization as these sites are more readily accessible by the oocyte for pronuclear formation [6,20].

A central current issue in male germ cell telomere biology is whether telomere length can be used as a biomarker for sperm quality and fertility. The parameters set by the World Health Organization (WHO) used to assess male fertility do not capture information about sperm chromatin quality [21]. Although measuring sperm DNA integrity is considered an important

endpoint [22], many of the methods have been classically challenging in a clinical setting as they require a high level of technical expertise. As a result, there is a demand for a quick reproducible test that would examine a new sperm parameter. Telomere length is a desirable measure, as preliminary studies are beginning to suggest links between fertility outcomes and sperm telomere length. However, there is some controversy in this field regarding which methods measure telomere length in a reliable and accurate way. The methods employed include Southern blotting, fluorescence in situ hybridization, and the quantitative polymerase chain reaction (qPCR). Both Verhulst [23] and Eisenberg [24] have discussed the issues as they relate to each method's reliability, pointing out the inherent cost–benefit analysis that must be done when deciding on a method. When assessing telomere length as a biomarker for fertility in humans, it would be most appropriate to use qPCR as it is relatively simple, inexpensive, and allows for a high-throughput analysis of many samples.

As previously mentioned, preliminary data on the links between sperm telomere length and well-established fertility parameters are beginning to emerge [25]. Several studies have found an association between a shorter telomere length and infertility or oligozoospermia [26–30], but not with classical WHO semen parameters. Interestingly, Garolla et al. found a positive association between sperm telomere length and protamination status [31]. This finding suggests that an error in chromatin packaging results in telomere dysregulation in mature sperm. Additionally, more loosely packaged chromatin could result in an increase in exposure to reactive oxygen species.

There are many factors known to increase male factor infertility, including smoking, alcohol, toxicant exposure, and being overweight [32]. These lifestyle factors, in addition to the aging process, greatly increase the presence of reactive oxygen species; several studies have found an association between these lifestyle factors and disrupted sperm telomere integrity [33,34].

Telomeres are particularly susceptible to oxidative damage as they are highly rich in guanine, allowing for the oxidization to 8-oxo-2'-deoxyguanosine (8-oxo-dG) [35]. In vitro results suggest that oxidative insult results not only in disrupted telomere integrity, but also in telomere shortening [36]. Additionally, the retention of histones in telomeric regions makes these regions more sensitive to oxidative insult [20]. The DNA damage that may be incurred from these oxidative insults can further lead to telomeric instability and telomere–telomere interactions may be lost [37].

Telomere length decreases in somatic cells with advanced age, but there are varying species-dependent effects on sperm telomere length. In studies examining telomere length in mice, the trend with advanced paternal age is a decrease in telomere length, similar to that seen in somatic cells [38]. However, when similar studies were done using human sperm, the telomere length appeared to increase with age [16,39]. There are two main hypotheses addressing the potential cause of telomere lengthening in species with longer life spans. The first is that because telomerase is active in spermatogonia and throughout spermatogenesis, it has ample time to act and build on telomeres as the pool of stem cells is aging. The second is that there is a selection of germ cells for those with the longest telomeres over the course of a man's lifespan, resulting in only those with long telomeres remaining at an advanced age [40].

Telomere homeostasis may exist, where there is a balance for the optimal telomere length. When the telomeres are dysregulated, meiosis can be more error-prone, with chromosome segregation being incomplete and higher rates of aneuploidy [41]. Supporting this hypothesis, Cariati et al. have shown data that there are pregnancy failures when male partners have short telomeres [28]. It is also interesting to note that these studies have explored the association between sperm telomere length and offspring leukocyte telomere length. Few studies have

directly studied both sperm telomere length and offspring telomere length; however, in rodents, birds, primates, and humans, there is a clear paternal age effect on telomere length, where older fathers produce offspring with longer telomeres [40,42–45]. These results are in favour of the hypothesis that telomeres are an epigenetic feature.

Although we are gaining insight into several aspects of the length of telomeres in the context of male reproduction, no study to date has related the effects of the phase of spermatogenesis and epididymal sperm maturation to telomere length with advancing paternal age, or established whether observed differences can be accounted for by the use of inbred and outbred rodent strains.

Materials and Methods

Animals

All studies were conducted on Brown Norway (BN) and Sprague Dawley (SD) transgenic rat strains bred in-house, with initial breeding pairs kindly provided by Dr. Hamra at UT Southwestern. The rats were transgenic for td-Tomato red (BN) and e-GFP (SD) expression in the germline. All animals had access to food and water ad libitum, and were kept in a 12-hour light, 12-hour dark, temperature- and humidity-controlled environment. BN and SD rats (n = 3–5) were sacrificed at young and aged time points. The average ages for the inbred BN rats were 5.6 months ± 0.2 and 19.2 ± 0.06 months for young and aged populations, respectively. For outbred SD rats, the average ages were 5.6 ± 0.18 and 18.7 ± 0.32 months for young and aged populations, respectively. Eighteen to twenty months of age in a rat is the age prior to germ cell loss and testicular atrophy [46]. Animal care and handling were done in accordance with the

guidelines put forth by the Canadian Council on Animal Care (McGill Animal Resources Centre protocol 4687).

Germ Cell Separation

Young and aged rats were euthanized by CO_2 asphyxiation. Testes were removed and weighed to assess the regression status associated with advanced aging, and rat testes less than 1.5 grams were considered regressed and not used in this study. When a testis was excluded, the attached epididymis was not used for sperm collection. Of 11 aged animals, four possessed only one testis that was not regressed. No animals had both testes regressed. Germ cells were obtained using the STA-PUT method for cell velocity sedimentation [47]. Briefly, testes were decapsulated prior to enzymatic digestion with 0.5 mg mL−1 collagenase (C9722-50MG; Sigma Aldrich, Oakville, Canada), followed by subsequent digestion with 0.5 mg mL−1 trypsin (Type I, T8003; Sigma-Aldrich, Oakville, Canada) and DNase I (Type I, DN-25; Sigma-Aldrich, Oakville, Canada). The dissociated germ cell suspension was then filtered through a 70 μM nylon mesh before being washed three times with 0.5% bovine serum albumin (A4612; Sigma Aldrich, Oakville, Canada) in RPMI 1640 (Life Technologies, Grand Island, USA) and pelleted at 233 g for 5 min. Cells were filtered once more with a 55 μM mesh to prevent clumping and 5.5 × 108 mixed germ cells in 25 mL of 0.5% BSA in RPMI were loaded into the STA-PUT (Proscience, Toronto, Canada) and separated on a gradient of 2–4% BSA/RPMI. The gradient was established over 50 min, and the cells were separated through unit gravity sedimentation for 1 h 45 min. A fraction collector was then used to collect the germ cells in individual populations of pachytene spermatocytes (PS), and round (RS) and elongating spermatids. Fractions that met at least 80% purity by phase-contrast microscopy identification were spun down, flash frozen, and kept at −80 ◦C for future experiments. Spermatozoa from the caput and cauda epididymidis

were isolated in PBS after 2 h of agitation. They were filtered through a 100 μM nylon mesh before being centrifuged and washed six times with 0.45% saline solution.

Telomere Measurement

DNA was extracted from 1.5 × 106 PS, RS, and spermatozoa from the caput and cauda epididymidis using the QiaAMP DNA mini kit (51304; Thermo Fisher Scientific, Mississauga, Canada), with the substitution of a separate sperm lysis buffer including 40 mM dithiothreitol (DTT). Extracted sperm DNA may have different recoverability at the telomeres, as in these regions, it is packaged primarily with histones, while the remainder of the DNA is bound to protamine. Given our protocol for sperm DNA extraction, which disrupts the bound protamines, we did not anticipate that this would be an issue. DNA was diluted to a working concentration of 5 ng μL−1 for telomere measurement by qPCR [48] for telomeric repeats and 36B4 single copy gene amplification measured by ΔCt. The mastermix for a final reaction volume of 20 μL per well was prepared using 10 μL per reaction SYBR Green MM solution (4367659; Thermo Fisher Scientific, Mississauga, Canada). For each 36B4 reaction, 1 μL of 2 μM forward and reverse primers for 36B4 and 5 μL PCR grade water were used. For each telomeric DNA reaction, 0.5 μL 2 μM forward and reverse primers for telomeric DNA with 4.5 μL of PCR grade water were used (Table S1). For all reactions, 20 ng/well DNA was used. The standard curve for telomeric repeats follows a 1:5 dilution, beginning with 4000 picograms (pg) of telomere oligomer (Table S2), corresponding to 7.6 × 109 kb. The 36B4 standards begin with a concentration of 2 pg (Table S2), following a 1:10 dilution, corresponding to 3.6 × 109 genome copies. Standards were brought to a total of 20 ng of DNA by spiking with pBR322 DNA. All samples presented herein fall along the presented standard curves. A four-step PCR amplification protocol was used. First, denaturation occurred at 95 ◦C for ten minutes (one cycle), followed by 40 cycles of denaturation

at 95 ◦C for 15 s and annealing at 60 ◦C for 1 min. The melt curve conditions were 95 ◦C for 15 s, and annealing at 60 ◦C for 1 min, with a temperature increase of 0.5 ◦C per cycle to 95 ◦C for 15 s. The final step was an infinite hold at 4 ◦C. By taking the telomeric repeats, relative to the genome copies, the telomere length per genome was represented. DNA taken from H1301 cells (#01051619-DNA-5UG; Sigma-Aldrich, Oakville, Canada) with a known telomere length of 70 kb was then used for the normalization of all samples. The calculations are as follows:

Calculating telomeric repeats in log scale:

$$\log(Tel) = \frac{\Delta Ct - B}{m}$$

Where telomere standard curve produces slope: $\Delta Ct = m\ (logTel) + B$

Calculating genome copies (GC) represented by single copy gene 36B4

$$\log(GC) = \frac{\Delta Ct - B}{m}$$

Where 36B4 standard curve produces slope: $\Delta Ct = m\ (logGC)$ + B

Calculating telomeric repeats per genome (telomere/single copy gene):

$$\log(telomeric\ repeats\ per\ genome) = \frac{\log(Tel)}{\log(GC)}$$

$$telomeric\ repeats\ per\ genome = \log(telomeric\ repeats\ per\ genome)^{10}$$

Calculating telomere length relative to H1301 cell DNA, with predicted telomere length of 70 kb:

$$70\ kb = \frac{telomeric\ repeats\ per\ genome\ H1301}{x}$$

$$relative\ telomere\ length = \frac{telomeric\ repeats\ per\ genome}{x}$$

All experiments were done in triplicate, with intra-class correlation coefficients of 0.82 and 0.85 for young and aged BN sperm telomere lengths, respectively. To control for inter-plate variation, H1301 and standard curves were run on each plate. An inherent limitation of this protocol is the normalization of samples to H1301, as different methods of DNA extraction and handling can alter the apparent measure of telomere length. Though this was controlled for with samples processed in house, H1301 DNA was extracted and purified by Sigma.

Statistical Analysis

To calculate the telomere length, telomere kb and 36B4 genome copies were extrapolated from the standard curves and ΔCt values (Equations (1) and (2)). The telomere kb was divided

by the genome copies represented by 36B4 (Equation (3)). These values were then normalized to the positive control H1301 DNA (Equation (4)), with a known telomere length of 70 kb, to give a measurement of relative telomere length (rTL). The median and interquartile range were calculated in Excel. Further statistics and data analysis were conducted using Graph-Pad Prism 6. Where appropriate, t-tests were used for statistical comparisons between groups; however, where variances were significantly different, a Mann Whitney U test was used as a replacement. Statistical significance of $p \leq 0.05$ has been indicated with an asterisk (*).

Results and Discussion

Telomere Dynamics Show Rat Strain Specificity Between Brown Norway and Sprague Dawley Rats

Telomere length for the outbred SD rats is in the range of 200–350 across spermatogenesis, while that for the inbred BN rats is shorter and has a decreased range of 115–160. Comparative studies of germ cell telomere length across varying species and strains have not been conducted. Although one would anticipate less variance in the lengths of telomeres from an inbred than outbred strain due to decreased genetic heterogeneity, it has also been proposed that inbred strains may have shorter somatic telomeres due to the increased oxidative stress and reduced evolutionary fitness [49]. The fact that this trend is maintained in the germline reveals potential long-term effects in an inbred rat strain as sperm telomere length is correlated with offspring telomere length [40,42–45]. Both strains show no difference in PS or RS rTL, a trending decrease in the caput sperm, and the subsequent recovery of telomere length in sperm from the cauda epididymidis. The most striking difference between strains is that both the interquartile range (IQR) and standard errors calculated for BN sperm telomere lengths are much smaller than those for the SD sperm (Table 2-1). The interquartile range represents the spread of data, by

showing where 50% of the data points lie in a given sample set. The smaller IQR values for BN rats are likely due to the inbred nature of BN rats and the level of their genetic similarity. The homogeneity in rTL further validates them as a model for epigenetic studies in rodents. With both the inherent variability seen in the SD telomere length measurements and the exclusive use of BN rats for epigenetic studies, data for BN rats will be presented throughout the remainder of the text. The SD data is presented in Supplemental Figure S2-A1.

Telomere Lengths During Spermatogenesis in Brown Norway Rats

Examining germ cell telomere dynamics has been done extensively in the context of telomerase activity, with a well-defined pattern of high telomerase activity in early germ cells that tapers off as spermatogenesis progresses. However, the existing literature that examines telomere length is less complete, mainly examining fully mature sperm and operating under the assumption that germ cell telomere length is strongly correlated with telomerase activity. When measuring rTL in BN rats, we find that there is no significant difference in the telomere length from PS to RS, with lengths measured at 155.4 (±11.6) and 159.2 (±20.1), respectively (Figure 2-1). This observation suggests that the length of telomeres remains relatively constant throughout the meiotic stages of spermatogenesis, independent of the apparent increase in telomerase activity [4]. An important component of understanding telomere dynamics throughout spermatogenesis that is missing is the measurement of telomere length in the spermatogonial stem cells; however, a methodology for the isolation of rat spermatogonial stem cells has yet to be developed.

Interestingly, when entering the epididymis, the length of telomeres shows a decrease of approximately 25% from what is observed for earlier stages of spermatogenesis. The length of telomeres from the spermatozoa obtained from the caput epididymidis of any species has not

been measured previously, so it is difficult to determine if this novel observation can be generalized beyond the rat. This finding suggests altered telomere organization during chromatin condensation and crosslinking through epididymal maturation. However, what is apparent is that by the time sperm reach the cauda epididymidis, the sperm telomere length reaches a length of 142 (±14.6), comparable to the germ cell telomere length prior to entering the epididymis (Figure 2-1). The epididymis is a tissue that has received relatively little attention; understanding how the environment of the caput, corpus, and cauda epididymidis alters sperm chromatin is a major challenge that needs to be addressed by the scientific community. It is possible that telomere organization is impacted by micro and non-coding RNAs that are passed to the sperm through epididymosomes [50,51]. As more interactions are being elucidated for non-coding RNAs and telomeric regions, the functional role of these interactions will become clearer [52]. Telomeric repeat containing RNA (TERRA) is a non-coding RNA transcribed from telomeric regions that is able to bind telomeric DNA. The proposed function of TERRA binding is to control telomere structure and elongation; this has been shown in various species [53–56]. TERRA has also been shown to modify polycomb repressive complex binding, and modify histone marks across the genome and in telomeres [57]. Though there is limited literature on TERRA in male germ cells, Reig-Viader et al. have shown that it is present in spermatocytes and spermatids [58]. They have also shown that telomeres and TERRA levels were disrupted in germ cells from men with idiopathic infertility [59] Taken together, these observations indicate the need for further studies to resolve the effects of non-coding RNAs during epididymal maturation.

Age-Dependent Decrease in Sperm Telomere Length

There is a significant age-dependent decrease in rTL from 115.6 (±6.5) to 93.3 (±6.3) in caput sperm ($p = 0.04$), which remained consistent for cauda sperm, with a decrease observed

from 142.4 (±14.6) to 105.3 (±2.5) in cauda sperm ($p = 0.02$; Figure 2-2). This decrease is consistent with mouse models of paternal aging presented in the literature [38]. Interestingly, the trend for increased telomere length during epididymal transit is seemingly reduced with aging. A modest increase in rTL is observed from 93.3 (±6.3) to 105.3 (±2.5) in the caput sperm. If, during epididymal transit, non-coding RNAs contribute to affecting telomere length, it is possible that the epididymosome payload changes with advancing age, though no study to date exists on epididymosomes and aging.

There are currently no hypotheses to address the decrease in telomere length observed in rodent models of paternal aging. However, it seems probable that hypotheses proposed to explain germ cell telomere lengthening in humans may not apply to the much shorter lifespan of a rodent.

Conclusion

Understanding telomere length in the varying contexts that influence male reproductive function and spermatogenesis is critical to understanding their epigenetic implications. As telomeres are associated with the nuclear envelope, it remains plausible that they are sites initially recognized by the egg after fertilization to aid in chromatin anchoring; telomere length may also influence offspring health in this way [20]. Altered telomere length, either increased or decreased, may lead to a disruption in chromatin reorganization events following fertilization [28]. Studies by our group have shown several effects of aging on male reproductive outcomes, including increased time to pregnancy, higher resorption rates, and an increased instance of infertility [46]. It is difficult to conclude if the negative outcomes are associated with one specific pathology of aging, such as telomere length, as these cells are also exposed to increased oxidative stress and decreased DNA damage repair, and thus show increased DNA damage. The

presence of increased DNA damage with aging has not been examined within telomeric regions; however, it may provide additional insight into sperm telomere dynamics during aging. Here, we have shown that sperm telomere length decreases with age in inbred Brown Norway rats. This poses an interesting question, and by examining telomere dynamics in embryos fertilized with young and aged sperm, we may begin to understand this relationship more clearly. Additionally, using RNA sequencing and chromatin conformation capture methods will elucidate how telomere dynamics are altered across spermatogenesis with aging.

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Author Contributions

Conceptualization, B.R. and H.E.F.; methodology, H.E.F.; investigation, H.E.F.; resources, B.R.; data curation, H.E.F.; writing—original draft preparation, H.E.F.; writing—review and editing, B.R.; supervision, B.R.; project administration, B.R.; funding acquisition, B.R.

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Conflicts of Interest

The authors declare no conflict of interest.

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Figures

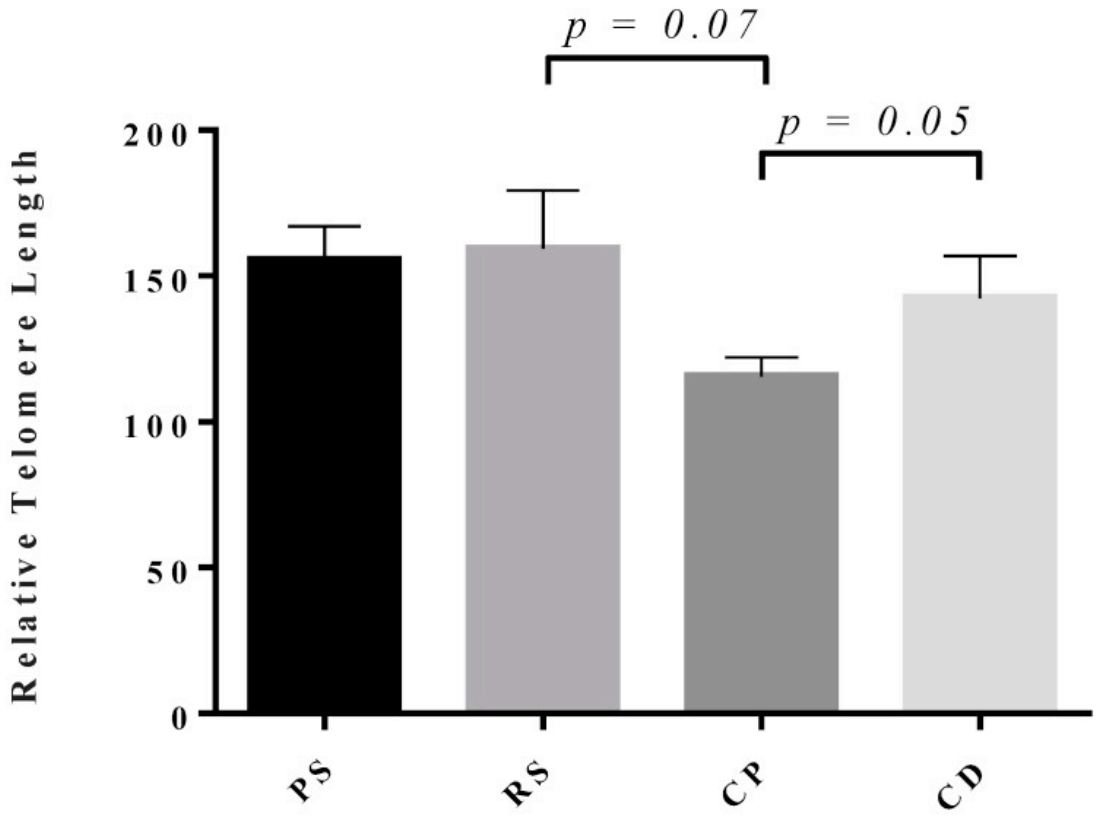

Figure 2-1: Telomere length in the young Brown Norway male germline. Relative telomere length (rTL) shown on the y-axis measured by quantitative polymerase chain reaction (qPCR) relative to H1301 cell DNA of a known telomere length, for pachytene spermatocytes (PS), round spermatids (RS), caput sperm (CP), and cauda sperm (CD). Each bar represents the mean ± SEM, n = 5. Sprague Dawley data shown in Figure A1.

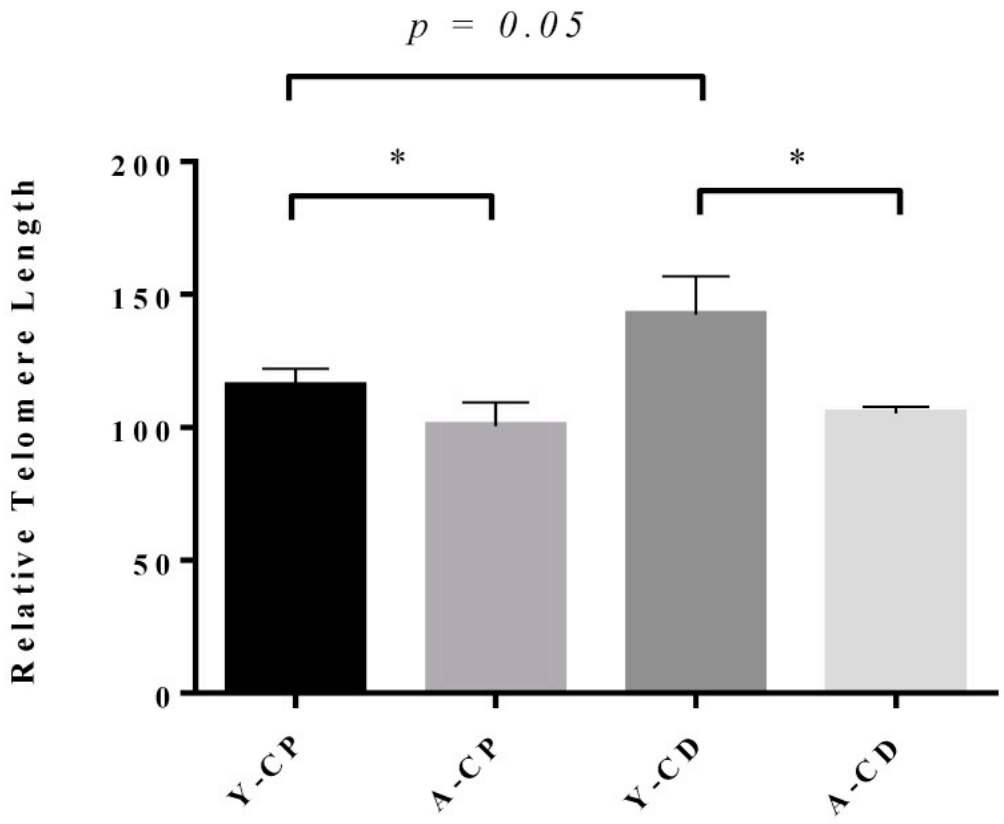

Figure 2-2: Telomere length for Brown Norway sperm during aging. Relative telomere length (rTL) shown on the y-axis measured by quantitative polymerase chain reaction (qPCR) relative to H1301 cell DNA of a known telomere length, for young caput sperm (Y-CP; n = 5), aged caput sperm (A-CP; n = 4), young cauda sperm (Y-CD; n = 5), and aged cauda sperm (A-CD; n = 4). Each bar represents the mean ± SEM. $p \leq 0.05$ is indicated by an asterisk. Sprague Dawley data shown in Figure S2-A1.

Table

Table 2- 1: Species variation for telomere length measurement in sperm

Rat Strain	Cell Type	N	Median	IQR	SEM
SD – Young	CP	6	230.47	79.17	25.54
	CD	10	396.81	253.57	54.94
SD - Aged	CP	3	116.93	205.51	133.00
	CD	12	302.82	132.82	28.49
BN - Young	CP	5	116.61	17.56	6.49
	CD	5	129.67	17.42	14.61
BN - Aged	CP	4	97.47	13.40	6.35
	CD	4	106.54	4.64	2.52

Species differences in telomere length variability shown between Sprague Dawley (SD) and Brown Norway (BN) rats for caput (CP) and cauda (CD) sperm for both young and aged samples. N: Sample Size. IQR: Interquartile Range. SEM: Standard Error of the Mean

Supplemental Materials

Appendix A

Table S2- A1. Telomere Length qPCR Oligomer Standard Sequences

Standard	Oligomer Sequence
Telomere	$(TTAGGG)^{14}$
36B4	CGACCTGGAAGTCCAACTACTTCCTT AAGATCATCCAACTTTTGGATGACTACC CAAAATGCTTCATTGTGGGAGCAGACA ATGTGGGCTCCAAGCAGATGCAGCAGA

Table S2-A2. Telomere Length qPCR Primers

Repeat Region	Forward Primer	Reverse Primer
Telo	CGGTTTGTTTGGGTTTG GGTTTGGGTTTGGGTTTGG GTT	GGCTTGCCTTACCCTTACCCTTA CCCTTACCCTTACCCT
36B4	CGACCTGGAAGTCCAA CTAC	ATCTGCTGCATCTGCTTG

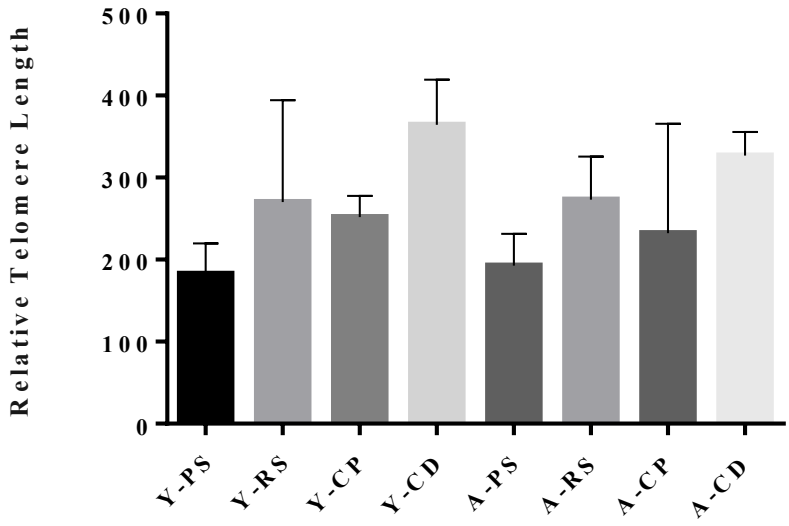

Figure S2-A1. Relative telomere length (rTL) for the Sprague Dawley germline during aging. rTL for: Pachytene Spermatocyte, RS: Round Spermatid, CP: Caput Sperm, CD: Cauda Sperm. Young samples presented as Y- (cell type), aging samples presented as A- (cell type).

Connecting Text

The research presented in Chapter 2 of this thesis examined telomere length during spermatogenesis and within the context of aging in a rat model. We measured telomere length in two strains of rats, the outbred Sprague-Dawley and inbred Brown Norway. Using quantitative PCR, we measured the telomere length in pachytene spermatocytes, round spermatids, and spermatozoa from the caput and cauda of the epididymis. We found that in the Sprague-Dawley rat, there was a great deal of variation in the telomere length regardless of germ cell stage. In the Brown Norway rat, this variation was greatly reduced, allowing us to observe trends in telomere length across spermatogenesis and sperm maturation, as well as with aging. Across spermatogenesis we saw that telomere length remains relatively consistent, with a decrease in telomere length upon sperm entry into the epididymis. The apparent decrease upon entry into the epididymis though, is recovered to previous length by the time sperm maturation is complete in the cauda of the epididymis. With aging, we saw a decrease in both caput and cauda sperm telomere length. Interestingly, we also noted that the increase in sperm telomere length during epididymal sperm maturation was lost with aging. This is the first study to report telomere length in early germ cells, and the first in rat to measure telomere length with aging. Given the apparent shift in telomere length with age, and our knowledge that telomeres aid in genome stabilization and help to regulate gene expression within the sperm nucleus by anchoring the DNA, we hypothesized that this may alter gene expression in germ cells with aging. We were also wanted to determine whether any gene related to telomere maintenance or chromatin packaging were altered with aging. In Chapter 3, we examine round spermatids from Brown Norway rats as they have a fully sequenced genome. The round spermatid stage is the final germ cell stage with transcriptional activity, and thus the last stage at which they may obtain necessary genes for telomere maintenance

CHAPTER 3

AGING AFFECTS GENE EXPRESSION IN SPERMATIDS OF BROWN NORWAY RATS

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Aging affects gene expression in spermatids of Brown Norway rats

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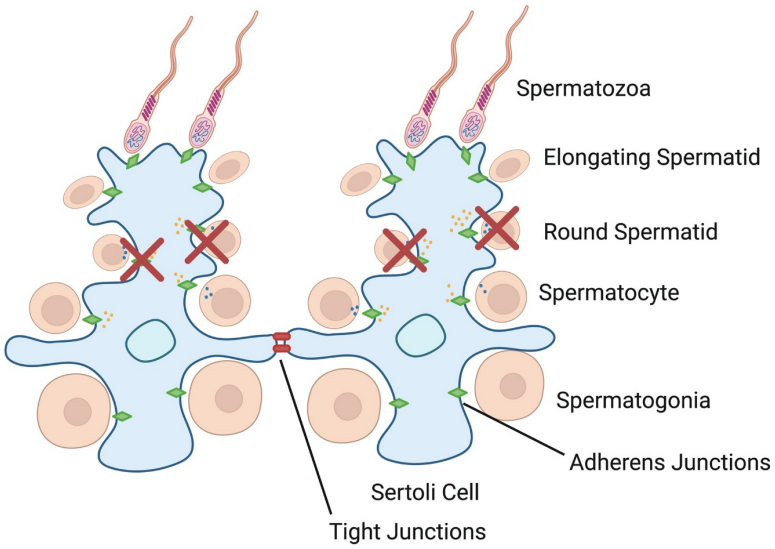
Highlights

- Aging causes a dysregulation in gene expression of round spermatids
- Significant DEGs are implicated in traditional reproductive pathways
- DEGs are involved in the interaction between germ cells and Sertoli cells

Abstract

The effects of aging on the reproductive health of men and the consequences for their offspring are becoming more widely recognized. Correlative epidemiological studies examining paternal age and offspring health suggest there are more frequent occurrences of genetic disorders in the children of older fathers. Given the genetic basis for paternal age-related disorders, we aim to characterize gene expression in developing germ cells. Round spermatids (RS) were collected from young (mean=5.3 months) and aged (mean=19.5 months) Brown Norway rats, representative of humans aged 20-30 years and 55+ years, respectively. Gene expression data were obtained by mRNA sequencing (n=5), and were analysed for differential expression. Sequencing data display 211 upregulated and 9 downregulated transcripts in RS of aged rats, compared to young ($log2FC > 1$, $p < 0.05$). Transcripts with increased expression are involved in several processes including sperm motility/morphology, sperm-egg binding, capacitation, and epigenetic inheritance. In addition, there are numerous dysregulated transcripts that regulate germ cell epigenetic marks and Sertoli-germ cell binding and communication. These results show an overall increase in RS gene expression with age, with spermatogenic functions being perturbed. Taken together, these findings help identify the genetic origin of the fertility, germ cell niche, and epigenetic effects observed with advanced paternal aging.

Graphical Abstract



Keywords

Paternal aging, spermatids, spermatogenesis, gene expression

Abbreviations

BP: base pairs
BN: Brown-Norway
BTB: Blood-Testis Barrier
DEG: differentially expressed gene
FPKM: Fragments Per Kilobase of transcript per Million reads mapped
GO: Gene ontology
IPA: Ingenuity Pathway Analysis
NIA: National Institute on Ageing
PCA: Principle component analysis
PS: Pachytene spermatocyte
ROS: Reactive oxygen species
RNA-Seq: RNA-Sequencing
RS: Round spermatid
SC: Sertoli cell
SSC: Spermatogonial stem cell

3-1. Introduction

The age at which individuals are choosing to have children has been steadily increasing since the 1970's. This trend remains true across geographic location, ethnicity, and education (Khandwala et al., 2017). The decline in oocyte quality with increasing maternal age is well established with the consequent implications on the offspring, such as trisomy 21 (Yoon et al., 1996; Crawford and Steiner, 2015). However, only recently have we begun to explore the impact of delayed paternity on sperm quality and progeny outcome. Initial studies have shown that with advanced paternal age there are decreases in sperm count, motility and morphology, with adverse effects on a couple's fecundity and the time to pregnancy (Dong et al., 2022; Eskenazi et al., 2003; Ng et al., 2004). Clinical fertility data have also shown that there is a decrease in the fertilizing ability of sperm from older men (Bartolacci et al., 2018). When pregnancy is achieved in instances of advanced paternal age, these pregnancies are at risk of higher incidences of stillbirth and preterm births, and low birth weight (Alio et al., 2012). The offspring born to older fathers also have higher incidences of disorders, such as achondroplasia, schizophrenia, and autism and increased risk of some cancers (Kovac et al., 2013; Paul and Robaire, 2013).

A well-established rodent model for studying reproductive aging is the Brown Norway (BN) rat (Wang et al., 1993; Zirkin et al., 1993). This is an inbred rat strain, with a fully-sequenced genome (Gibbs et al., 2004). BN rats are unique as they do not develop many of the pathologies associated with aging, such as obesity and cancers. However, similar to humans, BN rats experience a decrease in serum testosterone despite no change in luteinizing hormone with advancing age (Zirkin et al., 1993). Using this model, various groups have studied the effects of advanced age on spermatogenesis and fertility end-points. Serre et al., (Serre and Robaire, 1998)

established that there was an increase in preimplantation loss and neonatal death, along with an overall decrease in fetal weight. There are also substantiated age related disruptions in spermatogenesis in the BN rat (Wang et al., 1993; Wright et al., 1993).

Spermatogenesis is the continuous process by which spermatozoa are produced in the testes. The spermatogonial stem cells (SSC's) remain throughout the lifespan providing a major source of potential error with advanced age, as they may accumulate de novo mutations (Risch et al., 1987). Round spermatids are the post-meiotic germ cells; they contain half of the genetic material and are the final transcriptionally active germ cell type during spermatogenesis. These cells differentiate as they begin to form the acrosome and undergo chromatin compaction through the process of spermiogenesis. Spermiogenesis is characterized by the gradual replacement of DNA-binding histones with protamines, allowing for a tight toroidal chromatin conformation (O'Donnell, 2014). After spermiogenesis, the spermatozoa transit to the epididymis where they gain functional motility and undergo the final steps of chromatin compaction. The epididymis has been studied in the context of paternal age, with a marked disruption in epididymal gene expression (Jervis and Robaire, 2002). Mature spermatozoa are transcriptionally inert, with a small RNA payload delivered after fertilization.

During spermatogenesis, germ cells are supported by Sertoli cells (SCs) that function as the nurse cells of spermatogenesis. Sertoli cells are situated between the germ cells, connected to one another by tight junctions. The tight junctions function to form the major component of the blood-testis-barrier (BTB) and help create the immune environment of the testis. Sertoli cells provide support by forming crypts to 'hold' the developing germ cells, binding directly to them through adherens junctions. Through these junctions, the Sertoli cells provide important signaling information to the germ cells, predominantly through glycoproteins and cytokines

(Hedger and Meinhardt, 2003; Griswold, 1995). Similarly, the germ cells are able to signal back to the Sertoli cells. As the germ cells develop, Sertoli cells are constantly remodeled to allow for the progressive movement of the cells from the basal compartment of the seminiferous tubules to its adluminal compartment. The remodeling of Sertoli cells is cyclical and signaling information for this cycle is provided to the Sertoli cells by the germ cells (Jegou, 1991; Wright et al., 1989). Sertoli cells have been studied in the context of aging, with overall disruptions in Sertoli-germ cell communication, and metabolic dysfunction observed in aged Sertoli cells (Wright et al., 1993; Nie et al., 2022; Serre et al., 1999; Syed and Hecht, 2001; Syed and Hecht, 2002).

Taking together the expression of numerous genes required to create functional, high-quality sperm, and the genetic bases for offspring disease, understanding gene expression in germ cells is of critical importance. Previously, several studies have attempted to elucidate the genetic bases of age-related reproductive pathologies in testicular germ cells (Paul et al., 2011; Paul et al., 2013; Selvaratnam et al., 2015). Recently, this has been done at the whole testis level where overall transcriptomics show altered gene expression with aging (Han et al., 2021). In the rodent model, there have been few analyses done to understand and assess transcriptomic differences in specific cell types during spermatogenesis. This makes it difficult to determine if effects are in the germ cells or the somatic cells of the testis. As the last transcriptionally active germ cell type, we examined the impact of paternal age on the mRNA transcriptome of round spermatids of BN rats.

3-2. Materials and methods

3-2.1 Animals

Animal care and handling were done in accordance with the guidelines put forth by the Canadian Council on Animal Care (McGill Animal Resources Centre: Protocol 4687). All studies were done using wild-type Brown Norway rats from Charles River laboratories or bred in-house. Animals had access to food and water ad libitum and were kept on a 12-hour light, 12-hour dark cycle, and an environment controlled for temperature (21±3° C) and humidity (30-70%).

Animals were euthanized at 4-6 months for young (average=5.3 months) and aged at 18-20 months (average=19.2 months) with a sample size of n=5. Eighteen to twenty months of age in a rat is the age prior to germ cell loss and testicular atrophy (Paul and Robaire, 2013). These populations represent human populations of approximately 20-30 years of age (young), and 45-60 years (aged). The relative age approximations are based on the timing of testicular failure observed in rats relative to adult males, and the timing of life events such as sexual maturity and general reproductive senescence (Gruenewald et al., 1994; Agoston, 2017). Euthanasia was done by carbon dioxide asphyxiation, followed by collection of the testes. Testes were weighed upon sacrifice to assess testis regression defined as less than 1.5 g per testis. Animals with two regressed testes were not included in this study. Regressed testes were not used, two animals had one regressed testis and only the healthy testis was used for sample collection. The average weight of the testes were 1.76 g and 1.67 g for young and aged, respectively.

3-2.2 Germ Cell Separation

Germ cells were obtained using the STA-PUT method for cell velocity sedimentation (Bryant et al., 2013). Briefly, testes were decapsulated and mechanically separated prior to enzymatic digestion with 0.5 mg mL−1 collagenase (C9722-50MG; Sigma Aldrich, Oakville, Canada), to digest the collagen between seminiferous tubules. Initial digestion was followed by

subsequent digestion with 0.5 mg mL−1 trypsin (Type I, T8003; Sigma-Aldrich) and DNase I (Type I, DN-25; Sigma-Aldrich). Trypsin begins to digest the seminiferous tubules themselves, breaking them into small pieces and releasing the germ cells. DNase is used to aid in the breakdown of any free DNA to prevent cell clumping. Both enzymatic digestions were done at 34◦C for 16 minutes with physical stirring. The dissociated germ cell suspension was then filtered through a 70 μm nylon mesh before being washed three times with 0.5% bovine serum albumin (A4612; Sigma-Aldrich) in RPMI 1640 (Life Technologies, Grand Island, USA) and pelleted at 233 g for 5 min. Cells were filtered once more with a 55 μm mesh to prevent clumping, and 5.5 × 108 mixed germ cells in 25 mL of 0.5% BSA in RPMI were loaded into the STA-PUT (Proscience, Toronto, Canada) and separated on a gradient of 2–4% BSA/RPMI. The gradient was established over 50 min, and the cells were separated by unit gravity sedimentation for 1 h 45 min. Fractions were collected, and the relative purity of germ cell populations was assessed. Round spermatid (RS) fractions with a purity of at least 88% by phase-contrast microscopy identification were pelleted at 73 g for 5 mins, flash frozen, and kept at −80◦ C for RNA-extraction. No larger cells, such as Sertoli cells or spermatogonia, were ever identified in this cell fraction.

3-2.3 RNA-Sequencing

Total RNA was extracted from 1.5 x 10^7- 3.0 x 10^7 round spermatids from each of the 10 independent samples collected. RNA extraction was done using the Qiagen RNeasy Mini kit following manufacturer guidelines (74104; Qiagen, Mississauga, Canada). RNA library preparation and sequencing were done by Novogene Corporation Inc. using a minimum of 50 μL of 40-45 ng/μL total RNA (Sacramento, California, USA). Sequencing was done using their

NovaSeq 6000 platform (Illumina, San Diego, California) for paired-end sequencing at a read depth of 150 base pairs (bp).

3-2.4 Data Analysis

Preliminary data analysis was done by Novogene Corporation Inc. Beginning with quality control, raw data are processed to remove low quality reads, and those which contained adaptors, poly-N. The clean reads were next mapped to the BN rat reference genome Rnor_6.0 (HISAT2 v 2.0.5). Clean reads were counted for each mapped gene, and the Fragments Per Kilobase of transcript per Million reads mapped (FPKM) was calculated (featureCounts v 1.5.0-p3). The FPKM provides an estimate of gene expression level.

Differential gene expression (calculated using DESeq2 1.20. 0; Love et al. 2014); was taken as a log2 fold change in gene expression that is equal to or greater than 1.0, with an adjusted p-value equal to or less than 0.05 when corrected for multiple comparisons using the Benjamini and Hochberg approach. To determine gene family patterns, we conducted Gene Ontology (GO) enrichment analysis of differentially expressed genes (DEG's) (clusterProfiler). Ingenuity Pathway Analysis (IPA) software (Qiagen, Valencia, California, USA) was used to examine pathways, and transcription factor families observed with differentially expressed genes. GeneSpring (Agilent, Santa Clara, CA, USA) software was used to generate 3D Principle Component Analysis (PCA) plot. These data have been uploaded to Gene Expression Omnibus; the GEO identification number is: GSE219040.

3-3. Results

3-3.1 Age dependent differential gene expression in Round Spermatids

We found abundant gene expression dysregulation in round spermatids. RS from young and aged clustered independently when principle component analysis (PCA) was applied, mainly separating on PC1 accounting for 27.4% of the variability (Fig. 3-1A). When examining differential gene expression, of the differentially expressed (log2FC > 1) and non-significant genes (Fig. 3-1B), there were 344 transcripts expressed exclusively in the young, 592 expressed exclusively in the aged, and 12,493 that were expressed in both age groups. After applying the Benjamini-Hochberg p-value adjustment for multiple comparisons (padj < 0.05), we assessed the transcripts that met both the log2FC cut off of 1, and met significance. Overall, there were 220 transcripts that were differentially expressed with aging; 211 upregulated transcripts, and 9 downregulated transcripts (Fig. 3-2A). There were 3 transcripts expressed only in the young, 82 to the aged, and 136 that were expressed in both (Fig. 3-1C, Supp. Table 3-1).

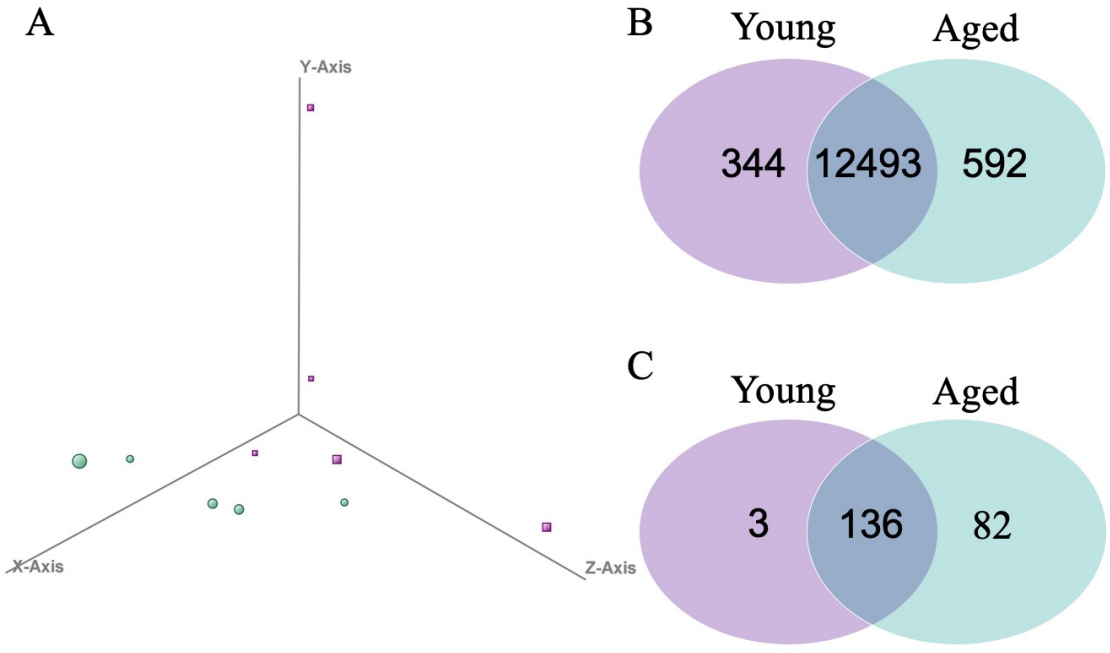


Figure 3-1: Round spermatid RNA-sequencing sample clustering and differential expression. A) Principle component analysis of all samples. Young RS: purple squares, Aged RS: blue

circles. X-axis:PC1=23%, Y-axis:PC2=17%, Z-axis:PC3=13%. B) Genes expressed in young or aged RS. C) Differentially expressed genes in young or aged RS.

When examining transcripts with a log2FC > 3.5, 18 transcripts met this cut-off. Of these, nine have specific relevance to male reproductive aging (Fig. 3-2B), with seven increasing in expression and two decreasing. '*LOC100363064*' and *Clec4a3* are mannose-binding genes, potentially implicated in sperm-egg fusion during fertilization. *Lrrn4* encodes for a gene that aids in development in the early embryo. *Folr2* and *Folr1*, coding for folate receptors 1 and 2, are both implicated in DNA methylation, as part of the folate metabolism pathway. *Gnrhr* has been shown to have function in spermatogenesis progression, germ cell apoptosis, sperm release and potentially fertilization. There was a down-regulation of both *'LOC100911154'* and *AC117925.6*. These are genes that have been associated with motile sperm or ciliated cell movement respectively, lending to a potential alteration in sperm motility.

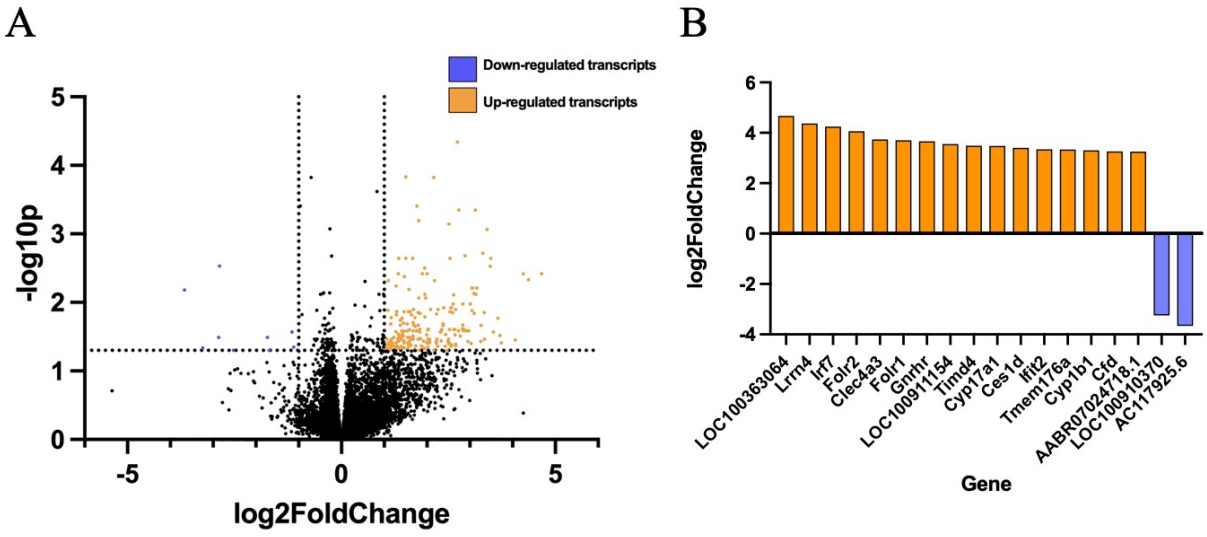


Figure 3-2: Differentially expressed genes in round spermatids. A) Volcano plot showing differential expression with a log2FC >|1|, and padj <0.05. Orange: up-regulated transcripts, Purple: down-regulated transcripts. B) Transcripts with log2FC >|3.5|.

3.3.2 Age dependent alterations in IGF-1 and senescence signalling pathways

Pathway analysis with IPA revealed significantly altered pathways, transcript families with shared molecular functions, and potential upstream regulators. There were 66 canonical pathways (Supp. Fig. 3-1), with significant alteration due to aging in RS. The canonical pathways with significant alterations ($p < 0.01$) but no predicted directionality ($Z = 0$) were: insulin-like growth factor 1 (IGF-1) signaling ($p=2.54x10^{-4}$) and calcium signaling ($p=4.82x10^{-3}$). The pathways with significant alterations but no predicted directionality had too few molecules to predict activation or inhibition of the pathway, this is likely a product of the relatively small number of altered transcripts observed in our study.The pathways with predicted activation were hypercytokinemia and hyperchemokinemia in pathogenesis (Z=2.24), NAD signaling (Z=1.89), cellular senescence (Z=1.15), and the coagulation system (Z= 1) (Fig. 3-3A). The cellular senescence pathway included the significantly altered transcripts *Araf*, *Capn1, Cebpb, Gadd45g* (Supp. Table 3-2). Seven non-significant DEGs also contribute to the overall alteration in the senescence pathway. In our data, pathways for which IPA could not predict pathway activation (Z=NA) include: caveolar-mediated endocytosis signaling, and agranulocyte adhesion and diapedesis.

The pathways with predicted activation with a Z-score > 2 ($p <0.05$) were: toll-like receptor signaling, hypercytokinemia/hyperchemokinemia in pathogenesis, acute phase response signaling, SNARE signaling, white adipose tissue browning, neuroinflammation signaling, pulmonary healing signaling, cardiac hypertrophy signaling, adrenomedullin signaling, ID1 signaling, and hepatic fibrosis signaling (Fig. 3-3B).

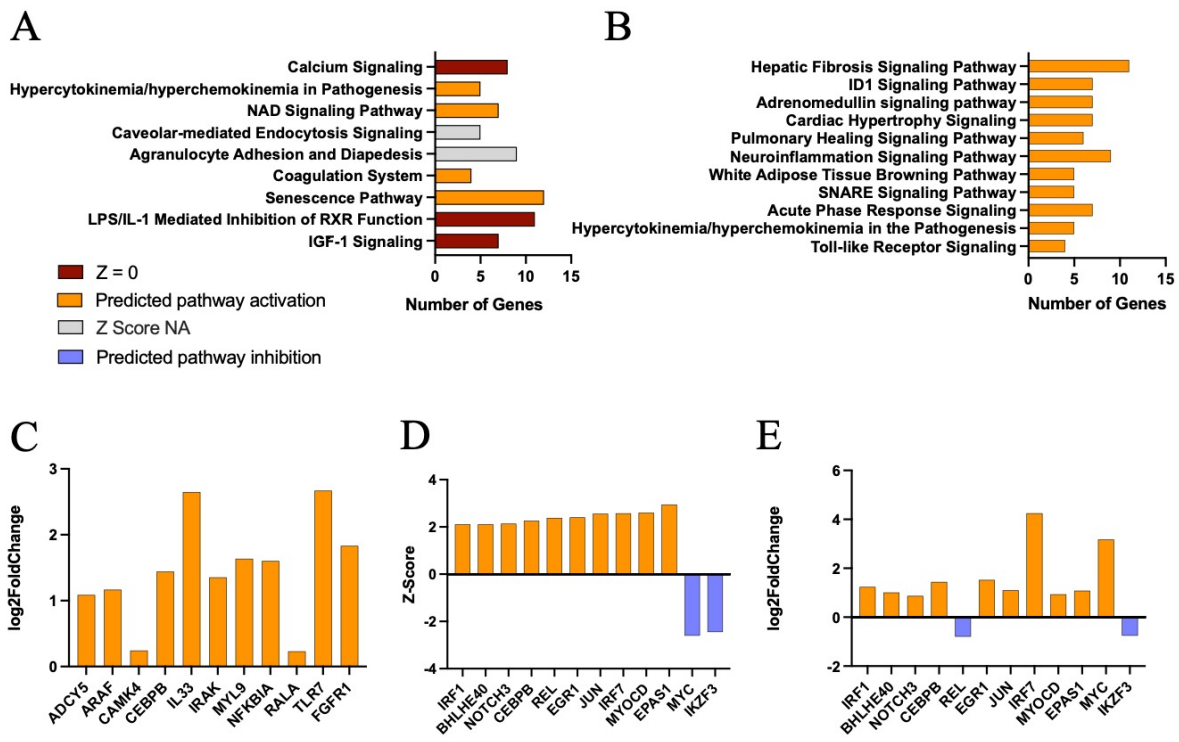


Figure 3-3: Ingenuity pathway analysis of DEGs in round spermatids. A) Pathways with predicted alteration (Z-score), with -log10(padj) > 2.3. Red: Z=0, Orange: Predicted pathway activation, Grey: Z-score cannot be calculated, Purple: Predicted inhibition. B) Pathways with predicted alteration with a Z-score > |2|. C) Transcripts which appear in these pathways more than 4 times. D) Transcription factors with predicted activation (orange) or inhibition (purple). E) Gene expression of transcription factors in log2FC, increased expression (orange) and decreased expression (purple).

When examining canonical pathways for their contributing transcripts there is overlap, with some transcripts contributing to various separate pathways. The following transcripts were

present in four or more of the presented canonical pathways: *Adcy5, Araf, Camk4, Cebpb, Il33, Irak, Nfkbia, Rala, Tlr7, Fgfr1* (Fig. 3-3C). These genes represent pathways with overlapping function in the immune system, through immune response and inflammation.

When examining upstream transcription regulators with a Z-score > 2 (Fig. 3-3D), p-value of overlap < 0.05, and differential expression log2FC > 0.8 (Fig. 3-3E), there are 12 transcripts that have downstream effects. *Myc*, with a log2FC of 3.178 and a predicted transcriptional activation of downstream targets, has been implicated in spermatogenesis and germ cell development. IRF1, BHLHE40, CEBPB, IRF7, MYC, and IKZF3 are transcription factors for many cytokines, and inflammation/immune response genes. Many of these transcription factors also have direct roles in Sertoli cell signaling and Sertoli gene activation.

When we examined the differentially expressed transcripts with GO analysis, two pathways with abundant representation were the oxidoreductase activity and process pathways, fitting with what is well-established with male reproductive aging (Fig. 3-4). There were many transcripts that sorted to immune, cytokine, and chemokine signaling, further supporting the findings of IPA. Interestingly, these pathways represent potential disruptions in communication between cells within the testes, specifically Sertoli and germ cells. Further supporting the notion that there are disruptions in the germ cell niche, there were also transcripts involved in the cytoskeleton, membrane organization, and extracellular regions. These pathways are necessary to allow for both the binding of germ cells through various junctions, and the remodelling of cell types as they progress through spermatogenesis.

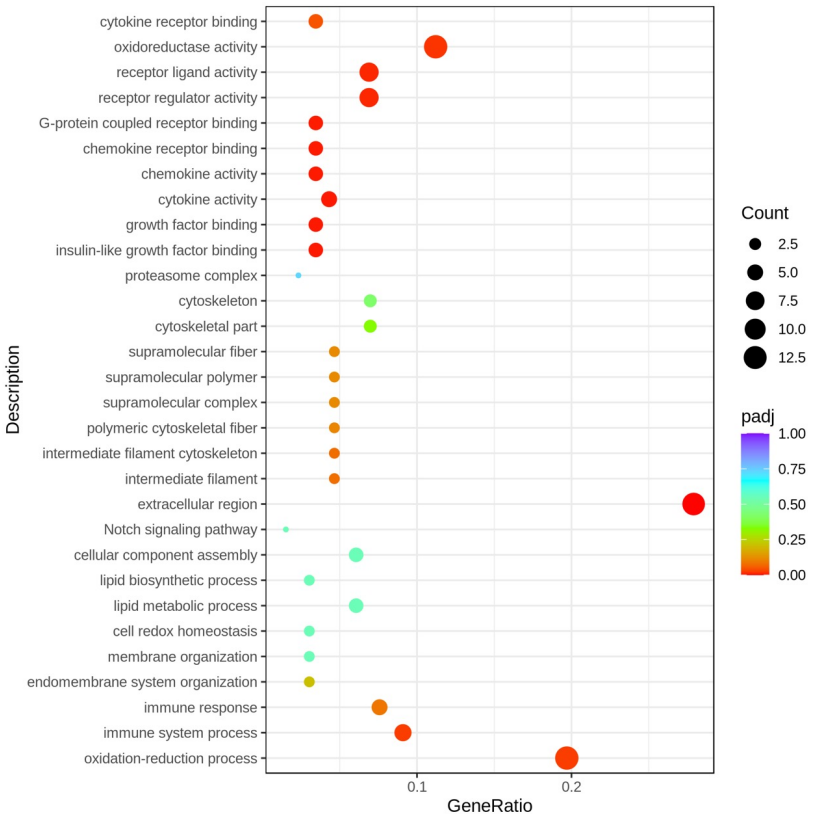


Figure 3-4: Gene ontology analysis of DEG's in round spermatids. Size of dot: number of genes contributing to the pathway. Significant pathways shown in red/orange, insignificant pathways shown in green-blue. Gene ratio: ratio of genes in pathway to total DEGs.

Taken together, these results suggest age dependent disruptions in cellular inflammation and immune responses. The canonical pathways with a p-value < 0.01 (Fig. 3-3A), and Z-score > 2 (Fig. 3-3B), represent a dysregulation in immune and inflammation pathways, and the upstream regulatory elements identified through IPA represent those which control downstream elements of the inflammation and immune systems. Given the biases of GO, and IPA towards

pathways that have been most studied, we independently examined the functions of affected transcript within the reproductive system. Specifically, we examined the action and function of dysregulated transcripts in round spermatid and spermatozoa development and function.

3-3.3 Differential gene expression with aging in reproduction specific pathways

The 220 differentially expressed genes were researched and sorted for their reproductive function based on the existing literature in July 2022 (Supp. Table 3-1). There were 169 transcripts that were studied in the context of reproduction, with a known function related to: reactive oxygen species regulation, DNA damage response, sperm motility, sperm-egg binding/fertilization, the cytoskeleton, epigenetics, Sertoli-germ cell interactions, sperm capacitation, metabolism, lipid regulation, transcription, apoptosis, steroidogenesis, cell cycle, ion binding or miscellaneous (Fig. 3-5A). Approximately 21% of these transcripts showed overlap, having more than one function within the reproductive system, the overlap is often seen with groups that involve motility and morphology. Concordant with what is known about reproductive aging, there were many transcripts involved in ROS, DNA damage, and sperm-egg binding/fertilization. Given that both the cytoskeleton, and immune signaling pathways observed with pathway analysis, it was interesting that the two largest reproductive groups were the Sertoli-germ cell interaction, and Sertoli-immune signalling, with 25, and 29 and transcripts contributing to these groups, respectively.

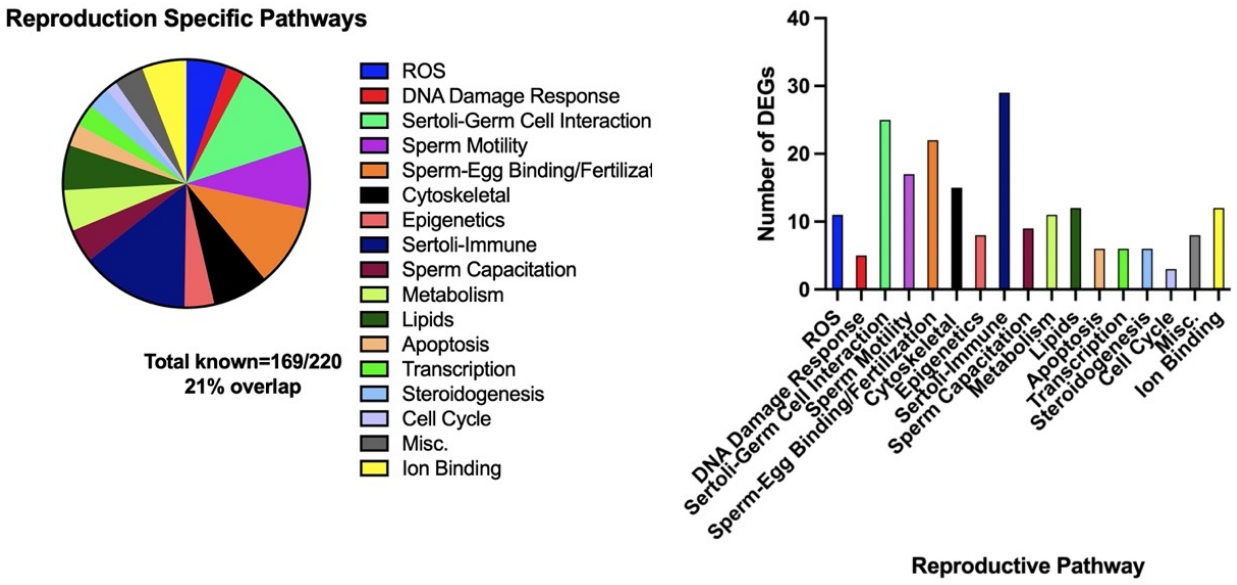


Figure 3-5: Age-related differential gene expression in reproductive specific pathways.

3-3.4 Age dependent increases in gene expression

We observed an increase in gene expression in 96% (211 of 220) of the age dependent dysregulated transcripts in RS ($log2FC > 1$, $padj < 0.05$).

3-3.4.1 Sperm function related transcripts

The most prominent reproductive pathways were those contributing to overall sperm structure and function. These pathways are: sperm motility, sperm-egg binding/fertilization, and sperm capacitation (calcium regulation). Seventeen transcripts were associated with sperm motility, including: *Aqp1, Vim,* and *Septin* (Fig. 3-6A). Twenty-two transcripts have been studied for their effects in sperm-egg binding or overall fertilization, including: *Serpinb9*, *Gas6*, and

LOC10036306. Of the nine transcripts that were formerly shown to contribute to capacitation or the acrosome reaction, *Rhob* and *Timd4* were best characterized. There are additional important functions in sperm for transcripts belonging to the pathways ROS (11 transcripts), cytoskeletal (15 transcripts), ion binding (12 transcripts), metabolism (11 transcripts), and lipids (12 transcripts).

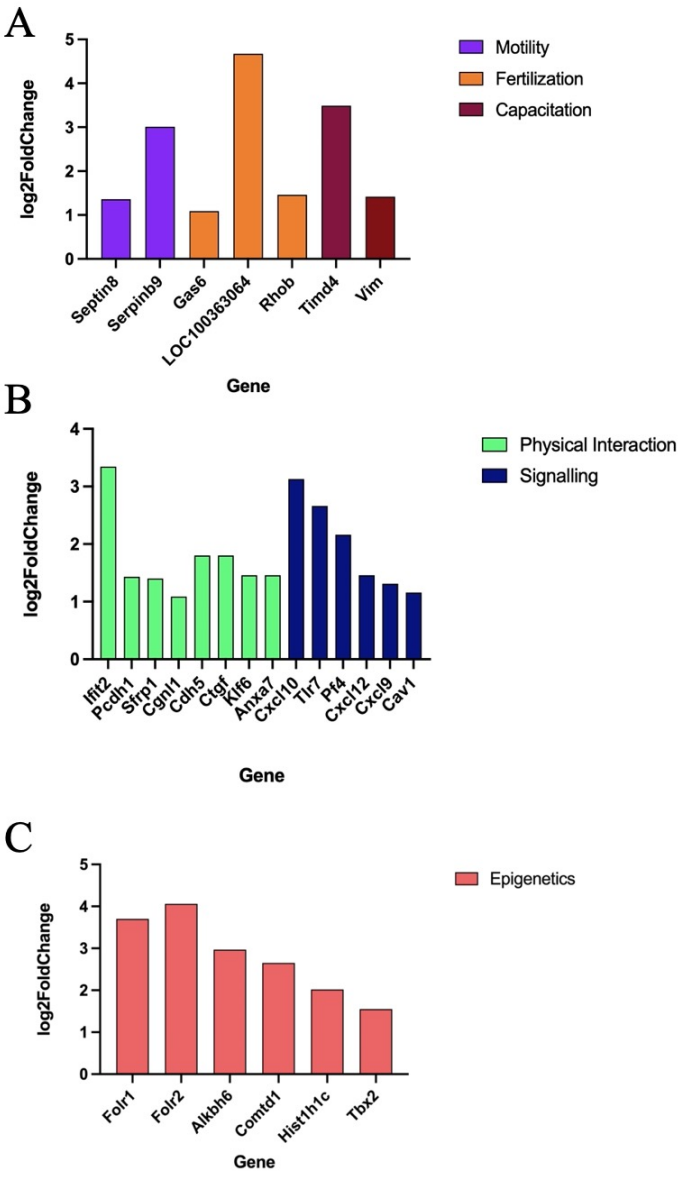


Figure 3-6: Differential gene expression of round spermatids in reproductive pathways. X-axis:

Gene name, Y-axis: log2FC. All transcripts are significant at padj<0.05. A) Sperm motility, morphology and fertilizing ability. B) Sertoli-Germ Cell Interaction. C) Epigenetics.

3-3.4.2 Sertoli-Germ cell interaction related transcripts

Transcripts for the interaction between Sertoli and germ-cells represent a total of 54 of the 220 dysregulated transcripts, and can be divided into genes that directly facilitate the binding of the two cell types (25 transcripts), or genes that are likely involved in the signaling and immune response (29 transcripts). Dysregulated genes responsible for the binding of Sertoli cells and round spermatids includes genes that bind actin, cadherin, and help form adherens junctions, such as: *Ifit2, Pcdh1, Cdh5, Sfrp1, Cgn11, Klf6, Anxa7,* and *Ctgf* (Fig 3-6B). The transcripts involved in the pathway regulating the Sertoli cell immune response, were mainly cytokines and elements that interact with SC signaling: *Cxcl10, Cxcl12, Cxcl9, Pf4, Tlr7*, and *Cav1*. The dysregulated interferons (*Irf7, Ifit2, Slfn2, Ifi47, Ifitm2*) in RS due to aging also point to alterations in SC-RS signaling.

3-3.4.3 Epigenetic related transcripts

Due to the overall disruptions in gene expression observed with aging, we examined whether there were effects of epigenetic related transcripts as a function of aging of round spermatids. We found eight such transcripts, including: *Folr1, Folr2, Alkbh6, Comtd1, Hist1h1c,* and *Tbx2* (Fig. 3-6C). These transcripts represent multiple epigenetic mechanisms through histone modifications, telomere maintenance, and DNA methylation. Hist1h1c, is a linker histone which may regulate chromatin compaction as it interacts with linker DNA between nucleosomes. Hist1h1c also has known function in binding with telomeric repeat elements and

elongation factors, a newly proposed epigenetic regulator. Folr1 and Folr2 are two folate receptors that regulate intracellular folate, a rate-limiting step in the folate and DNA methylation cycles. Comtd1, is a catechol-O-methyltransferase, with predicted functionality in S-adenosylmethionine-dependent methyltransferase activity. Alkbh6 has protective effects on DNA methylation. Alkbh6 is an enzyme with dioxygenase activity, and is able to recognize and reverse DNA methylation damage.

3.5 Age dependent decreases in gene expression

Interestingly, only 9 measured DEGs had a decrease in gene expression with four of these down-regulated transcripts having no known function. Two of these transcripts represent non-coding RNAs, a microRNA (*Mir3074*) potentially involved in DNA damage response, and a long intergenic non-coding RNA (*AABR07028258.1)* with no predicted function. Two transcripts have predicted or known zinc regulatory function: *RGD1566138,* and *Zfp14.* The remaining five transcripts showed no pattern in pathway activity including: *Atp13a3*, *Fam181a, AC117925.6, LOC100910370,* and *AABR07002741.1*. Though together these transcripts do not represent a major reproductive or canonical pathway, as they have predicted functions in DNA damage response, zinc binding, transcription, and ciliated cell movement, it is not unreasonable to predict they may also have specific functions in RS with aging.

4. Discussion

By examining genomics at the round spermatid level, we observed overall changes in gene expression due to aging in round spermatids of the Brown Norway rat. There is an overall increase in gene expression, suggesting that many genes are turned on as a result of aging in RS.

There exist no known age-related gene expression programmes that have been conserved across evolution or across tissues within an organism. However, hallmarks of aging give us insight into trends for the behaviour of certain classes of genes. The hallmarks of aging include: downregulation of mitochondrial proteins, dysregulation of the immune system genes, response to DNA damage and stress, reduction in growth factors, and dysregulation of overall gene expression and mRNA processing (Frenk and Houseley, 2018). Our data fit within these hallmarks of aging, showing a disruption in genes related to: the immune system, DNA damage and stress response, and overall gene expression or mRNA processing. As previously noted, one of the theories of aging is that there is general genome instability which could be characterized by aberrant gene expression observed in RS. Given the tissue specificity of age related disruptions in gene expression, it remains difficult to predict transcripts that would be targeted by the aging process in RS outside of our own data.

We are able to appreciate the dysregulation of spermatogenesis and fertility related genes within the context of reproductive aging pathologies. There are decreases in sperm motility, capacitation, and normal morphology with aging that are concordant with disruptions in transcripts necessary to make functional sperm (Dong et al., 2022; Eskenazi et al., 2003; Ng et al., 2004). Of note, vimentin (*Vim*) has been shown to be present on the sperm head in an asymmetric manner with higher levels of vimentin being present in abnormal spermatozoa (Markova et al., 2002). It is unclear if the asymmetry is a result of structural abnormalities, or if it causes them, but here we do see an increase in vimentin expression. Vimentin has also been shown to be highly detectable in the developing spermatid flagella (Mali et al., 1987). Septin8, is a transcript with potential involvement in sperm motility through interaction with septin family

bundles (Lin et al., 2011); high expression of this transcript has been observed in normozoospermic patients who did not achieve pregnancy (Azpiazu et al., 2014).

We found dysregulation of expression of transcripts that are required for successful sperm-egg interactions leading to fertilization, another pathology noted with advanced paternal age (Bartolacci et al., 2018). Particularly, transcripts within our dataset were involved in mannose binding and interactions. Mannose binding sites on the sperm membrane initiate the acrosome reaction and allow for sperm fertilizing ability (Rosano et al., 2007). Mannose binding has been also studied with binding partners such as cholesterol (Benoff et al., 1993), which is of particular interest given that we saw a disruption in lipid synthesis or homeostasis within our data. Additional genes with differential expression potentially contribute to capacitation and the acrosome reaction, including *Rhob, Timd4,* and many calcium ion channels. *Gas6* has been studied in the context of fertilization, and it has been reported that in oocytes lacking *Gas6* the sperm pronucleus is unable to form (Kim et al., 2019). It remains to be determined whether sperm functionally contribute *Gas6* to the oocyte at the time of fertilization.

Beyond the role Sertoli cells play in providing support to the developing germ cells, they also establish and maintain the unique immune system within the testis. The testis is immune privileged, and must maintain a balance between protecting the body from infection through the reproductive tract while simultaneously protecting the germ cells from the systemic immune system (Zhao et al., 2014). The immune privilege is maintained by the blood-testis barrier, formed by Sertoli cells. The Sertoli cells form tight-junctions, and with cyclical remodelling allow for the transit of developing germ cells from within the protection of the systemic immune environment, into the external compartment of the testis. This requires many physical transformations of both the Sertoli cells and germ cells to allow the progression through the

epithelium (Vogl et al., 2013). Thus, transcripts involved in the direct binding of germ cells to Sertoli cells through junctions as well as the immune interaction are of importance. Transcripts directly involved in remodelling of the germ cell cytoskeleton or progression through the epithelium, such as cadherins (*Pcdh1, Cdh5)*, *Sfrp1* and *Ctgf* were disrupted within our data. Additionally, we saw disruptions in transcripts which help form the junctions between germ cells and Sertoli-cells, such as *Cgn11* and *Klf6* (Mazaud-Guittot et al., 2010; Wang et al., 2019).

We observe disruptions in many transcripts that either signal to Sertoli cells, or are otherwise involved in germ cell-Sertoli cell interaction. Given the necessary and extensive supporting roles provided by Sertoli cells, it is of particular interest to note that the relationship between these cell types begins to deteriorate with aging. Studies have reported the suspected loss of Sertoli cell support to developing germ cells (Levy et al., 1999; Syed and Hecht, 2001; Syed and Hecht, 2002). Levy et al., (1999) reported that there were empty crypts, classically occupied by round spermatids, while Syed and Hecht (2001;2002) reported that there were age related decreases in mRNAs that signal between Sertoli and germ cells, with a focus on the proteins coming from Sertoli cells. However, there is much less evidence that developing germ cells are able to provide signals to the Sertoli cells. Haugen et al., (1994) showed the presence of IL-1 alpha in rat germ cells, and De et al., (1993) showed the presence of TNF-alpha in mouse germ cells. These are known signalling proteins that direct Sertoli cell action. Our data show that there are increases in many factors that participate in the signalling cascade between germ and Sertoli cells, including; *Cxcl10, Cxcl12, Cxcl9, Pf4, Tlr7*, and *Cav1 Irf7, Ifit2, Slfn2, Ifi47, Ifitm2*. Additional transcripts in our dataset suggest altered signalling to Sertoli cells, including *Notch3*. Notch3 expression has been observed in germ cells, with the main effectors being in Sertoli cells

through activating expression of *Hes1* and *Hes5* (Murta et al., 2013). We can hypothesize from these data that the developing spermatids are signalling to the Sertoli cells.

As we mentioned, there are additional transcripts of note that appear to be multifunctional within round spermatids. Vimentin, mentioned earlier in the context of morphology and motility, is also a protein involved in junctions between Sertoli and germ cells, with a prediction that the vimentin expression in germ cells may have a role in embedding them within the deep pockets of Sertoli cells (Mali et al., 1987). A disruption in vimentin then, would alter not only the morphology of sperm but the potential for spermatids to sit in Sertoli-cell crypts as seen by Levy et al. (1999). Additionally, *Gas6*, noted for its necessity in sperm pronuclear formation in the oocyte, may also have function in regulating phagocytosis of germ cells by Sertoli cells through their binding (Xiong et al., 2008). Though beyond the scope of the present study, exploring the interaction between Sertoli cells and germ cells during aging is evidently important. A next step will be confirming if these transcripts specifically direct action in Sertoli cells within the context of aging.

Age related alterations in DNA methylation and the epigenome are vast, with species dependent effects. In humans, there is an overall increase in global DNA methylation observed with age (Cao et al., 2020). Cao et al., showed a clear correlation between age and increased DNA methylation; indeed, this correlation is so strong that it can be used to predict the age of the individual within 2 years. In rodents, thousands of differentially methylated regions due to aging have also been identified (Pilsner et al., 2021). We observed increases in transcripts: *Folr1, Folr2,* and *Comtd1,* all key transcripts in the folate and DNA methylation cycle. If there are disruptions in folate metabolism, the availability of methyl groups may alter overall DNA methylation. Altered folate metabolism has been noted with age in other contexts (Amenyah et

al., 2020). It will be interesting in the future to explore if these changes in gene expression are correlated with altered DNA methylation.

Our results show that with aging there is dysregulated expression of genes involved in making functional sperm. This is the first study showing alterations in germ cell transcripts involved in binding and signaling to Sertoli cells, providing an additional avenue for age related defects in sperm production and function.

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Supplemental Material

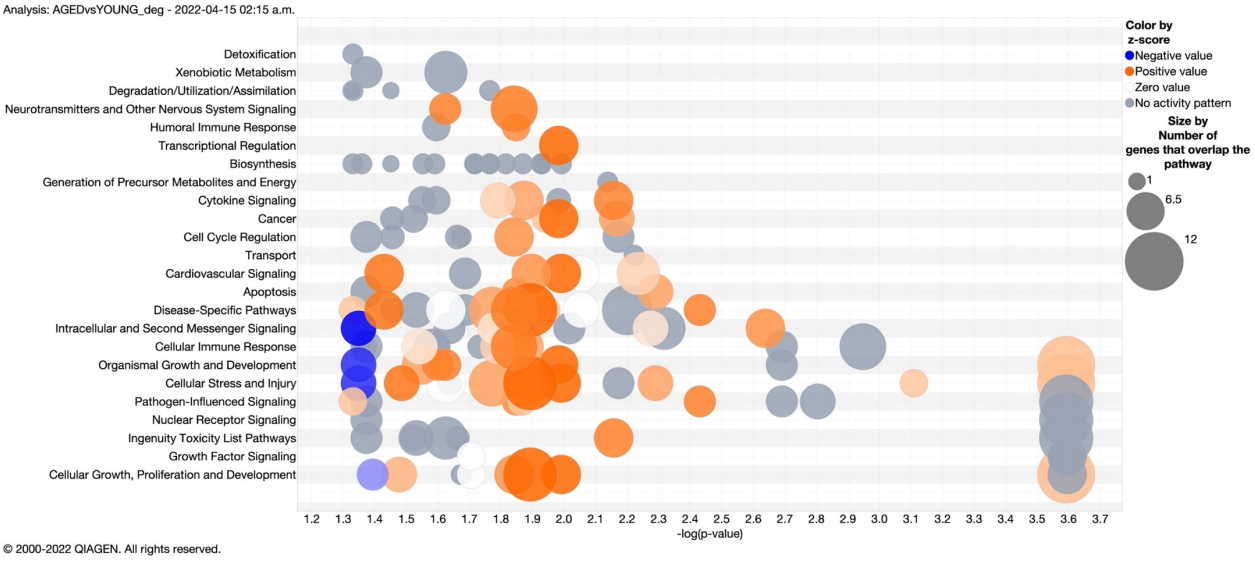

Figure S3-1: Ingenuity Canonical Pathways. Dot color represents the z-score and predicted action of the pathway. Blue: negative value and predicted inhibition. Orange: positive value and predicted activation. White: no value, not able to predict action. Grey: no activity pattern given the nature of the pathway. Dot size represents the number of contributing genes in the dataset to that canonical pathway.

Table S3-1: Differentially Expressed Gene List.

Ensembl Gene ID	Gene Name	log2FC	padj	Sorting Reference
ENSRNOG00000001027	LOC100363064	4.674262	0.003809	Rosano, G., Caille, A. M., Gallardo-Ríos, M., & Munuce, M. J. (2007). D-Mannose-binding sites are putative sperm determinants of human oocyte recognition and fertilization. *Reproductive biomedicine online*, *15*(2), 182–190.
ENSRNOG00000032989	Lrrn4	4.36877	0.004674	Lee, E. C., & Hu, V. W. (2020). Phenotypic Subtyping and Re-Analysis of Existing Methylation Data from Autistic Probands in Simplex Families Reveal ASD Subtype-Associated Differentially Methylated Genes and Biological Functions. *International journal of molecular sciences*, *21*(18), 6877. https://doi.org/10.3390/ijms21186877
ENSRNOG00000017414	Irf7	4.249671	0.003809	Nodari, A., Scambi, I., Peroni, D., Calabria, E., Benati, D., Mannucci, S., Manfredi, M., Frontini, A., Visonà, S., Bozzato, A., Sbarbati, A., Schena, F., Marengo, E., Krampera, M., & Galiè, M. (2021). Interferon regulatory factor 7 impairs cellular metabolism in aging adipose-derived stromal cells. *Journal of cell science*, *134*(11), jcs256230. https://doi.org/10.1242/jcs.256230
ENSRNOG00000019890	Folr2	4.062292	0.035167	1) Holm, J., & Hansen, S. I. (2020). Characterization of soluble folate receptors (folate binding proteins) in humans. Biological roles and clinical potentials in infection and malignancy. *Biochimica et biophysica acta. Proteins and proteomics*, *1868*(10), 140466. https://doi.org/10.1016/j.bbapap.2020.140466 2) Greene, N. D., Stanier, P., & Moore, G. E. (2011). The emerging role of epigenetic mechanisms in the etiology of neural tube defects. *Epigenetics*, *6*(7), 875–883. https://doi.org/10.4161/epi.6.7.16400
ENSRNOG00000010018	Clec4a3	3.736793	0.039023	1) Oatley, J. M., Oatley, M. J., Avarbock, M. R., Tobias, J. W., & Brinster, R. L. (2009). Colony stimulating factor 1 is an extrinsic stimulator of mouse spermatogonial stem cell self-renewal. *Development (Cambridge, England)*, *136*(7), 1191–1199. https://doi.org/10.1242/dev.032243 2) https://www.uniprot.org/uniprotkb/Q5YIS0/entry2=
ENSRNOG00000019902	Folr1	3.701829	0.030217	1) Holm, J., & Hansen, S. I. (2020). Characterization of soluble folate receptors (folate binding proteins) in humans. Biological roles and clinical potentials in infection and malignancy. *Biochimica et biophysica acta. Proteins and proteomics*, *1868*(10), 140466. https://doi.org/10.1016/j.bbapap.2020.140466 2) Greene, N. D., Stanier, P., & Moore, G. E. (2011). The emerging role of epigenetic mechanisms in the etiology of neural tube defects. *Epigenetics*, *6*(7), 875–883. https://doi.org/10.4161/epi.6.7.16400
ENSRNOG00000002011	Gnrhr	3.658623	0.017004	Wang, G., Hao, L., Cheng, Y., Li, S., Zhang, Y., Lv, C., Wei, W., Huang, S., Shi, H., Dong, L., Zhang, Y., Yu, H., & Zhang, J. (2017). Effects of GnRHR polymorphisms on sperm quality in Chinese water buffalo. *Animal reproduction science*, *186*, 37–43. https://doi.org/10.1016/j.anireprosci.2017.09.001
ENSRNOG00000042884	LOC100911154	3.552005	0.026998	
ENSRNOG00000038894	Timd4	3.488809	0.002266	Rival, C.M., Xu, W., Shankman, L.S. *et al.* Phosphatidylserine on viable sperm and phagocytic machinery in oocytes regulate mammalian fertilization. *Nat Commun* **10**, 4456 (2019). Xu, L. Y., Qi, J. N., Liu, X., Ma, H. X., Yuan, W., Zhao, P. Q., Liang, X. H., Xu, Y., Wang, H. X., Xu,

				X. Y., Wang, W., Ma, C. H., & Gao, L. F. (2015). Tim-4 Inhibits NO Generation by Murine Macrophages. *PloS one*, *10*(4), e0124771.
ENSRNOG00000020035	Cyp17a1	3.479593	0.002968	Yadav, R., Petrunak, E. M., Estrada, D. F., & Scott, E. E. (2017). Structural insights into the function of steroidogenic cytochrome P450 17A1. *Molecular and cellular endocrinology*, *441*, 68–75.
ENSRNOG00000015519	Ces1d	3.4016	0.00086	Legoff, L., D'Cruz, S. C., Lebosq, M., Gely-Pernot, A., Bouchekhchoukha, K., Monfort, C., Kernanec, P. Y., Tevosian, S., Multigner, L., & Smagulova, F. (2021). Developmental exposure to chlordecone induces transgenerational effects in somatic prostate tissue which are associated with epigenetic histone trimethylation changes. *Environment international*, *152*, 106472.
ENSRNOG00000036604	Ifit2	3.344882	0.034232	Satie, A. P., Mazaud-Guittot, S., Seif, I., Mahé, D., He, Z., Jouve, G., Jégou, B., & Dejucq-Rainsford, N. (2011). Excess type I interferon signaling in the mouse seminiferous tubules leads to germ cell loss and sterility. *The Journal of biological chemistry*, *286*(26), 23280–23295.
ENSRNOG00000023708	Tmem176a	3.33908	0.014095	1) Lewandowski, J. P., Dumbović, G., Watson, A. R., Hwang, T., Jacobs-Palmer, E., Chang, N., Much, C., Turner, K. M., Kirby, C., Rubinstein, N. D., Groff, A. F., Liapis, S. C., Gerhardinger, C., Bester, A., Pandolfi, P. P., Clohessy, J. G., Hoekstra, H. E., Sauvageau, M., & Rinn, J. L. (2020). The Tug1 lncRNA locus is essential for male fertility. Genome biology, 21(1), 237. 2) Yu Y, Fuscoe JC, Zhao C, et al. A rat RNA-Seq transcriptomic BodyMap across 11 organs and 4 developmental stages. Nature Communications. 2014 ;5:3230.
ENSRNOG00000040287	Cyp1b1	3.30258	0.001907	Nixon, B. J., Katen, A. L., Stanger, S. J., Schjenken, J. E., Nixon, B., & Roman, S. D. (2014). Mouse spermatocytes express CYP2E1 and respond to acrylamide exposure. *PloS one*, *9*(5), e94904.
ENSRNOG00000033564	Cfd	3.26202	0.021622	Sinnar, S. A., Small, C. L., Evanoff, R. M., Reinholdt, L. G., Griswold, M. D., Kopito, R. R., & Ryu, K. Y. (2011). Altered testicular gene expression patterns in mice lacking the polyubiquitin gene Ubb. *Molecular reproduction and development*, *78*(6), 415–425.
ENSRNOG00000033014	AABR07024718.1	3.257112	0.037074	
ENSRNOG00000018757	Insl3	3.165382	0.006158	https://www.uniprot.org/uniprotkb/Q9WUK0/entry
ENSRNOG00000012879	Fabp3	3.143647	0.0076	1) Oko, R., & Morales, C. R. (1994). A novel testicular protein, with sequence similarities to a family of lipid binding proteins, is a major component of the rat sperm perinuclear theca. *Developmental biology*, *166*(1), 235–245. 2) Smathers, R.L., Petersen, D.R. The human fatty acid-binding protein family: Evolutionary divergences and functions. *Hum Genomics* **5**, 170 (2011).
ENSRNOG00000022256	Cxcl10	3.126923	0.000449	1) Jiang, Q., Wang, F., Shi, L., Zhao, X., Gong, M., Liu, W., Song, C., Li, Q., Chen, Y., Wu, H., & Han, D. (2017). C-X-C motif chemokine ligand 10 produced by mouse Sertoli cells in response to mumps virus infection induces male germ cell apoptosis. *Cell death & disease*, *8*(10), e3146. 2) Nagpal, M. L., Chen, Y., & Lin, T. (2004). Effects of overexpression of CXCL10 (cytokine-responsive gene-2) on MA-10 mouse Leydig tumor cell steroidogenesis and proliferation. *The Journal of endocrinology*, *183*(3), 585–594.

ENSRNOG00000012749	C1qb	3.101721	0.014489	Galvan, M. D., Foreman, D. B., Zeng, E., Tan, J. C., & Bohlson, S. S. (2012). Complement component C1q regulates macrophage expression of Mer tyrosine kinase to promote clearance of apoptotic cells. *Journal of immunology (Baltimore, Md. : 1950)*, *188*(8), 3716–3723.
ENSRNOG00000008465	Tmem176b	3.094428	0.007424	Kang, C., Rostoker, R., Ben-Shumel, S., Rashed, R., Duty, J. A., Demircioglu, D., Antoniou, I. M., Isakov, L., Shen-Orr, Z., Bravo-Cordero, J. J., Kase, N., Cuajungco, M. P., Moran, T. M., LeRoith, D., & Gallagher, E. J. (2021). TMEM176B Regulates AKT/mTOR Signaling and Tumor Growth in Triple-Negative Breast Cancer. *Cells*, *10*(12), 3430.
ENSRNOG00000017123	B2m	3.06615	0.006158	Güssow, D., Rein, R., Ginjaar, I., Hochstenbach, F., Seemann, G., Kottman, A., & Ploegh, H. L. (1987). The human beta 2-microglobulin gene. Primary structure and definition of the transcriptional unit. *Journal of immunology*
ENSRNOG00000013484	Gsta1	3.050983	0.013179	Schlecht, U., Demougin, P., Koch, R., Hermida, L., Wiederkehr, C., Descombes, P., Pineau, C., Jégou, B., & Primig, M. (2004). Expression profiling of mammalian male meiosis and gametogenesis identifies novel candidate genes for roles in the regulation of fertility. *Molecular biology of the cell*, *15*(3), 1031–1043.
ENSRNOG00000012436	Adh6	3.050466	0.006171	Han, L., Wang, J., Zhao, T., Wu, Y., Wei, Y., Chen, J., Kang, L., Shen, L., Long, C., Yang, Z., Wu, S., & Wei, G. (2021). Stereological analysis and transcriptome profiling of testicular injury induced by di-(2-ethylhexyl) phthalate in prepubertal rats. *Ecotoxicology and environmental safety*, *220*, 112326.
ENSRNOG00000004302	Pah	3.030469	0.039475	Doan, R. N., Lim, E. T., De Rubeis, S., Betancur, C., Cutler, D. J., Chiocchetti, A. G., Overman, L. M., Soucy, A., Goetze, S., Autism Sequencing Consortium, Freitag, C. M., Daly, M. J., Walsh, C. A., Buxbaum, J. D., & Yu, T. W. (2019). Recessive gene disruptions in autism spectrum disorder. *Nature genetics*, *51*(7), 1092–1098.
ENSRNOG00000033772	Serpinb9	3.011401	0.010515	1) Heit, C., Jackson, B. C., McAndrews, M., Wright, M. W., Thompson, D. C., Silverman, G. A., Nebert, D. W., & Vasiliou, V. (2013). Update of the human and mouse SERPIN gene superfamily. *Human genomics*, *7*(1), 22. 2) Zhao, Y., Sun, W., Zhang, P., Chi, H., Zhang, M. J., Song, C. Q., Ma, X., Shang, Y., Wang, B., Hu, Y., Hao, Z., Hühmer, A. F., Meng, F., L'hernault, S. W., He, S. M., Dong, M. Q., & Miao, L. (2012). Nematode sperm maturation triggered by protease involves sperm-secreted serine protease inhibitor (Serpin). *Proceedings of the National Academy of Sciences of the United States of America*, *109*(5), 1542–1547.
ENSRNOG00000047943	Alkbh6	2.97423	0.02593	1) Zhao, S., Devega, R., Francois, A., & Kidane, D. (2021). Human ALKBH6 Is Required for Maintenance of Genomic Stability and Promoting Cell Survival During Exposure of Alkylating Agents in Pancreatic Cancer. *Frontiers in genetics*, *12*, 635808. 2) Ma, L., Lu, H., Tian, Z., Yang, M., Ma, J., Shang, G., Liu, Y., Xie, M., Wang, G., Wu, W., Zhang, Z., Dai, S., & Chen, Z. (2022). Structural insights into the interactions and epigenetic functions of human nucleic acid repair protein ALKBH6. *The Journal of biological chemistry*, *298*(3), 101671.
ENSRNOG00000037853	Rarres1	2.954813	0.037219	Agarwal, A., Panner Selvam, M. K., & Baskaran, S. (2020). Proteomic Analyses of Human Sperm Cells: Understanding the Role of Proteins and Molecular Pathways Affecting Male Reproductive

				Health. *International journal of molecular sciences*, *21*(5), 1621.
ENSRNOG00000038319	Akr1c19	2.926468	0.02593	
ENSRNOG00000020480	Fads1	2.891562	0.002078	Castellini, C., Mattioli, S., Moretti, E., Cotozzolo, E., Perini, F., Dal Bosco, A., Signorini, C., Noto, D., Belmonte, G., Lasagna, E., Brecchia, G., & Collodel, G. (2022). Expression of genes and localization of enzymes involved in polyunsaturated fatty acid synthesis in rabbit testis and epididymis. *Scientific reports*, *12*(1), 2637.
ENSRNOG00000053086	Sepp1	2.858271	0.025971	Burk, R. F., & Hill, K. E. (2009). Selenoprotein P-expression, functions, and roles in mammals. *Biochimica et biophysica acta*, *1790*(11), 1441–1447.
ENSRNOG00000059776	AABR07024382.1	2.857993	0.024796	
ENSRNOG00000016488	Pltp	2.838011	0.021426	Drouineaud, V., Lagrost, L., Klein, A., Desrumaux, C., Le Guern, N., Athias, A., Ménétrier, F., Moiroux, P., Sagot, P., Jimenez, C., Masson, D., & Deckert, V. (2006). Phospholipid transfer protein deficiency reduces sperm motility and impairs fertility of mouse males. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, *20*(6), 794–796.
ENSRNOG00000019120	Hmgcs2	2.837202	0.010515	
ENSRNOG00000061304	Atp2b3	2.832632	0.024259	Yoshida, K., Shiba, K., Sakamoto, A., Ikenaga, J., Matsunaga, S., Inaba, K., & Yoshida, M. (2018). Ca^{2+} efflux via plasma membrane Ca^{2+}-ATPase mediates chemotaxis in ascidian sperm. *Scientific reports*, *8*(1), 16622.
ENSRNOG00000008837	Ass1	2.776289	0.030835	
ENSRNOG00000060381	LOC100909752	2.744747	0.000449	
ENSRNOG00000018454	Apoe	2.737924	0.025874	Setarehbadi, R., Vatannejad, A., Vaisi-Raygani, A., Amiri, I., Esfahani, M., Fattahi, A., & Tavilani, H. (2012). Apolipoprotein E genotypes of fertile and infertile men. *Systems biology in reproductive medicine*, *58*(5), 263–267.
ENSRNOG00000011494	Rpl36al	2.709679	4.55E-05	Zou, Q., Yang, L., Shi, R., Qi, Y., Zhang, X., & Qi, H. (2021). Proteostasis regulated by testis-specific ribosomal protein RPL39L maintains mouse spermatogenesis. *iScience*, *24*(12), 103396.
ENSRNOG00000014011	Dll4	2.669559	0.040778	Murta, D., Batista, M., Silva, E., Trindade, A., Henrique, D., Duarte, A., & Lopes-da-Costa, L. (2013). Dynamics of Notch pathway expression during mouse testis post-natal development and along the spermatogenic cycle. *PloS one*, *8*(8), e72767.
ENSRNOG00000004517	Igf1	2.668554	0.012662	Yao, J., Zuo, H., Gao, J., Wang, M., Wang, D., & Li, X. (2017). The effects of IGF-1 on mouse spermatogenesis using an organ culture method. *Biochemical and biophysical research communications*, *491*(3), 840–847.
ENSRNOG00000004249	Tlr7	2.66815	0.042639	Ren, F., Xi, H., Qiao, P., Li, Y., Xian, M., Zhu, D., & Hu, J. (2022). Single-cell transcriptomics reveals male germ cells and Sertoli cells developmental patterns in dairy goats. *Frontiers in cell and developmental biology*, *10*, 944325.
ENSRNOG00000013968	Comtd1	2.658881	0.029783	González, C. R., & González, B. (2021). Exploring the Stress Impact in the Paternal Germ Cells Epigenome: Can Catecholamines Induce Epigenetic Reprogramming?. *Frontiers in endocrinology*, *11*, 630948.

ENSRNOG00000016456	Il33	2.647847	0.024796	
ENSRNOG00000024159	Fcer1g	2.625412	0.024796	Feig, C., Kirchhoff, C., Ivell, R., Naether, O., Schulze, W., & Spiess, A. N. (2007). A new paradigm for profiling testicular gene expression during normal and disturbed human spermatogenesis. *Molecular human reproduction*, *13*(1), 33–43.
ENSRNOG00000021206	Pla2g16	2.624272	0.007754	Gómez-Torres, M. J., García, E. M., Guerrero, J., Medina, S., Izquierdo-Rico, M. J., Gil-Izquierdo, Á., Orduna, J., Savirón, M., González-Brusi, L., Ten, J., Bernabeu, R., & Avilés, M. (2015). Metabolites involved in cellular communication among human cumulus-oocyte-complex and sperm during in vitro fertilization. *Reproductive biology and endocrinology : RB&E*, *13*, 123.
ENSRNOG00000010210	Slc7a11	2.609258	0.010502	
ENSRNOG00000009068	Phlda3	2.598579	0.014095	
ENSRNOG00000019112	Chp2	2.575903	0.041417	https://www.ncbi.nlm.nih.gov/gene/63928
ENSRNOG00000012682	Nr5a1	2.56683	0.011922	Bashamboo, A., Ferraz-de-Souza, B., Lourenço, D., Lin, L., Sebire, N. J., Montjean, D., Bignon-Topalovic, J., Mandelbaum, J., Siffroi, J. P., Christin-Maitre, S., Radhakrishna, U., Rouba, H., Ravel, C., Seeler, J., Achermann, J. C., & McElreavey, K. (2010). Human male infertility associated with mutations in NR5A1 encoding steroidogenic factor 1. *American journal of human genetics*, *87*(4), 505–512.
ENSRNOG00000037563	Cd68	2.565547	0.043537	Liguori, G., De Pasquale, V., Della Morte, R., Avallone, L., Costagliola, A., Vittoria, A., & Tafuri, S. (2015). Expression of the CD68 glycoprotein in the rat epididymis. *Biochimie*, *118*, 221–224.
ENSRNOG00000037113	Slfn2	2.555881	0.011889	Katsoulidis, E., Carayol, N., Woodard, J., Konieczna, I., Majchrzak-Kita, B., Jordan, A., Sassano, A., Eklund, E. A., Fish, E. N., & Platanias, L. C. (2009). Role of Schlafen 2 (SLFN2) in the generation of interferon alpha-induced growth inhibitory responses. *The Journal of biological chemistry*, *284*(37), 25051–25064.
ENSRNOG00000000186	Tst	2.535288	0.002266	Kruithof, P. D., Lunev, S., Aguilar Lozano, S. P., de Assis Batista, F., Al-Dahmani, Z. M., Joles, J. A., Dolga, A. M., Groves, M. R., & van Goor, H. (2020). Unraveling the role of thiosulfate sulfurtransferase in metabolic diseases. *Biochimica et biophysica acta. Molecular basis of disease*, *1866*(6), 165716.
ENSRNOG00000019470	Hao2	2.533508	0.013614	
ENSRNOG00000004210	Osr1	2.520933	0.024796	1) Liu, Y. L., Yang, S. S., Chen, S. J., Lin, Y. C., Chu, C. C., Huang, H. H., Chang, F. W., Yu, M. H., Lin, S. H., Wu, G. J., & Sytwu, H. K. (2016). OSR1 and SPAK cooperatively modulate Sertoli cell support of mouse spermatogenesis. *Scientific reports*, *6*, 37205. 2) https://www.ncbi.nlm.nih.gov/gene/23967
ENSRNOG00000002470	Ifi47	2.514034	0.038718	
ENSRNOG00000012787	Tmem164	2.512381	0.000719	
ENSRNOG00000005861	Hsd11b1	2.510074	0.039941	

ENSRNOG00000012881	Fgl2	2.506239	0.034232	Olson, G. E., Winfrey, V. P., NagDas, S. K., & Melner, M. H. (2004). Region-specific expression and secretion of the fibrinogen-related protein, fgl2, by epithelial cells of the hamster epididymis and its role in disposal of defective spermatozoa. *The Journal of biological chemistry*, *279*(49), 51266–51274.
ENSRNOG00000010331	Ctsb	2.49095	0.037564	
ENSRNOG00000020935	Capn1	2.486865	0.037219	
ENSRNOG00000003510	Fmo2	2.466803	0.049827	
ENSRNOG00000004699	Fibin	2.438356	0.029308	
ENSRNOG00000015957	F13a1	2.426548	0.043537	Xing, K., Chen, Y., Wang, L., Lv, X., Li, Z., Qi, X., Wang, X., Xiao, L., Ni, H., Guo, Y., & Sheng, X. (2022). Epididymal mRNA and miRNA transcriptome analyses reveal important genes and miRNAs related to sperm motility in roosters. *Poultry science*, *101*(1), 101558.
ENSRNOG00000042620	Marveld1	2.387818	0.011922	Mruk, D. D., & Cheng, C. Y. (2015). The Mammalian Blood-Testis Barrier: Its Biology and Regulation. *Endocrine reviews*, *36*(5), 564–591.
ENSRNOG00000029682	Clic1	2.378644	0.021622	Myers, K., Somanath, P. R., Berryman, M., & Vijayaraghavan, S. (2004). Identification of chloride intracellular channel proteins in spermatozoa. *FEBS letters*, *566*(1-3), 136–140.
ENSRNOG00000011648	Aqp1	2.374836	0.020546	1) Ito, J., Kawabe, M., Ochiai, H., Suzukamo, C., Harada, M., Mitsugi, Y., Seita, Y., & Kashiwazaki, N. (2008). Expression and immunodetection of aquaporin 1 (AQP1) in canine spermatozoa. *Cryobiology*, *57*(3), 312–314. 2) Nicòtina, P. A., Romeo, C., Arena, S., Arena, F., Maisano, D., & Zuccarello, B. (2005). Immunoexpression of aquaporin-1 in adolescent varicocele testes: possible significance for fluid reabsorption. *Urology*, *65*(1), 149–152.
ENSRNOG00000027552	Tapbpl	2.343452	0.024796	
ENSRNOG00000024410	Blvrb	2.333156	0.036511	
ENSRNOG00000009112	Pon2	2.328892	0.025971	Ferreira, C. E. R., Haas, C. S., Goularte, K. L., Rovani, M. T., Cardoso, F. F., Schneider, A., Gasperin, B. G., & Lucia, T., Jr (2018). Expression of paraoxonase types 1, 2 and 3 in reproductive tissues and activity of paraoxonase type 1 in the serum and seminal plasma of bulls. *Andrologia*, *50*(3), 10.1111/and.12923.
ENSRNOG00000013014	Cyba	2.313157	0.026998	https://www.ncbi.nlm.nih.gov/gene/1535
ENSRNOG00000031743	Gbp2	2.253538	0.012662	Stenz, L., Escoffier, J., Rahban, R., Nef, S., & Paoloni-Giacobino, A. (2017). Testicular Dysgenesis Syndrome and Long-Lasting Epigenetic Silencing of Mouse Sperm Genes Involved in the Reproductive System after Prenatal Exposure to DEHP. *PloS one*, *12*(1), e0170441.
ENSRNOG00000052294	Spint3	2.24746	0.043811	Robertson, M. J., Kent, K., Tharp, N., Nozawa, K., Dean, L., Mathew, M., Grimm, S. L., Yu, Z., Légaré, C., Fujihara, Y., Ikawa, M., Sullivan, R., Coarfa, C., Matzuk, M. M., & Garcia, T. X. (2020). Large-scale discovery of male reproductive tract-specific genes through analysis of RNA-seq datasets. *BMC biology*, *18*(1), 103.

ENSRNOG00000055962	Bgn	2.231015	0.034232	Mayer, C., Adam, M., Glashauser, L., Dietrich, K., Schwarzer, J. U., Köhn, F. M., Strauss, L., Welter, H., Poutanen, M., & Mayerhofer, A. (2016). Sterile inflammation as a factor in human male infertility: Involvement of Toll like receptor 2, biglycan and peritubular cells. *Scientific reports*, *6*, 37128.
ENSRNOG00000014936	Ifitm2	2.220269	0.014742	Pisarek, A., Pośpiech, E., Heidegger, A., Xavier, C., Papież, A., Piniewska-Róg, D., Kalamara, V., Potabattula, R., Bochenek, M., Sikora-Polaczek, M., Macur, A., Woźniak, A., Janeczko, J., Phillips, C., Haaf, T., Polańska, J., Parson, W., Kayser, M., & Branicki, W. (2021). Epigenetic age prediction in semen - marker selection and model development. *Aging*, *13*(15), 19145–19164.
ENSRNOG00000017672	Akr1c14	2.216825	0.037564	1) Barski, O. A., Tipparaju, S. M., & Bhatnagar, A. (2008). The aldo-keto reductase superfamily and its role in drug metabolism and detoxification. *Drug metabolism reviews*, *40*(4), 553–624. 2) https://www.ncbi.nlm.nih.gov/gene/105387
ENSRNOG00000059538	Clec2g	2.215238	0.039385	
ENSRNOG00000003228	Mid1ip1	2.175385	0.004807	
ENSRNOG00000028015	Pf4	2.162222	0.049855	
ENSRNOG00000002322	RGD1310587	2.154792	0.00015	
ENSRNOG00000021380	Fads6	2.082165	0.012662	Stroud, C. K., Nara, T. Y., Roqueta-Rivera, M., Radlowski, E. C., Lawrence, P., Zhang, Y., Cho, B. H., Segre, M., Hess, R. A., Brenna, J. T., Haschek, W. M., & Nakamura, M. T. (2009). Disruption of FADS2 gene in mice impairs male reproduction and causes dermal and intestinal ulceration. *Journal of lipid research*, *50*(9), 1870–1880.
ENSRNOG00000050869	Cebpd	2.062067	0.037552	El-Sokary, M., Ibrahim, S., El-Naby, A. S., Sosa, A., Mahmoud, K., & Nawito, M. (2021). New insights into molecular aspects of sperm-oviductal binding in Egyptian buffaloes using an in vitro model: Effects of oviductal segments and media. *Andrologia*, *53*(4), e13984.
ENSRNOG00000016064	Tkt	2.057729	0.03436	Cannarella, R., Condorelli, R. A., Mongioì, L. M., La Vignera, S., & Calogero, A. E. (2020). Molecular Biology of Spermatogenesis: Novel Targets of Apparently Idiopathic Male Infertility. *International journal of molecular sciences*, *21*(5), 1728.
ENSRNOG00000019968	Trim8	2.054431	0.039023	
ENSRNOG00000021079	Fxyd1	2.053107	0.039023	
ENSRNOG00000018237	Gstp1	2.042742	0.034232	Llavanera, M., Mateo-Otero, Y., Delgado-Bermúdez, A., Recuero, S., Olives, S., Barranco, I., & Yeste, M. (2021). Deactivation of the JNK Pathway by GSTP1 Is Essential to Maintain Sperm Functionality. *Frontiers in cell and developmental biology*, *9*, 627140.
ENSRNOG00000043007	Rcn3	2.030755	0.024569	
ENSRNOG00000007304	Herc3	2.023773	0.034232	
ENSRNOG00000054978	Hist1h1c	2.017203	0.044885	1) Ivanyi-Nagy, R., Ahmed, S. M., Peter, S., Ramani, P. D., Ong, P. F., Dreesen, O., & Dröge, P. (2018). The RNA interactome of human telomerase RNA

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ENSRNOG00000007545	Angptl4	2.016495	0.025151	Lee, S., Jang, H., Moon, S., Lee, O. H., Lee, S., Lee, J., Park, C., Seol, D. W., Song, H., Hong, K., Kim, J. H., Uhm, S. J., Lee, D. R., Lee, J. W., & Choi, Y. (2019). Differential Regulation of *TLE3* in Sertoli Cells of the Testes during Postnatal Development. *Cells*, *8*(10), 1156.
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ENSRNOG00000033261	Fam107a	2.000973	0.003809	
ENSRNOG00000015880	Dpep1	1.99165	0.029783	
ENSRNOG00000005478	Fkbp9	1.987033	0.038026	
ENSRNOG00000020173	Tie1	1.969374	0.008625	
ENSRNOG00000058006	Sncg	1.950879	0.0076	
ENSRNOG00000013949	Idh2	1.950195	0.016215	Zhu, S., Huang, J., Xu, R., Wang, Y., Wan, Y., McNeel, R., Parker, E., Kolson, D., Yam, M., Webb, B., Zhao, C., Sigado, J., & Du, J. (2022). Isocitrate dehydrogenase 3b is required for spermiogenesis but dispensable for retinal viability. *The Journal of biological chemistry*, *298*(9), 102387.
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ENSRNOG00000034198	Wbp5	1.933518	0.047222	
ENSRNOG00000011946	Ptn	1.926331	0.046334	Zhang, N., Yeh, H. J., Zhong, R., Li, Y. S., & Deuel, T. F. (1999). A dominant-negative pleiotrophin mutant introduced by homologous recombination leads to germ-cell apoptosis in male mice. *Proceedings of the National Academy of Sciences of the United States of America*, *96*(12), 6734–6738.
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ENSRNOG00000013954	Alpl	1.893242	0.003809	
ENSRNOG00000007728	Gsdmd	1.871445	0.043228	
ENSRNOG00000054344	AC120246.2	1.845253	0.033697	
ENSRNOG00000012081	Txn1	1.842676	0.03361	Al-Kandari, N., Fadel, F., Al-Saleh, F., Khashab, F., & Al-Maghrebi, M. (2019). The Thioredoxin System is Regulated by the ASK-1/JNK/p38/Survivin Pathway During Germ Cell Apoptosis. *Molecules (Basel, Switzerland)*, *24*(18), 3333.
ENSRNOG00000051977	Mmrn2	1.835193	0.014489	Lorenzon, E., Colladel, R., Andreuzzi, E., Marastoni, S., Todaro, F., Schiappacassi, M., Ligresti, G., Colombatti, A., & Mongiat, M. (2012). MULTIMERIN2 impairs tumor angiogenesis and growth by interfering with VEGF-A/VEGFR2 pathway. *Oncogene*, *31*(26), 3136–3147.
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ENSRNOG00000017283	Kcnt1	1.812743	0.034232	
ENSRNOG00000059900	Bst2	1.811309	0.049855	
ENSRNOG00000013324	Cdh5	1.802499	0.008625	Marín-Briggiler, C. I., Veiga, M. F., Matos, M. L., Echeverría, M. F., Furlong, L. I., & Vazquez-Levin, M. H. (2008). Expression of epithelial cadherin in the human male reproductive tract and gametes and evidence of its participation in fertilization. *Molecular human reproduction*, *14*(10), 561–571.
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ENSRNOG00000023830	Dnase2	1.792209	0.013992	
ENSRNOG00000033402	LOC501110	1.789069	0.034232	
ENSRNOG00000051548	Lmod1	1.763371	0.000393	Mahyari, E., Guo, J., Lima, A. C., Lewinsohn, D. P., Stendahl, A. M., Vigh-Conrad, K. A., Nie, X., Nagirnaja, L., Rockweiler, N. B., Carrell, D. T., Hotaling, J. M., Aston, K. I., & Conrad, D. F. (2021). Comparative single-cell analysis of biopsies clarifies pathogenic mechanisms in Klinefelter syndrome. *American journal of human genetics*, *108*(10), 1924–1945.
ENSRNOG00000042838	Junb	1.755832	0.02593	Zhu, Z., Li, C., Yang, S., Tian, R., Wang, J., Yuan, Q., Dong, H., He, Z., Wang, S., & Li, Z. (2016). Dynamics of the Transcriptome during Human Spermatogenesis: Predicting the Potential Key Genes Regulating Male Gametes Generation. *Scientific reports*, *6*, 19069.
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ENSRNOG00000001544	Cyyr1	1.723448	0.022281	
ENSRNOG00000013469	Txndc5	1.718145	0.016642	
ENSRNOG00000002050	Igfbp7	1.708181	0.048364	1) Cannarella, R., Condorelli, R. A., Mongioì, L. M., La Vignera, S., & Calogero, A. E. (2020). Molecular Biology of Spermatogenesis: Novel Targets of Apparently Idiopathic Male Infertility. *International journal of molecular sciences*, *21*(5), 1728. 2) Hwa, V., Oh, Y., & Rosenfeld, R. G. (1999). The insulin-like growth factor-binding protein (IGFBP) superfamily. *Endocrine reviews*, *20*(6), 761–787.
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ENSRNOG00000057713	Cav2	1.652422	0.032473	Miranda, P. V., Allaire, A., Sosnik, J., & Visconti, P. E. (2009). Localization of low-density detergent-resistant membrane proteins in intact and acrosome-reacted mouse sperm. *Biology of reproduction*, *80*(5), 897–904.
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ENSRNOG00000010805	Fabp4	1.625537	0.034232	Yamane, T., Shimizu, T., Takahashi-Niki, K., Takekoshi, Y., Iguchi-Ariga, S. M. M., & Ariga, H. (2015). Deficiency of spermatogenesis and reduced expression of spermatogenesis-related genes in prefoldin 5-mutant mice. *Biochemistry and biophysics reports*, *1*, 52–61.
ENSRNOG00000009625	Dpysl2	1.623064	0.041901	Prokai, D., Pudasaini, A., Kanchwala, M., Moehlman, A. T., Waits, A. E., Chapman, K. M., Chaudhary, J., Acevedo, J., Keller, P., Chao, X., Carr, B. R., & Hamra, F. K. (2020). Spermatogonial Gene Networks Selectively Couple to Glutathione and Pentose Phosphate Metabolism but Not Cysteine Biosynthesis. *iScience*, *24*(1), 101880.
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ENSRNOG00000010872	Ckb	1.566324	0.013614	Lee, M. S., Liu, C. H., Lee, T. H., Wu, H. M., Huang, C. C., Huang, L. S., Chen, C. M., & Cheng, E. H. (2010). Association of creatin kinase B and peroxiredoxin 2 expression with age and embryo quality in cumulus cells. *Journal of assisted reproduction and genetics*, *27*(11), 629–639.
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ENSRNOG00000005332	Csdc2	1.563779	0.045406	
ENSRNOG00000002449	Maged2	1.555189	0.046088	Florke Gee, R. R., Chen, H., Lee, A. K., Daly, C. A., Wilander, B. A., Fon Tacer, K., & Potts, P. R. (2020). Emerging roles of the MAGE protein family in stress response pathways. *The Journal of biological chemistry*, *295*(47), 16121–16155.
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ENSRNOG00000054695	Calcrl	1.543855	0.032473	Chan, Y. F., Tang, F., & O, W. S. (2008). Adrenomedullin in the rat testis. II: Its production, actions on inhibin secretion, regulation by follicle-

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ENSRNOG00000019810	Des	1.469427	0.026273	Arenas, M. I., Bethencourt, F. R., De Miguel, M. P., Fraile, B., Romo, E., & Paniagua, R. (1997). Immunocytochemical and quantitative study of actin, desmin and vimentin in the peritubular cells of the testes from elderly men. *Journal of reproduction and fertility*, *110*(1), 183–193.
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ENSRNOG00000007136	Anxa7	1.460786	0.038795	Chojnacka, K., Bilinska, B., & Mruk, D. D. (2017). Annexin A2 is critical for blood-testis barrier integrity and spermatid disengagement in the mammalian testis. *Biochimica et biophysica acta. Molecular cell research*, *1864*(3), 527–545.
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ENSRNOG00000046560	AC109096.1	1.447706	0.020081	
ENSRNOG00000057347	Cebpb	1.442037	0.030835	
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ENSRNOG00000023465	LOC500300	1.423994	0.029783	
ENSRNOG00000018087	Vim	1.417971	0.017004	Mali, P., Virtanen, I., & Parvinen, M. (1987). Vimentin expression in spermatogenic and Sertoli cells is stage-related in rat seminiferous epithelium. *Andrologia*, *19*(6), 644–653.
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ENSRNOG00000003772	Csrp2	1.382304	0.046088	
ENSRNOG00000042848	Jam2	1.377424	0.021426	Paul, C., & Robaire, B. (2013). Impaired function of the blood-testis barrier during aging is preceded by a decline in cell adhesion proteins and GTPases. *PloS one*, *8*(12), e84354.
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ENSRNOG00000030712	RT1-A2	1.368368	0.035167	
ENSRNOG00000007462	SEPTIN8	1.358622	0.030165	Lin, Y. H., Kuo, Y. C., Chiang, H. S., & Kuo, P. L. (2011). The role of the septin family in spermiogenesis. *Spermatogenesis*, *1*(4), 298–302.
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ENSRNOG00000003209	Pcp4l1	1.343193	0.032583	
ENSRNOG00000054890	Flna	1.341978	0.035856	Su, W., Mruk, D. D., Lie, P. P., Lui, W. Y., & Cheng, C. Y. (2012). Filamin A is a regulator of blood-testis barrier assembly during postnatal development in the rat testis. *Endocrinology*, *153*(10), 5023–5035.
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ENSRNOG00000020865	Ano1	1.329865	0.046043	Saberiyan, M., Mirfakhraie, R., Gholami, D., Dehdehi, L., & Teimori, H. (2020). Investigating the regulatory function of the ANO1-AS2 on the ANO1 gene in infertile men with asthenozoospermia and terato-asthenozoospermia. *Experimental and molecular pathology*, *117*, 104528.
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ENSRNOG00000051307	Aoc3	1.314435	0.037301	Romauch M. (2020). Zinc-α2-glycoprotein as an inhibitor of amine oxidase copper-containing 3. *Open biology*, *10*(4), 190035.
ENSRNOG00000015941	Fkbp10	1.298748	0.043228	
ENSRNOG00000047706	RT1-CE16	1.2909	0.013614	
ENSRNOG00000043387	Cpe	1.280075	0.015178	Cawley, N. X., Wetsel, W. C., Murthy, S. R., Park, J. J., Pacak, K., & Loh, Y. P. (2012). New roles of carboxypeptidase E in endocrine and neural function and cancer. *Endocrine reviews*, *33*(2), 216–253.
ENSRNOG00000050630	RGD1565368	1.276153	0.005777	Miki, K., Qu, W., Goulding, E. H., Willis, W. D., Bunch, D. O., Strader, L. F., Perreault, S. D., Eddy, E. M., & O'Brien, D. A. (2004). Glyceraldehyde 3-phosphate dehydrogenase-S, a sperm-specific glycolytic enzyme, is required for sperm motility and male fertility. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(47), 16501–16506.
ENSRNOG00000047768	Lamb2	1.273085	0.039023	
ENSRNOG00000003875	Ocrl	1.272401	0.049855	
ENSRNOG00000016164	Fcrl2	1.270505	0.026652	
ENSRNOG00000015496	Tpm4	1.263077	0.044885	
ENSRNOG00000010830	Slc25a4	1.221078	0.032473	Brower, J. V., Rodic, N., Seki, T., Jorgensen, M., Fliess, N., Yachnis, A. T., McCarrey, J. R., Oh, S. P., & Terada, N. (2007). Evolutionarily conserved mammalian adenine nucleotide translocase 4 is essential for spermatogenesis. *The Journal of biological chemistry*, *282*(40), 29658–29666.
ENSRNOG00000015295	Ctdsp1	1.219611	0.043228	
ENSRNOG00000008738	Tp53i11	1.203128	0.049855	Tian, R., Yao, C., Yang, C., Zhu, Z., Li, C., Zhi, E., Wang, J., Li, P., Chen, H., Yuan, Q., He, Z., & Li, Z. (2019). Fibroblast growth factor-5 promotes spermatogonial stem cell proliferation via ERK and AKT activation. *Stem cell research & therapy*, *10*(1), 40.

ENSRNOG00000014453	Anxa5	1.18037	0.039023	Lavorato, H. L., Markoff, A., Altholz, V., Bogdanova, N., Wieacker, P., Kliesch, S., & Schlatt, S. (2019). The relevance of ANXA5 genetic variants on male fertility. *Journal of assisted reproduction and genetics*, *36*(7), 1355–1359.
ENSRNOG00000056836	Cav1	1.16893	0.034232	Baltiérrez-Hoyos, R., Roa-Espitia, A. L., & Hernández-González, E. O. (2012). The association between CDC42 and caveolin-1 is involved in the regulation of capacitation and acrosome reaction of guinea pig and mouse sperm. *Reproduction (Cambridge, England)*, *144*(1), 123–134.
ENSRNOG00000010838	Araf	1.165425	0.043228	Goriely, A., & Wilkie, A. O. (2012). Paternal age effect mutations and selfish spermatogonial selection: causes and consequences for human disease. *American journal of human genetics*, *90*(2), 175–200.
ENSRNOG00000010107	Palld	1.161805	0.016642	https://www.ncbi.nlm.nih.gov/gene/72333
ENSRNOG00000037850	Mtarc2	1.151475	0.020546	
ENSRNOG00000001295	S100b	1.151259	0.036511	
ENSRNOG00000006052	Sulf2	1.1441	0.046088	Langsdorf, A., Schumacher, V., Shi, X., Tran, T., Zaia, J., Jain, S., Taglienti, M., Kreidberg, J. A., Fine, A., & Ai, X. (2011). Expression regulation and function of heparan sulfate 6-O-endosulfatases in the spermatogonial stem cell niche. *Glycobiology*, *21*(2), 152–161.
ENSRNOG00000009592	Cyb5r3	1.123913	0.049855	
ENSRNOG00000014030	Synm	1.121031	0.039475	Russell M. A. (2020). Synemin Redefined: Multiple Binding Partners Results in Multifunctionality. *Frontiers in cell and developmental biology*, *8*, 159.
ENSRNOG00000018233	Gas6	1.093336	0.004807	1) Xiong, W., Chen, Y., Wang, H., Wang, H., Wu, H., Lu, Q., & Han, D. (2008). Gas6 and the Tyro 3 receptor tyrosine kinase subfamily regulate the phagocytic function of Sertoli cells. *Reproduction (Cambridge, England)*, *135*(1), 77–87. 2) Kim, K. H., Kim, E. Y., Lee, S. Y., Ko, J. J., & Lee, K. A. (2018). Oocyte Cytoplasmic Gas6 and Heparan Sulfate (HS) are Required to Establish the Open Chromatin State in Nuclei During Remodeling and Reprogramming. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology*, *45*(1), 37–53.
ENSRNOG00000054080	Cgnl1	1.088505	0.039023	Mazaud-Guittot, S., Meugnier, E., Pesenti, S., Wu, X., Vidal, H., Gow, A., & Le Magueresse-Battistoni, B. (2010). Claudin 11 deficiency in mice results in loss of the Sertoli cell epithelial phenotype in the testis. *Biology of reproduction*, *82*(1), 202–213.
ENSRNOG00000002229	Adcy5	1.085403	0.012972	Balbach, M., Ghanem, L., Rossetti, T., Kaur, N., Ritagliati, C., Ferreira, J., Krapf, D., Puga Molina, L. C., Santi, C. M., Hansen, J. N., Wachten, D., Fushimi, M., Meinke, P. T., Buck, J., & Levin, L. R. (2021). Soluble adenylyl cyclase inhibition prevents human sperm functions essential for fertilization. *Molecular human reproduction*, *27*(9), gaab054.
ENSRNOG00000013000	Ldhb	1.07401	0.043228	Odet, F., Duan, C., Willis, W. D., Goulding, E. H., Kung, A., Eddy, E. M., & Goldberg, E. (2008). Expression of the gene for mouse lactate dehydrogenase C (Ldhc) is required for male fertility. *Biology of reproduction*, *79*(1), 26–34.
ENSRNOG00000030963	LOC685186	1.0615	0.034232	Miki, K., Qu, W., Goulding, E. H., Willis, W. D., Bunch, D. O., Strader, L. F., Perreault, S. D., Eddy, E. M., & O'Brien, D. A. (2004). Glyceraldehyde 3-

				phosphate dehydrogenase-S, a sperm-specific glycolytic enzyme, is required for sperm motility and male fertility. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(47), 16501–16506.
ENSRNOG00000002403	Fam129a	1.040735	0.049855	
ENSRNOG00000021090	Pygm	1.031244	0.046583	
ENSRNOG00000001724	Atp13a3	-1.11751	0.044885	
ENSRNOG00000058781	AABR07028258.1	-1.15996	0.026998	
ENSRNOG00000030932	Zfp14	-1.6891	0.049855	https://www.ncbi.nlm.nih.gov/gene/57677
ENSRNOG00000045673	AABR07002741.1	-1.72878	0.032473	
ENSRNOG00000035568	Mir3074	-2.52657	0.049855	
ENSRNOG00000046677	Fam181a	-2.85161	0.002951	
ENSRNOG00000052199	RGD1566138	-2.87297	0.032473	
ENSRNOG00000048523	LOC100910370	-3.24369	0.046099	
ENSRNOG00000060514	AC117925.6	-3.66761	0.006571	https://rgd.mcw.edu/rgdweb/report/gene/main.html?id=1561494

Table S3-2: Ingenuity Canonical Pathways and Contributing Genes.

Ingenuity Canonical Pathways	-log(p-value)	Ratio	z-score	Molecules(Genes)
RHOGDI Signaling	1.35	0.0279	-1	CDH5,GNA13,MYH11,MYL9,PIKFYVE,RHOB
Autophagy	1.35	0.0279	-0.816	ATM,ATR,CAMK4,IGF1,RALA,SLC7A5
Epithelial Adherens Junction Signaling	1.39	0.0318	-0.447	ECT2,FGFR1,RALA,TNS1,YWHAG
Gα12/13 Signaling	2.27	0.0451	0.447	CDH5,F2R,GNA13,MYL9,NFKBIA,RALA
RHOA Signaling	1.78	0.0403	0.447	GNA13,IGF1,MYL9,PIKFYVE,SEPTIN8
Leukocyte Extravasation Signaling	1.54	0.0311	0.447	ACTN1,CDH5,CXCL12,CYBA,JAM2,TIMP3
Thrombin Signaling	2.24	0.0356	0.816	ADCY5,ARHGEF25,CAMK4,F2R,GNA13,MYL9,RALA,RHOB
CXCR4 Signaling	1.79	0.0355	0.816	ADCY5,CXCL12,GNA13,MYL9,RALA,RHOB
Coagulation System	3.11	0.114	1	F13A1,F2R,PLAT,VWF
Glioma Invasiveness Signaling	1.94	0.0548	1	F2R,RALA,RHOB,TIMP3
Role of MAPK Signaling in Inhibiting the Pathogenesis of Influenza	1.87	0.0519	1	ARAF,CXCL10,NFKBIA,PLAAT3
Role of MAPK Signaling in Promoting the Pathogenesis of Influenza	1.33	0.0354	1	ARAF,NFKBIA,PLAAT3,RALA
Senescence Pathway	3.59	0.0404	1.155	ARAF,ATM,ATR,CAMK4,CAPN1,CEBPB,GADD45G,NF1,RALA,RING1,YPEL3,ZFP36L1
ILK Signaling	1.48	0.03	1.342	ACTN1,FLNA,MYH11,MYL9,RHOB,VIM
Ferroptosis Signaling Pathway	2.29	0.0455	1.633	ANGPTL4,ARAF,CTSB,RALA,SLC39A8,SLC7A11
MSP-RON Signaling In Cancer Cells Pathway	2.17	0.0429	1.633	ARAF,FLNA,NFKBIA,RALA,VIM,YWHAG
IL-8 Signaling	1.87	0.0333	1.633	ARAF,GNA13,IRAK1,MYL9,NFKBIA,RALA,RHOB
Actin Cytoskeleton Signaling	1.55	0.0286	1.633	ACTN1,F2R,FLNA,GNA13,MYH11,MYL9,RALA
Pulmonary Fibrosis Idiopathic Signaling Pathway	1.77	0.0276	1.667	ARAF,CAV1,CCN2,CXCL12,F2R,FGFR1,GNA13,RALA,VIM
NAD Signaling Pathway	2.64	0.0464	1.89	CEBPB,HMGCS2,IDH2,IGF1,LDHB,SLC7A5,TNKS2
HIF1α Signaling	1.9	0.0337	1.89	ARAF,CAMK4,IGF1,LDHB,RACK1,RALA,VIM
Integrin Signaling	1.84	0.0329	1.89	ACTN1,CAPN1,CAV1,MYL9,PIKFYVE,RALA,RHOB
Signaling by Rho Family GTPases	1.81	0.0299	1.89	CDH5,DES,GNA13,MYL9,PIKFYVE,RHOB,SEPTIN8,VIM
Toll-like Receptor Signaling	1.85	0.0513	2	IL33,IRAK1,NFKBIA,TLR7

Role of Hypercytokinemia/hyperchemokinemia in the Pathogenesis of Influenza	2.43	0.0581	2.236	CXCL10,IFIT2,IL33,IRF7,TLR7
Acute Phase Response Signaling	2.16	0.0378	2.236	C1QB,CEBPB,IL33,IRAK1,NFKBIA,RALA,VWF
SNARE Signaling Pathway	1.62	0.0368	2.236	ADCY5,CAMK4,MYH11,MYL9,SNCG
White Adipose Tissue Browning Pathway	1.6	0.0362	2.236	ADCY5,CEBPB,FCER1G,FGFR1,LDHB
Neuroinflammation Signaling Pathway	1.84	0.0284	2.333	B2M,CD200,CXCL10,CXCL12,HLA-A,IRAK1,IRF7,S100B,TLR7
Pulmonary Healing Signaling Pathway	1.49	0.0302	2.449	ARAF,CXCL12,FGFR1,IDH2,NFKBIA,RALA
Cardiac Hypertrophy Signaling	1.43	0.0269	2.449	ADCY5,CAMK4,GNA13,IGF1,MYL9,RALA,RHOB
Adrenomedullin signaling pathway	1.99	0.0352	2.646	ADCY5,ARAF,CALCRL,CAMK4,CEBPB,IL33,RALA
ID1 Signaling Pathway	1.98	0.035	2.646	ARAF,CAV1,CCN2,FGFR1,MCAM,RALA,VIM
Hepatic Fibrosis Signaling Pathway	1.89	0.0261	2.714	ARAF,CAMK4,CCN2,CEBPB,FGFR1,IL33,IRAK1,MYL9,NFKBIA,RALA,RHOB
IGF-1 Signaling	3.6	0.0673	0	CCN1,CCN2,IGF1,IGFBP2,IGFBP7,RALA,YWHAG
LPS/IL-1 Mediated Inhibition of RXR Function	3.59	0.0433	0	APOE,FABP3,FABP4,FMO2,GSTA2,GSTP1,HMGCS2,IL33,IRAK1,MAOB,PLTP
Calcium Signaling	2.32	0.0367	0	ATP2B3,CAMK4,MYH11,MYL9,Tpm1,Tpm2,Tpm4,TRPM8
Calcium Transport I	2.22	0.2	0	ANXA5,ATP2B3
ATM Signaling	2.17	0.0505	0	ATM,ATR,GADD45G,NFKBIA,SMC2
Ketogenesis	2.14	0.182	0	ACAA2,HMGCS2
α-Adrenergic Signaling	2.02	0.0463	0	ADCY5,CAMK4,GNA13,PYGM,RALA
Oleate Biosynthesis II (Animals)	1.99	0.154	0	FADS1,FADS6
Mevalonate Pathway I	1.93	0.143	0	ACAA2,HMGCS2
Glucocorticoid Biosynthesis	1.87	0.133	0	CYP17A1,HSD3B2
D-myo-inositol (1,3,4)-trisphosphate Biosynthesis	1.76	0.118	0	OCRL,SYNJ1
iNOS Signaling	1.73	0.0638	0	CAMK4,IRAK1,NFKBIA
Bile Acid Biosynthesis, Neutral Pathway	1.72	0.111	0	Akr1c14,Akr1c19
UVC-Induced MAPK Signaling	1.64	0.0588	0	ARAF,ATR,RALA
Adipogenesis pathway	1.64	0.037	0	CEBPB,CEBPD,FABP4,FGFR1,LPL
Androgen Biosynthesis	1.59	0.0952	0	CYP17A1,HSD3B2
Superpathway of D-myo-inositol (1,4,5)-trisphosphate Metabolism	1.55	0.0909	0	OCRL,SYNJ1
IL-1 Signaling	1.55	0.0417	0	ADCY5,GNA13,IRAK1,NFKBIA
p53 Signaling	1.52	0.0408	0	ATM,ATR,GADD45G,STAG1
GADD45 Signaling	1.46	0.05	0	ATM,ATR,GADD45G

SPINK1 Pancreatic Cancer Pathway	1.46	0.05	0	CPD,CPE,CTSB
Aryl Hydrocarbon Receptor Signaling	1.37	0.0314	0	ATM,ATR,CYP1B1,GSTA2,GSTP1
Phagosome Maturation	1.37	0.0314	NA	B2M,CTSB,DYNC2H1,HLA-A,PIKFYVE
Activation of IRF by Cytosolic Pattern Recognition Receptors	1.37	0.0462	0	IFIT2,IRF7,NFKBIA
Glutathione Redox Reactions I	1.36	0.0714	0	GPX1,GSTP1
Methylglyoxal Degradation III	1.33	0.069	0	Akr1c14,Akr1c19
Superpathway of Cholesterol Biosynthesis	1.33	0.069	0	ACAA2,HMGCS2
Role of Tissue Factor in Cancer	1.3	0.0345	0	CCN1,CCN2,GNA13,RALA
Dilated Cardiomyopathy Signaling Pathway	2.05	0.0405	0	ADCY5,CAMK4,CNN1,DES,MYH11,MYL9
PDGF Signaling	1.71	0.0465	0	CAV1,OCRL,RALA,SYNJ1
Osteoarthritis Pathway	1.63	0.0297	0	ALPL,ANXA5,CEBPB,FGFR1,OCRL,SLC39A8,TIMP3

Connecting Text

The research presented in Chapter 3 of the thesis examined mRNA expression in round spermatids from young and aged Brown Norway rats using RNA-sequencing. This study suggests that with aging there is major upregulation of transcripts (211/220) involved in several processes associated with aging and with reproduction. Specifically, we observed an upregulation in transcripts associated with the immune system and inflammation, sperm motility morphology and functionality, and the interaction of germ cells with the supporting Sertoli cells of the testis. We observed very few transcripts with decreased expression due to aging, (9/220). This was the first study in Brown Norway rats to examine gene expression with aging using RNA-sequencing, and the first to report an alteration in the signalling molecules, with known involvement in germ cells-Sertoli cell signalling as a consequence of age. We also noticed an increase in transcripts known to alter DNA methylation, including folate receptors, transcripts with predicted DNA methylase activity, and transcripts with protective roles in DNA methylation. As we noticed a general decrease in expression of mRNA, and altered transcripts associated with DNA methylation, we hypothesized that altered gene expression may be the result of altered DNA methylation. Altered DNA methylation also has consequences for telomere dynamics, and telomere length changes may alter DNA methylation. In Chapter 4, we examine DNA methylation in round spermatids and mature spermatozoa from Brown Norway rats using whole-genome bisulfite sequencing.

CHAPTER 4

DIFFERENTIAL DNA METHYLATION DURING SPERMATOGENESIS IN YOUNG AND AGED BROWN NORWAY RATS

Heather E, Fice, Bernard Robaire

(In preparation)

Differential DNA Methylation during spermatogenesis in young and aged Brown Norway Rats

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Abstract

The trend of delayed parenthood, and the aging population in North America are leading to more fathers of advanced paternal age. Epidemiological data suggests that the children of older fathers are susceptible to genetic disorders and diseases, suggesting that delaying parenthood is not without risk. There are limited studies to suggest epigenetic alterations in the sperm of aged fathers, and few studies that link what we know about reproductive aging from rodent models with epigenetic mechanisms. We hypothesize that due the aging process in Brown Norway rats causes alterations in the germ cell epigenome through DNA methylation. We have used whole-genome bisulfite sequencing to assess germ cell DNA methylation in post-meiotic round spermatids and mature spermatozoa from young (4-6 months) and aged (18-20 months) Brown Norway rats (n=3). We found that there are major disruptions in DNA methylation, with 5486 differentially methylated regions (DMRs) in spermatids, and 1888 DMRs in spermatozoa. The majority of these DMRs are hypermethylated and found in intergenic regions within the genome. We examined the transition from spermatid to spermatozoa and found that with aging the inherent process of DNA methylation was perturbed. These findings suggest that DNA methylation is altered with aging in germ cells, and may have consequences for the offspring.

Introduction

Delayed parenthood is a trend observed across ethnicities since the 1970s in North America and westernized countries (Khandwala et al., 2017). This trend can be attributed to individuals increased career or personal aspirations, and increased costs of living. The delay in parenthood is observed in both biological sexes though potentially more striking in maternal aging with more frequent pathologies such as trisomy (Pellestor et al., 2003). However, the effects of paternal aging are worth note as epidemiological findings suggest genetic consequences for the offspring of older fathers. Primarily, genetic disorders of neurodevelopment arise in the children of older fathers; these include attention deficit hyperactivity disorder (ADHD), autism, and schizophrenia (Bray et al., 2006; D'Onofrio et al., 2014; Malaspina et al., 2015; Frans et al., 2013). Additionally, with advanced paternal age there are alterations to overall male fertility, with reported decreases in sperm quality accompanied by

decreased fecundity and poor pregnancy outcomes (Dong et al., 2022; Bartolacci et al., 2018; Beguería et al., 2014; Pino et al., 2020).

Spermatogenesis, the process by which spermatozoa (sperm) are continuously produced in the seminiferous tubules of the testis, occurs from puberty until death. The sperm arise from a spermatogonial stem cell pool, which may be vulnerable to the effects of aging. The stem cells differentiate to derive a germ cell lineage, in which cells undergo meiosis to eventually produce haploid sperm (Griswold, 2016). Each germ cell stage has distinct characteristics, for example the pre-meiotic spermatocytes undergo gene crossovers and have active DNA damage repair machinery. The post-meiotic spermatids (haploid) undergo the repackaging of DNA, spermiogenesis (Paul et al., 2011). Spermiogenesis is characterized by the gradual replacement of histones, to protamines, allowing for the tight DNA packaging required for sperm to develop their: transcriptional inactivity, small size, and streamlined motility (O'Donnell, 2015). During spermatogenesis, and DNA reorganization, the germ cell epigenome is dynamic.

The 'epigenome' includes all mechanisms that regulate the genome without altering the primary DNA sequence. This includes noncoding RNAs, histone modifications, and DNA methylation. DNA methyltransferases (DNMTs) catalyze this reaction through the addition of a methyl group to 5-carbon position of cytosine nucleotides, forming 5-methyl cytosine (5-mC) (Trasler, 2009). Methylation mainly occurs on cytosines paired with guanine (CpG), but can occur on cytosines separated from guanine by one nucleotide (CHG), or entirely on their own (CHH). DNA methylation regulates gene expression, with hypermethylated regions conferring transcriptional inactivity and hypomethylation conferring transcription activity (Trasler, 2009). In addition, DNA methylation is critical for the development of X-inactivation and genomic imprinting of paternally or maternally inherited genes. To confer totipotency in the embryo, DNA methylation must be erased and reestablished except in select imprinted regions (Hirasawa et al., 2008; Cirio et al., 2008a; Cirio et al., 2008b). The erasure of DNA methylation occurs after fertilization, increasing only after the blastocyst stage. During embryonic development in males, the primordial germ cells undergo a wave of demethylation, and male germ cell *de novo* DNA methylation is partly established in the prospermatogonia before birth (Smallwood and Kelsey, 2011). DNA methylation is further modified, gained and lost, in the mitotic and meiotic germ cells, and is complete by the secondary spermatocyte stage after meiosis I (Oakes et al., 2007).

The methylation of some testis-specific genes become demethylated to facilitate their expression during spermatogenesis (Geyer et al., 2004).

Due to the dynamic nature of genomic methylation during germline development and spermatogenesis, we may examine germ cell DNA methylation as a mode of epigenetic inheritance. Toxicant exposure, nutritional status, lifestyle and aging have all been demonstrated to have effects on the sperm DNA methylome (Chan et al., 2012; Lambrot et al., 2013; Salas-Huetos et al., 2020; Jenkins et al., 2014; Cao et al., 2020). The effect of age on sperm DNA methylation was initially investigated by Jenkins et al., (2014) who reported that there were 140 consistent differential methylated regions (DMRs) when comparing sperm DNA from young and aged men, with predominantly hypermethylation. Cao et al., report that aging causes abundant differential DNA methylation, with slightly more hypermethylation than hypomethylation. Oluwayoise et al., (2021) also found an increase in DNA methylation in sperm from aged men in 91% of the CpG DMRs. These groups report that there is an association of the DMRs with genes that are necessary for fertility or neurodevelopment (Jenkins et al., 2014; Cao et al., 2020; Oluwayoise et al., 2021). The reliable alterations in DNA methylation have been used repeatedly to predict a man's age from his sperm epigenome, more recently getting accuracy within 2 years (Horvath, 2013; Jenkins et al., 2018; Cao et al., 2020).

In Brown Norway rats, a commonly used model for the study of reproductive aging (Wang et al., 1993), few studies have been done to suggest similar effects of aging. Oakes et al., (2003) found that there were alterations in ribosomal DNA methylation of rat sperm. The Pilsner group have reported that there are numerous sperm DMRs in aged rats treated with flame retardants, and that some were involved in embryonic development (Pilsner et al., 2021). The goal of this study is to examine whether there were alterations in post-meiotic spermatids prior to chromatin compaction, and in mature spermatozoa from the cauda of the epididymis through whole genome bisulfite sequencing (WGBS). We examined wild-type Brown Norway rats, at 4-6 months of age and 18-20 months of age, representing human populations of approximately 20-30, or 45+ years of age respectively.

Results

Differential DNA methylation in germ cells with aging

WGBS was used to assess the effects of aging on DNA methylation at CpG sites within the genome. We first examined differential methylation in round spermatids, and mature sperm. In round spermatids, the process of aging resulted in 5486 DMRs, with 70% hypermethylation and 30% hypomethylation at these regions (Fig 4-1). Mature sperm had fewer DMRs as a consequence of aging, with only 1888 DMRs. The proportions of DMR hyper and hypomethylation were similar to that of round spermatids, with 74% hypermethylation:26% hypomethylation. We mapped the DMRs to their chromosomal locations using Idiographica, and found that in either spermatids or sperm, the differential methylation was spread throughout the genome (Supp. Fig S4-1 and Supp. Fig S4-2). The majority of DMRs in both round spermatids and sperm were between 50-200 kb in length (Fig. 4-2B/D). However, sperm had 5 very long (> 1500 kb) DMRs that were not seen in the round spermatids.

Given that regions of low or intermediate methylation (20-80%) might be more susceptible to paternal exposures or lifestyle factors (diet, toxicants etc), we examined the percent change of methylation at DMRs (Fig 4-2). In round spermatids (Fig. 4-2A), just over half (60%) of DMRs had low methylation changes. The remaining DMRs had an intermediate change in methylation, ranging from 20-50% methylation. In spermatozoa (Fig. 4-2C), this was reversed as the majority of the DMRs were intermediate. Here, 35% of the DMRs had low DNA methylation changes, and 65% had intermediate DNA methylation changes.

Differential DNA methylation of genomic regions

Using HOMER annotation, we examined the genomic region of DMRs in round spermatids (Fig. 4-3A), and sperm (Fig. 4-3B). When examining all functional units of a gene, including: 3’ UTR, 5’ UTR, exon, intron, promoter-TSS and TTS there was more equal representation of hyper and hypomethylation types in round spermatids than in spermatozoa. There was also proportionally more DMRs in functional gene unit areas in round spermatids than there were in spermatozoa. This is to be expected as spermatids are transcriptionally active and require transcriptional control in gene regions, while sperm are transcriptionally inert. In sperm,

the DMRs in gene regions were more often hypermethylated with ~60% hypermethylation in gene regions. Specifically, in exons and promoters, there was 2/3 hypermethylation. This suggests that DNA methylation may play a role in transcription inactivation in sperm, as has been theorized previously. When examining the gene ontology (GO) of the DMRs in either promoters or gene regions, we see that there is little commonality amongt the pathways presented (Supp. Fig S4-3.). None of the promoter DMRs were significant in our analysis; however, in the spermatids, the promoters were hypermethylated at sites related to reproductive processes, while the sperm promoter DMRs were related to sexual reproduction and regulation of cellular processes. There were significant hypermethylated DMRs in GO pathways in spermatids for cell communication and signal transduction, and hypermethylated DMRs in GO pathways for membrane regulation, developmental processes, cell adhesion and calcium ion binding. In sperm, there was only one significant gene region DMR for cell morphogenesis.

Though there were DMRs affected by aging in all genomic regions, intergenic regions represented the majority of the genomic regions, 61% of DMRs in spermatids, and 71% of DMRS in sperm. This was overwhelmingly DNA hypermethylation, as approximately 3/4 of intergenic DMRs were hypermethylated. Introns were also areas with a high proportion of DMRs, with 18% of DMRs in spermatids, and 12% of DMRS in sperm.

Intergenic and LINE-1 differential DNA methylation

Though many of the intergenic DMRs in spermatids (Fig. 4-4A) and sperm (Fig. 4-4B) were in true intergenic regions, a fraction also mapped to simple repeat regions, satellite regions, SINE and LINE-1 elements, LTRs, rRNA, scRNA, and CpG islands. In sperm, there was also a tRNA region with differential methylation.

When examining the percent change of methylation in the DMRs, most of these again fell within the dynamic regions between 20-50% methylation difference. In round spermatids, there were 177 dynamic regions with intermediate methylation differences, and 103 regions with low DNA methylation changes. In sperm, there were 129 areas with intermediate methylation change, and 50 with low methylation change. Strikingly, approximately 80% of LINE-1elements in both spermatids and sperm were hypermethylated (% change greater than 0).

Differential methylation during spermatogenesis and sperm maturation

Recently advances have been made in understanding how DNA methylation changes during spermatogenesis and sperm maturation. Consequently, we designed this study such that we would be able to examine the effect of aging on these processes as well as in individual cell types. We examined the DNA methylation alterations due to aging in each individual cell type, round spermatids, or sperm, from aged rats compared to young rats. However, we also examined how the transition from round spermatid to sperm was impacted by aging at the DNA methylation level.

We found that when comparing sperm to round spermatids in a young BN rat, there were very few sites of differential methylation, there were 711 hypermethylated DMRS and 525 hypomethylated DMRS. When the same comparison was made in the aged rat though, there were almost seven times as many DMRs. When comparing the progression of spermatids to spermatozoa in aging, we observed 5387 hypermethylated DMRs and 3144 hypomethylated DMRs. This suggests that during the development of mature sperm from spermatids in BN rats, there is a major disruption in DNA methylation processes.

Discussion

The presented study demonstrates differential methylation in round spermatids and spermatozoa as a consequence of aging in Brown Norway rats through WGBS analysis. We found numerous sites of differential methylation across the genome, as a result of the aging process in these germ cells. These sites were predominantly hypermethylated DMRs. DMRs were found across genomic regions, some within functional gene regions.

The results presented here are consistent with other studies of paternal aging and sperm DNA methylation, in which hypermethylation was reported as an effect of aging in sperm. Most of the hypermethylation was found in distal or intergenic regions within the genome, which is consistent with research on DNA methylation in sperm with altered fertility status, aging, folate metabolism or lifestyle factors (Štiavnická et al., 2022; Cao et al., 2020; Chan et al., 2019; Youngson et al., 2016). Regions of particular interest include the satellite repeats, as Youngson et al. (2016) showed that obesity in rats altered DNA methylation at satellite repeats, but this

transmission was not conferred to the offspring. Additionally, we saw a number of DMRs within LINE-1 intergenic regions. Sperm LINE-1 hypermethylation has been observed in studies of exposure to alcohol or nicotine, and has been associated with overall chromatin inaccessibility and altered development in the early embryo (Zhang et al., 2019; Jachowicz et al., 2017). These results suggest that DNA methylation in intergenic regions may have consequences for the offspring.

In spermatids, these gene regions had potential involvement in cell communication, Ras protein signal transduction, membrane related genes, cell adhesion, calcium ion binding, and reproductive processes. Given that the round spermatids are still transcriptionally active, altered DMRs at this stage will alter the ability of the cells to develop functional spermatozoa. Interestingly, the methylation of the genes in similar pathways were altered in asthenozoospermic men (Du et al., 2016). In this way, genes involved in cell communication, cell adhesion, and reproductive processes may negatively impact the developing germ cells. Calcium ion binding gene regions having differential methylation may also impact the ability of the sperm to correctly undergo capacitation, a process required for fertilization (Hong et al., 1984).

It is striking how much of the differential methylation falls within intermediate levels of methylation within the context of aging in BN rats. Though, this dynamic methylation with 20-80% alteration, has been reported in other contexts for its susceptibility to the environment or lifestyle factors (Chan et al., 2019). However, this is the first study to examine DNA methylation during the transition of round spermatids to spermatozoa within the context of aging. Some studies (Chan et al., 2012; Ben et al., 2022) have examined the germ cells independently at each of these stages; however, the comparison has not often been made between the two cell types. Ben et al., (2022) examined germ cell DNA methylation during spermatogenesis in rats, but this group compared round spermatids to immature spermatozoa prior to transit of sperm through the epididymis. They reported that there were 710 significant DMRs during this transition, and that there were an additional 4 DMRs between caput and cauda sperm. These findings support the observation in our data that from round spermatid to cauda spermatozoa there are 1236 DMRs in a young Brown Norway rat. In our data though, we report that there are many more DMRs during this transition in the aged rat, thus suggesting that there are alterations in DNA

methylation processes as a consequence of aging in rat germ cells. Further studies are required to understand how these processes, and their machinery, are affected by aging. We can hypothesize based on the gross changes to sperm quality with aging, that there may be altered access to the chromatin due to the aging process that results in altered DNA methylation. There may also be disrupted DNA methylation/demethylation, or one-carbon metabolism that cause the altered methylation with aging. Chen et al., (2022) recently reported that in the mouse model the epididymis may actually confer a functional role in DNA methylation, as they saw DNA methylation patterns change during spermatozoa transit from testis to cauda epididymis.

In addition to further study on the process of DNA methylation during spermatogenesis and sperm maturation, there are additional avenues to be explored within our data. The stories present here are a brief analysis of the DNA methylation data. It will be advantageous to examine more closely the DMRs in gene rich regions, and to examine the DMRs that fall in CpG islands or shores. It will also be interesting to examine the proximity of DMRs. We also aim to explore the remaining methylated cytosines (CHH/CHG) and their locations or contexts within the genome, though it is presently predicted that they represent very little DNA methylation.

This is the first study to examine DNA methylation at the depth of WGBS in Brown Norway rats. It is also the first study to examine the transition of round spermatid to spermatozoa within the framework of aging, and provides insight into not only the cellular DNA methylation changes due to aging but how the inherent DNA methylation process may be altered during this crucial window.

Methods

Animal Care

Animal care and handling were done in accordance with the guidelines put forth by the Canadian Council on Animal Care (McGill Animal Resources Centre: Protocol 4687). Source of rats. All samples were collected from wild-type Brown Norway rats following euthanasia by carbon dioxide asphyxiation. Animals belonged to the young control group, 4-6 months of age, or the aged treatment group, 18-20 months of age. Testes were weighed upon sacrifice to assess

testis regression (weight < 1.5g), and regressed testes with accompanying epididymides were not used.

Sperm Collection

Epididymides were extracted from young and aged male rats. The epididymides were separated into the caput and cauda segments, and left in 1x phosphate buffered saline (PBS) to agitate on ice for 3 hours.. The sperm suspension was filtered on a 70 μM mesh. The sperm were then washed 10 times with 0.45% by volume saline solution (NaCl) to burst any remaining blood cells. The sperm were pelleted between washes at 2000 g for 10 minutes. After all washes were complete, the sperm were pelleted again at 2000 g for 10 minutes, and stored at -80◦C for DNA extraction.

Germ Cell Separation

Germ cells were separated using the STA-PUT method for cell velocity sedimentation (Bryant et al., 2013). Briefly, testes were decapsulated and mechanically separated prior to two 16-minute enzymatic digestion steps at 34 ◦C with agitation. The first digestion is done with 0.5 mg mL^{-1} collagenase (C9722-50MG; Sigma Aldrich, Oakville, Canada), the second with 0.5 mg mL^{-1} trypsin (Type I, T8003; Sigma-Aldrich) and DNase I (Type I, DN-25; Sigma-Aldrich). The first enzymatic digestion removes collagen between the seminiferous tubules, while the second breaks the seminiferous tubules into small pieces and releases the germ cells. DNase is used to aid in the breakdown of any free DNA to prevent cell clumping. The dissociated germ cell suspension was then filtered through a 70 μM nylon mesh before being washed three times with 0.5% bovine serum albumin (A4612; Sigma-Aldrich) in RPMI 1640 (Life Technologies, Grand Island, USA) and pelleted at 233 g for 5 min. Cells were filtered once more with a 55 μM mesh to prevent clumping, and 5.5×10^8 mixed germ cells in 25 mL of 0.5% BSA in RPMI were loaded into the STA-PUT over 50 minutes (Proscience, Toronto, Canada). The germ cells were then separated on a gradient of 2–4% BSA/RPMI, over 1.5 hrs. Using a fraction collector, the relative purity of germ cell fractions was assessed. Round spermatid (RS) fractions with a purity of at least 88% by phase-contrast microscopy identification were pelleted at 73 g for 5 mins, flash frozen, and kept at −80 ◦C for DNA extraction.

DNA Extraction

Total DNA was extracted using the QIAamp DNA mini kit (51304; Qiagen, Mississauga, Canada). DNA from spermatozoa was first treated with a sperm specific lysis buffer with final concentrations of: 0.15 M EDTA, 0.1 M Tris, and 40 mM dithiothreitol in sterile water. DNA from round spermatid was extracted as per user manual instructions.

Whole Genome Bisulfite Sequencing

Whole genome bisulfite sequencing was done by Novogene Corporation (Sacramento, California, USA). Briefly, the DNA is fragmented and undergoes a bisulfite conversion. The bisulfite conversion allows for the replacement of unmethylated cytosine with uracil, while 5-methyl-cytosine remains protected from conversion. The genomic DNA was then sequenced on the Novogene Illumina6000 platform and paired ended reads were generated. The samples are then processed and compared between treatment groups, any variation in uracil/cytosine allows for the detection of methylated or unmethylated cytosine.

Data Analyses

Initial data analyses were performed by Novogene Corporation (USA). Briefly, basic statistical analysis was done using FastQC (fastqc-v0.11.5), and clean reads were generated using the Trimmomatic (Trimmomatic-0.36) software using the parameters (SLIDINGWINDOW: 4:15; LEADING:3, TRAILING:3; ILLUMINACLIP: adapter.fa: 2: 30: 10; MINLEN:36). The clean bisulfite treated reads were then mapped to the bisulfite converted Brown Norway reference genome (rn_6.0) using Bismark software (version 0.16.3; Krueger F 2011). The methylation level was calculated by dividing the sequencing into bins, with bin size of 10kb. The sum of methylated and unmethylated read counts in each window was calculated, and corrected for the bisulfite non-conversion rate. Differentially methylated regions (DMRs) were identified using the DSS software (Hao Feng Hao Wu, 2014; Hao Wu 2015; Yongseok Park Hao Wu, 2016). DMRs were defined as related to genes, when the gene body region (TSS to TES) or promoter region (upstream 2kb from TSS) has an overlap with the DMR.

DMRs in the CpG context were annotated using HOMER annotation through R studio. DMRs were mapped to chromosomal regions using Idiographica (R-based). Graphs and frequency distributions were generated using GraphPad Prism (v. 9).

Data Availability

All data will be made accessible upon publication through GEO.

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Author contributions

HEF formulated the project, collected the cells, prepared the DNA for WGBS and wrote the manuscript. BR formulated the project, and wrote the manuscript.

Competing Interests

The authors declare no competing interests.

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Figure Captions

Figure 4-1. The number and proportion of DMRs in round spermatids from young (YRS) and aged (ARS), and sperm from young (YS) and aged (AS) of BN rats. Hypermethylation shown in orange. Hypomethylation shown in purple.

Figure 4-2. A) The percent differential methylation of the DMRs in round spermatids (blue), binned for 10% intervals (ex 10-20%). B) The size of DMRs in round spermatids, measured in kilobases (kb). C) The percent differential methylation of the DMRs in spermatozoa (purple), binned for 10% intervals (10-20% differential methylation). D) The size of DMRs in spermatozoa, measured in kilobases (kb).

Figure 4-3. A) Genomic locations of differential methylation in round spermatids, B) in sperm.

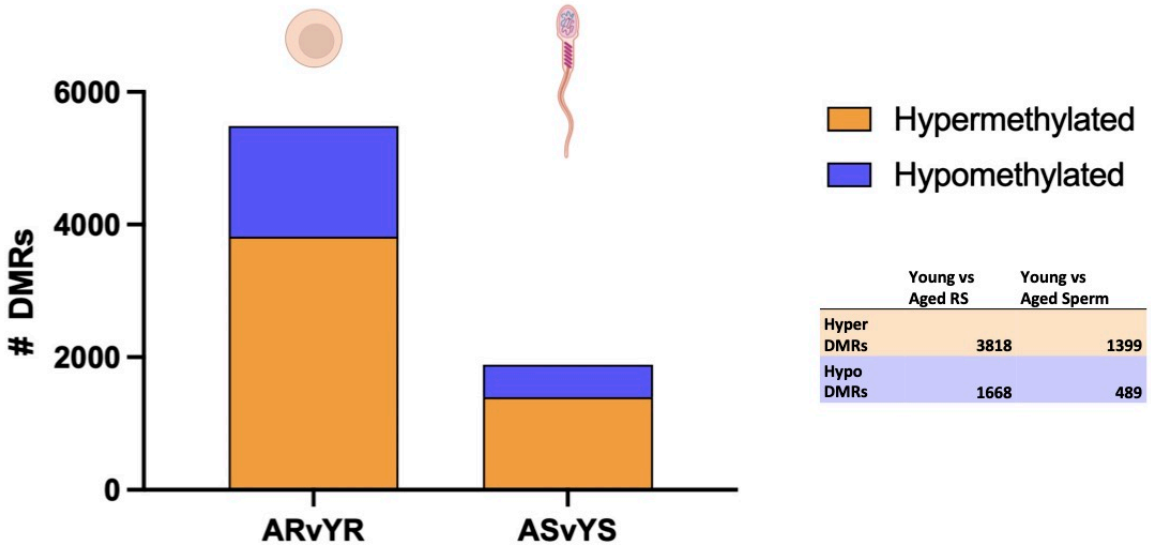
Hypermethylation shown in orange. Hypomethylation shown in purple.

Figure 4-4. Intergenic DMRs due to aging, broken down into proportion of type of intergenic region A) in round spermatids and B) in spermatozoa. LINE-1 percent differential methylation binned for 10% intervals in C) round spermatids (blue) and D) spermatozoa (purple).

Figure 4-5. The number and proportion of DMRs in independent germ cell types with age as a factor (left of dotted separation line), or with spermiogenesis and sperm maturation as a factor

(right of dotted separation line). Hypermethylation shown in orange. Hypomethylation shown in purple. ARS: round spermatid from aged, YRS: round spermatid from young, AS: sperm from aged, YS: sperm from young.

Figures



	Young vs Aged RS	Young vs Aged Sperm
Hyper DMRs	3818	1399
Hypo DMRs	1668	489

Figure 4-1. Differential methylation due to aging in round spermatids and spermatozoa.

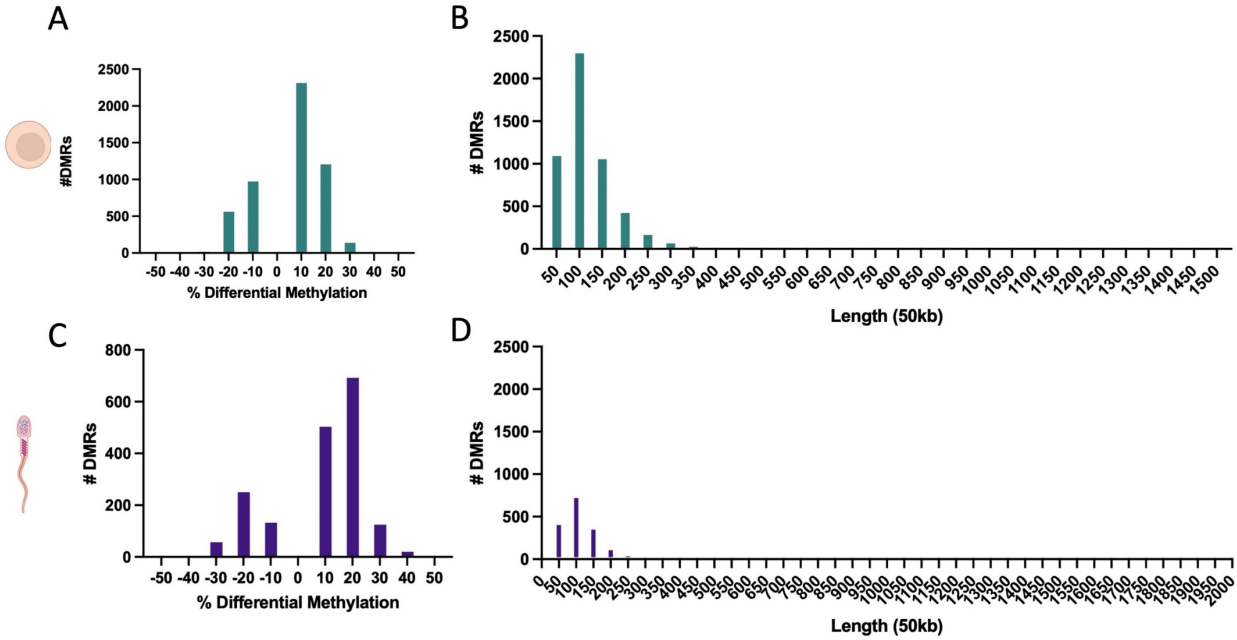


Figure 4-2. Characterization of differentially methylation regions due to aging.

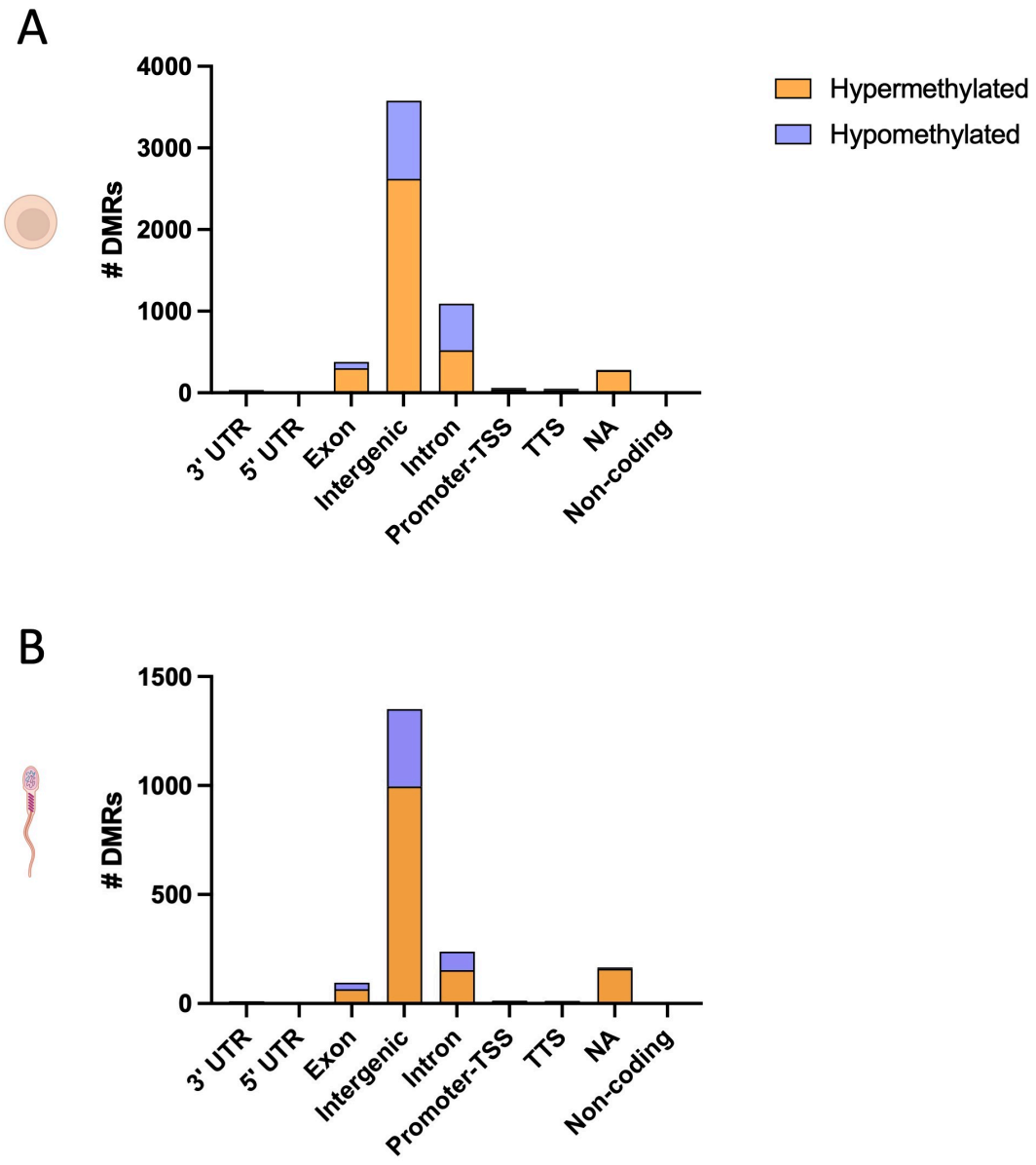


Figure 4-3. Location of differential methylation due to aging.

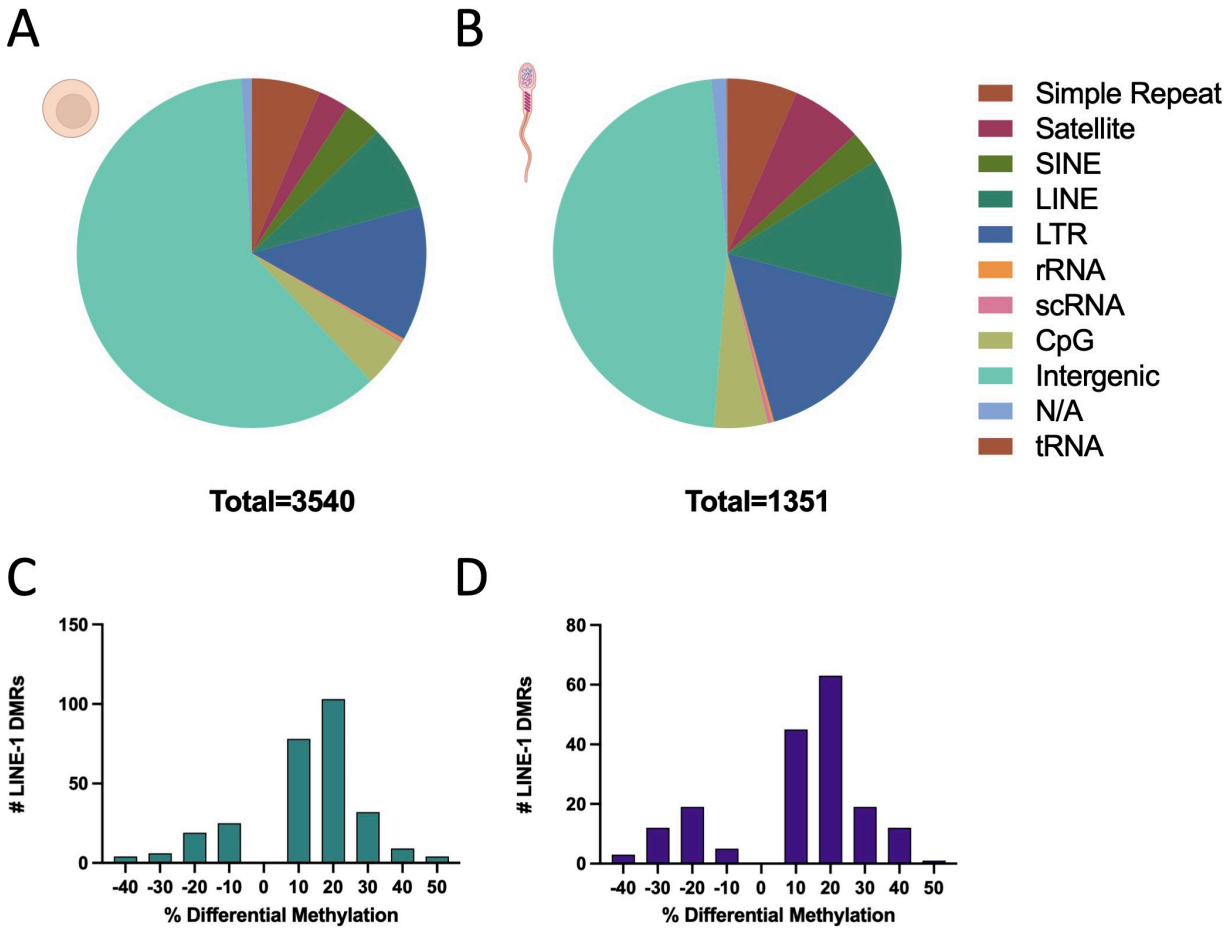
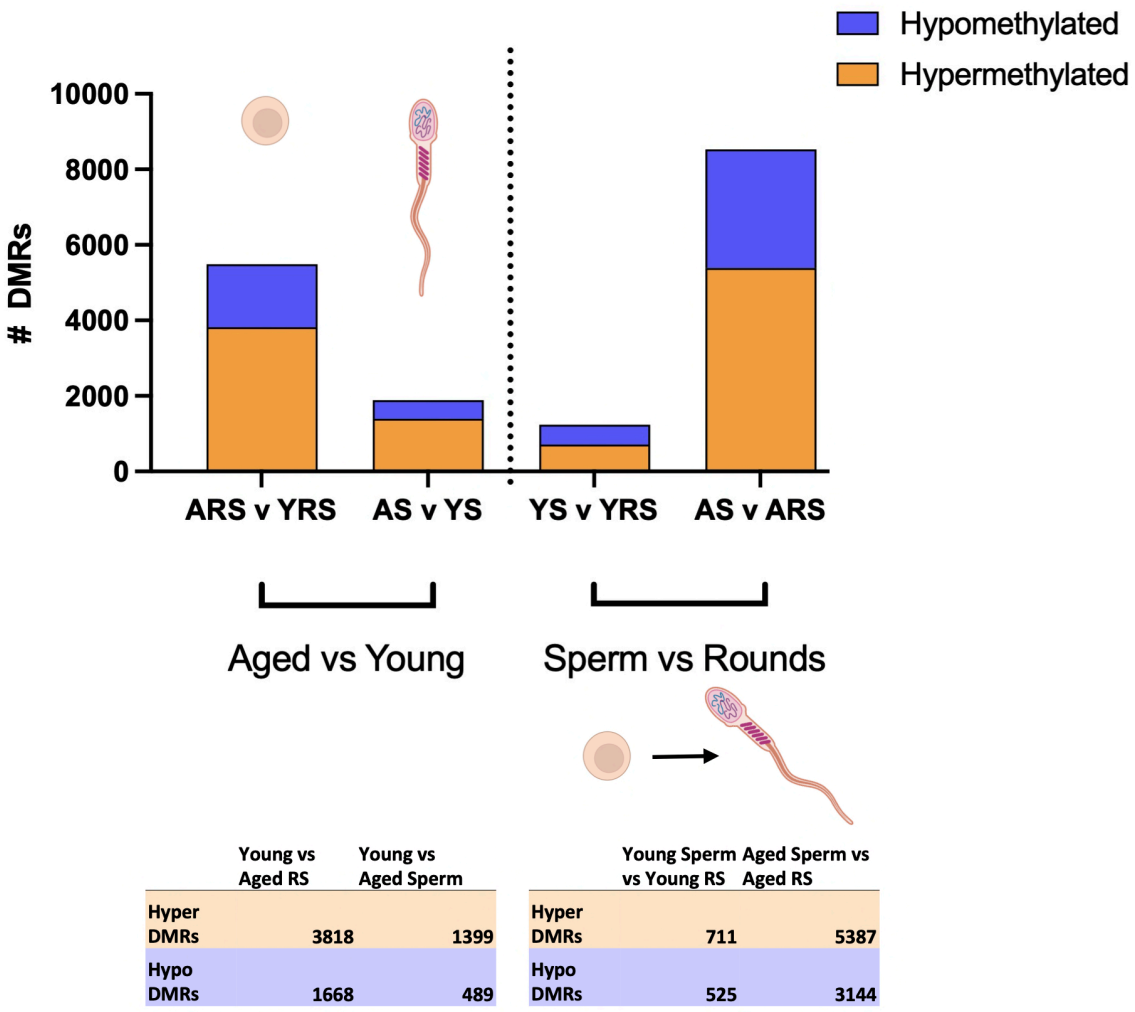


Figure 4-4. Differential methylation within intergenic regions of the genome.



	Young vs Aged RS	Young vs Aged Sperm
Hyper DMRs	3818	1399
Hypo DMRs	1668	489

	Young Sperm vs Young RS	Aged Sperm vs Aged RS
Hyper DMRs	711	5387
Hypo DMRs	525	3144

Figure 4-5. Differential methylation due to aging in germ cells, and due to the process spermiogenesis and sperm maturation.

Supplementary Figure Captions

Supplementary Figure 4-1: Differential DNA methylation in Brown Norway round spermatids mapped to each chromosome using Idiographica.

Supplementary Figure 4-2: Differential DNA methylation in Brown Norway spermatozoa mapped to each chromosome using Idiographica.

Supplementary Figure 4-3: Gene Ontology Analysis of differential DNA methylation sites in A) round spermatid promoters and B) gene regions, and in C) sperm promoters and D) gene regions.

Supplementary Figures

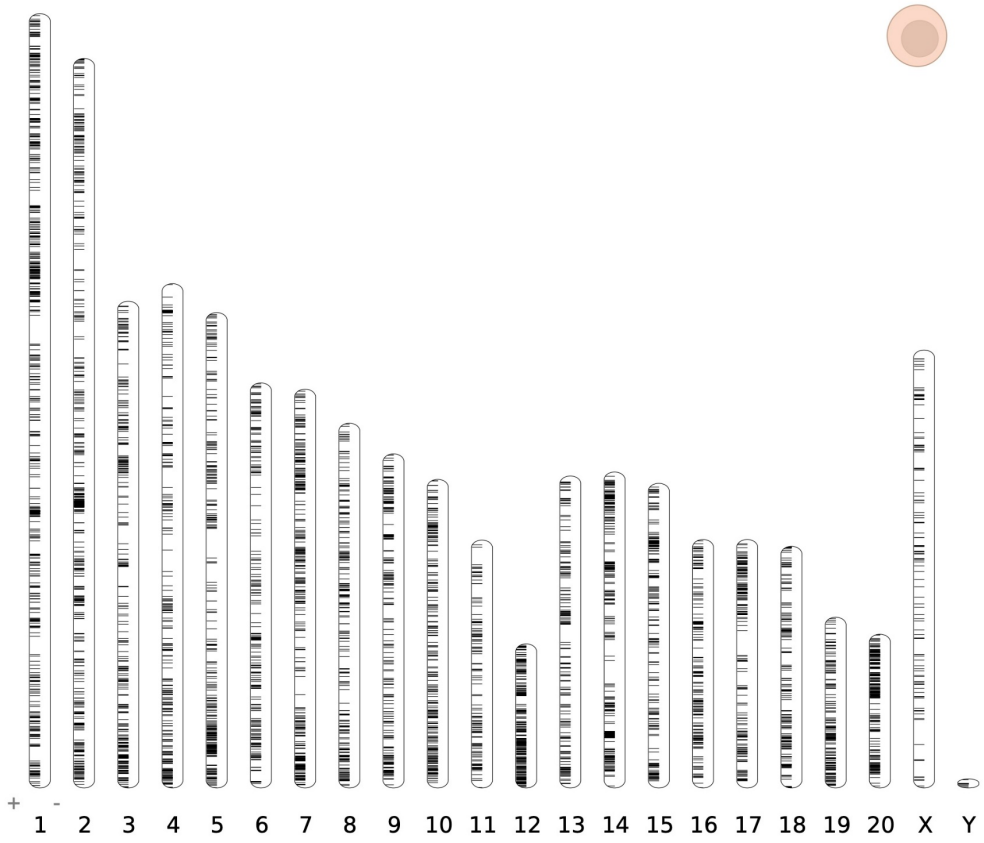


Figure S4-1. Genomic Location of Differential Methylation in round spermatids.

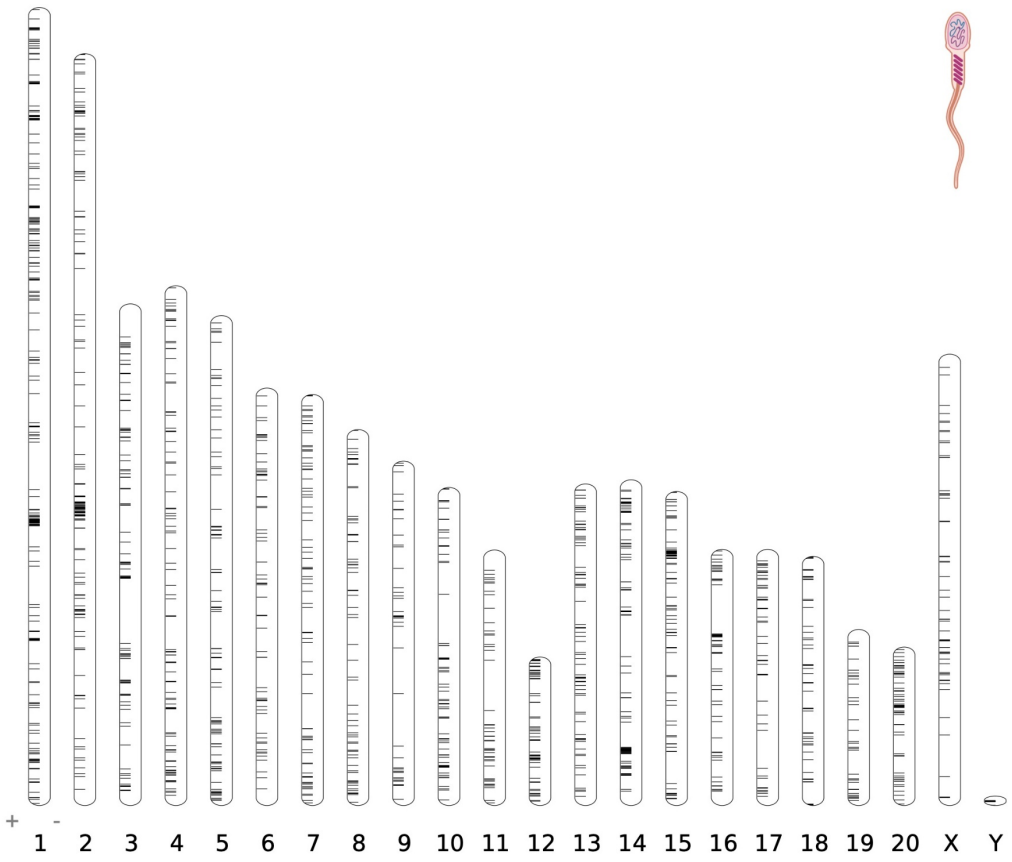


Figure S4-2. Genomic Location of Differential Methylation in sperm.

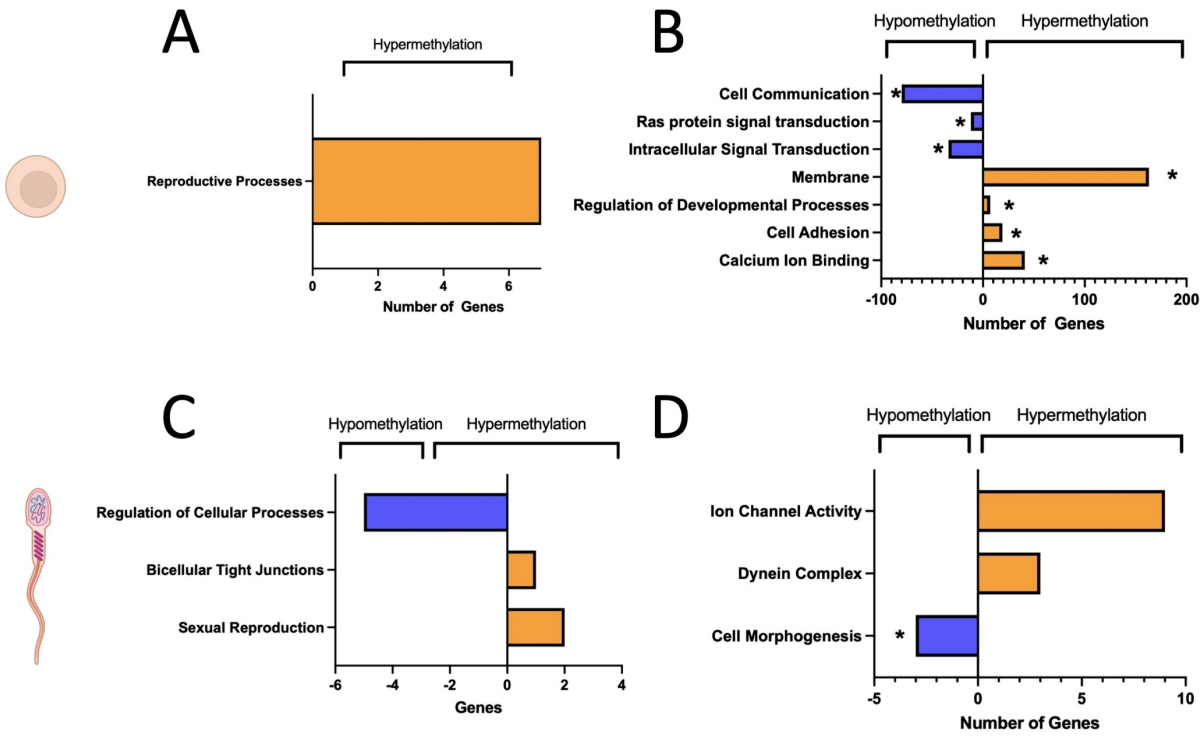


Figure S4-3. Gene Ontology of differential methylation in genes and promoters.

CHAPTER 5

DISCUSSION

5.1 Summary

The objective of this thesis is to examine how aging affects male germ cell DNA at various levels including telomere length, gene expression, and DNA methylation. The introduction to the thesis provides necessary background information on male reproduction, spermatogenesis, spermiogenesis, DNA organization and epigenetic mechanisms in male germ cells. Further, the introduction provides a review of the current knowledge of the effects of advanced paternal age on sperm quality, fertility, pregnancy outcomes, and effects on the offspring.

This work began by examining germ cell telomere length, both across spermatogenesis and in epididymal spermatozoa. We used two rat models, an outbred Sprague-Dawley rat and the inbred Brown Norway (BN) rat. In isolated pachytene spermatocytes and round spermatids, we capture the process of spermatogenesis from pre-meiotic and post-meiotic stages respectively. Through sperm from the caput of the epididymis we were able to examine sperm prior to DNA cross-linking and epididymal maturation, and in sperm from the cauda of the epididymis we examined mature sperm. To our knowledge, this is the first report of germ cell telomere length during spermatogenesis or epididymal transit. This is also the first study to examine sperm telomere length in a rat model of reproductive aging. Importantly, we noted that telomere length was consistent during spermatogenesis with a transient decrease upon entry to the epididymis that was recovered after maturation. With aging, we showed that 1) sperm telomere length in caput or cauda sperm was shorter with aging and 2) that the increase in telomere length during epididymal transit was lost.

In the 3rd chapter, gene expression analysis was done to compare isolated round spermatids from young and aged BN rat. We found that with aging there was a general up-regulation of gene expression for 211 of the 220 significant differentially expressed genes (DEGs). Many of these transcripts belonged to pathways involved in the immune response and inflammation, cytoskeletal processes, and various forms of cell signalling. After acknowledging the biases of pathway analyses, we sorted the transcripts based on their known reproductive function based on the literature as of July 2022. We found that 169/220 transcripts had predicted or known function in male reproduction. These functions included sperm motility and

morphology, sperm-egg binding, capacitation reaction and calcium homeostasis, Sertoli-germ cell interactions, and epigenetics. Importantly, we are the first to report a potential disruption of round spermatid signalling to the Sertoli cells with aging.

The 4th chapter presents the beginning of our analysis of the DNA methylome in round spermatids and spermatozoa from the BN rat with age. Here, we report the number of differentially methylated regions (DMRs) in round spermatids and sperm due to age. We highlight that with age there are many hyper-DMRs in both spermatids and sperm. We also suggest that these hyper-DMRs mainly fall in intergenic regions of the genome, such as in LINE-1 regions, though there are some DMRs within functional gene units. As of now, no such study has been done to examine DNA methylation in rat round spermatids or spermatozoa using whole-genome bisulfite sequencing.

This final chapter addresses the key findings of this thesis, the significance and clinical implications of the findings. Limitations of the studies presented will be discussed. This chapter will also highlight future research that can be done based off of the findings presented within the thesis.

5.2 Telomere dynamics with aging – a role for the epididymis?

Increasingly, associations are being drawn between sperm telomere length and classical semen parameters, such as sperm count. The overarching conclusion is that altered telomere length is associated with poor fertility, and this may have an effect in the potential offspring (Gentiluomo et al., 2021). Many studies examining telomere length and fertility have shown that infertile men typically have shorter sperm telomeres, and poor pregnancy outcomes (Ferlin et al., 2013; Thilagavathi et al., 2013; Rocca et al., 2016; Biron-Shental et al., 2018; Lafuente et al., 2018). With aging, other groups have reported that sperm telomere length increases with a man's age (Kimura et al., 2008; Laurentino et al., 2020). The hypothesis is that there must exist a telomere homeostasis that determines fertility (Reig-Viader et al., 2014). Once the boundaries around optimal telomere length are set, it is not unreasonable to predict that telomere length may

be an easy biomarker for fertility (Cariati et al., 2016). The majority of these studies have been done in human sperm samples, given the ease of access to sperm samples from men. Very few studies have been done examining sperm telomere length in rodents, with one study done in mature sperm from mouse (de Frutos et al., 2016). They reported that sperm telomere length decreased as a result of aging in the mouse, in contradiction with what had been observed in the human. We aimed to address this discrepancy in the most commonly used rodent model for the study of reproductive aging, the Brown Norway rat. The Brown Norway rat, has served as the model for many of the key findings that have shaped our knowledge of reproductive aging in man. It is thus important to understand how a robust rodent model compares to the human data. We found that with age in a rat model, sperm telomere length decreases as was seen in the mouse. This is in direct contradiction with what is seen in the human.

There exist two hypotheses about what is causing the discrepancy in telomere length patterns with aging, generated in *Drosophila melanogaster* (Wallenfang et al., 2006). The first is that the spermatogonial stem cells (SSCs) undergo a sort of natural selection, where over time only the strongest and best cells remain and these may have longer telomeres. The second hypothesis hinges on the fact that germ cells contain active telomerase. Here, the SSCs not only maintain telomeres from degradation, but with time actually increase their length. Each hypothesis is able to explain the discrepancy we see between rodent and man, due to the drastically different lifespan of each species. In humans, the stem cells would simply have more time (40+ years) to undergo selection or telomere lengthening, whereas rats have no more than 20 months.

In the future, it will be beneficial to examine the telomere length of SSCs from young and aged animals. With the development of novel methods to isolate SSCs, this will become more feasible. Presently the only method by which successful isolate of SSCs is accomplished, is with flow-cytometry analysis (FACs). Our concern in using this method was the potential for introducing DNA damage to the cells after subjecting them to lasers for sorting. We began to characterize telomere length during the remaining stages of spermatogenesis though, by examining the pachytene spermatocytes and round spermatids. Telomere length measurements had not been done before in these cells, though the presence of telomerase had been shown (Ozturk, 2015). We found that telomere length remained relatively consistent when comparing

pachytene spermatocytes and round spermatids, despite the previously reported presence of the enzyme. This suggests that despite the presence of telomerase in these cell types, it may not be actively elongating telomeres in these stages. The conflicting results support a need for further research examining telomerase activity in all germ cell types including the SSCs.

Though telomere length appeared stable during spermatogenesis, we noticed an interesting trend in control rats where there is an apparent decrease in telomere length when the sperm enter the caput of the epididymis before undergoing maturation. The apparent decrease in telomere length is transient, and when the sperm have undergone maturation and are being stored in the cauda, their telomere length is not significantly different from the testicular germ cells. With age however, there is no increase in telomere length during epididymal maturation. This posed an interesting question regarding what the epididymis may contribute to telomere organization or stabilization, and how that may change with age.

A study by Sharma et al., (2018) looked at small RNAs that are trafficked to sperm in the caput of the epididymis. They found that the proportions of small non-coding RNAs (sncRNA) in the sperm were drastically different in the testis, and the epididymis. In the testis, the majority of sncRNAs were piRNAs, while in the epididymis the majority were tRFs. The enrichment of tRFs was also stronger in sperm from the cauda, than from the caput of the epididymis. Caput sperm contained more microRNAs (Sharma et al., 2018). A particularly useful element of this study, was the use of co-culture of the isolated epididymosomes from the caput of the epididymis with testicular sperm. This would be particularly useful for future experiments to ascertain the role the epididymosomes may have in sperm telomere maintenance. Here, epididymosomes could be isolated from the caput, corpus, and cauda of the epididymis and co-cultured with testicular spermatozoa. The average telomere length could then be measured in sperm to see if epididymosomes are contributing something, and at which stage of maturation.

Additionally, non-coding RNAs from the epididymosomes could be sequenced. There are few known regulators of telomere length, mainly including TERRA and TERC. TERC is the lncRNA component of the telomerase enzyme complex. TERRA is another lncRNA, transcribed from telomeric regions and suppresses telomere elongation, while potentially protecting them from degradation (Grammatikakis et al., 2014). TERRA in particular has been recently

associated with infertility in men (Rocca et al., 2021). Additional ncRNAs can be found in the microRNA family, GUARDIN a lncRNA, the Shelterin complex, and tRFs (Rossi and Gorospe, 2021). Each of these RNAs should be examined within the context of sperm telomere maintenance, especially with age.

It is also possible that the reactive oxygen species (ROS) levels within the epididymis transiently alters telomere length. A study presented by Mishra et al. (2016) suggested that mild ROS is beneficial in the maintenance of telomeres. The epididymis itself contributes varying antioxidant enzymes during sperm maturation, in an attempt to protect the sperm from DNA damage (O'Flaherty, 2019). It is possible that the balance between necessary or beneficial ROS, and abundant ROS due to aging is altered in the epididymis as has been seen with other models, and may also have effects on telomere maintenance (Noblanc et al., 2020).

Another avenue to explore is the possibility that the DNA packaging is transiently modified upon entry into the epididymis, as proposed by Chen et al. (2022). This group found that sperm from the caput epididymidis displayed distinct DNA methylation marks, that render it at risk to DNA damage and able to bind extracellular DNA. It is feasible then, that the transient change in chromatin packaging may also alter the conformation of telomeric regions. To address this, differential DNA methylation could be examined between sperm from the caput and cauda of the epididymis – specifically examining the subtelomeric regions. The telomeric regions themselves have histone markers, but do not contain CpG islands or DNA methylation (Gadalla et al., 2012). However, subtelomeric regions are known to have increased DNA methylation and are enriched in CpG islands (Blasco, 2007). Mouse models have shown that DNA methylation in the subtelomere may have telomere length regulatory roles (Schoeftner and Blasco, 2009).

5.3 Gene expression with aging – a role for the Sertoli cell?

When considering the effects of advanced paternal age on the quality of the sperm, the viability of any potential embryos formed, and the genetic disorders observed in the offspring, understanding how the genetic contributions of the sperm may be altered with age is of critical importance. However, the limited studies of age-related alterations in germ cell gene expression have focused predominantly on specific sets of transcripts, such as those involved in the regulation of DNA damage repair (Paul et al., 2011). The other classic approach when studying germ cell gene expression is looking at the whole testis (Han et al., 2021). This approach makes it difficult to detect small scale changes in transcript levels, and to determine the origin of potential alterations. Recently, a study was done by Nie et al. (2022) examining single-cell RNA-sequencing of all testicular cells from human. They found that most testicular germ cells did not have major gene expression changes due to aging until the elongating spermatid stage (Nie et al., 2022). To our knowledge, our study examining gene expression in round spermatids with aging is the only study that examined the transcriptome of a single rodent cell type. It is likely that, though significant, the gene expression changes that we observed in round spermatids would be undiscernible in a whole testis experiment. Additionally, understanding the potential outcomes of these age dependent alterations would be completely lost.

As the majority of the alterations were classified to immune regulatory, and cell signalling pathways these would very likely have been attributed to the somatic cells of the testis. While it is logical that these pathways would be altered due to aging in the somatic cells, such as the Sertoli cells, it is entirely plausible that round spermatid signalling is also disrupted. Previous studies, examining either histology or a select few mRNAs, have suggested that there was an age-related impediment in communication between Sertoli and germ cells, resulting in failure of Sertoli cells to provide support to developing germ cells (Levy et al., 1999; Syed and Hecht, 2001; Syed and Hecht, 2002). Our study provides potential support for this hypothesis, by showing both an alteration in cell communication transcripts and transcripts required for the direct interaction or physical support between germ and Sertoli cells. Critical to these findings is an understanding of the method for germ cell isolation, STA-PUT gravity sedimentation, as there is less than 12% contamination of the round spermatid fractions. This contamination is entirely comprised of residual bodies or spermatids that have begun the elongation process and show early stages of acrosome development.

There are two major experiments that could be done to confirm the hypothesis of disrupted germ cell-Sertoli cell communication and interaction as a product of age. The first would be to directly examine the junctions between round spermatids and Sertoli cells in young and aged rats, potentially using immunohistochemistry. With aging, studies have been done examining tight junction component proteins and transcripts from spermatocytes (Paul and Robaire, 2013). However, it has been shown that round spermatids interact with Sertoli cells using adherens junctions, intermediate filament attachments (desomosomes and hemidesmosomes), and communication junctions (gap junctions) (Cheng and Mruk, 2002). It would thus be interesting to examine the following testis-confirmed junctional molecules: N and E-cadherin, catenins, alpha and F-actin, integrin, paxillin, vinculin, and connexins. It is also important to consider the cytoskeleton and extracellular matrix proteins within the germ cell, as these may contribute to the function and anchoring of junctional molecules (Cheng and Mruk, 2002).

The second major experiment that could be done is to address communication between round spermatids and Sertoli cells. The communication between cells may be assessed by a few approaches. One approach would be to perform RNA-sequencing in the Sertoli cell, and examine if there are transcriptional changes in the downstream effectors of altered transcripts observed in the round spermatid. This could be done using a pathway analysis software, or manually. If the RNA sequencing were done using single-cell RNA-seq (scRNA-seq), there exists a novel software tool through R, called CellChat (Jin et al., 2021). This tool is an integrated web-based program that allows examination of the interaction between two cell types either with ligand-receptor activity, or with their Cell-Cell communication atlas.

An area that has not been considered in the context of aging is the regulation of Sertoli-germ cell interactions via lipid and protein kinases. Siu et al. (2005) reported that there was an activation in many protein kinases when Sertoli and germ cells were co-cultured, suggesting that molecules such as phosphatidylinositol 3-kinase (PI3K), phosphorylated protein kinase B (PKB/Akt), p21-activated kinase-2 (PAK-2), their downstream effector (ERK) are all involved in regulating communication between these cells. They also reported that the ERK signalling pathway may be involved in regulating laminin-integrin in the adherens junctions between germ and Sertoli cells. When they perturbed members of these pathways, there was a resulting germ

cell loss. It remains possible that this pathway is an additional contributor to the deterioration of Sertoli cell support, and should be considered in future analyses.

A final area of exploration regarding round spermatid-Sertoli interactions, would be doing co-culture experiments. This could be designed to examine if the signalling breakdown may be originating with round spermatids, or with the Sertoli cells. There would be two controls, 1) young RS and young Sertoli cells 2) aged RS and aged Sertoli cells. The experimental groups would be 1) young RS and aged Sertoli cells and 2) aged RS and young Sertoli cells. Then using either transcriptomics or metabolomics, one could assess which cell groupings are similar to the communication alterations we observe with aging.

In addition, we observed increased expression of many genes related to sperm-egg binding and fertilization. Given that we saw altered transcripts with calcium regulatory functions, one major area to study is capacitation. Capacitation is a process which includes, among many other processes, an influx of calcium into the sperm, which allows for the hyperactivation and the acrosome reaction of the sperm. This cascade of events is necessary for proper fertilization (Ickowicz et al., 2012). This has not been studied in depth within the context of aging, however recent reports are suggesting that there is a reduction in sperm capacitation with aging. Specifically, in hamsters there was an age-dependent reduction in capacitation and hyperactivation (Miyashita and Fujinoki, 2022). Additionally, Sharara et al. (2022) reported that men seeking fertility assistance had equal reductions in capacitation ability regardless of age; however they did not compare with a fertile population. It is possible that the capacitation reaction is compromised in the sperm of aged males. Previous studies examining sperm capacitation from aged mammals have pointed to the epididymis as the problem, suggesting that inherent differences in the epididymis may alter the sperm's ability to undergo capacitation (Cuasnicu and Bedford, 1989). Our research suggests that the issue may be inherent to the sperm and a product of aberrant gene expression.

We also saw alterations in genes encoding mannose-binding proteins. This is of particular interest as loss of mannose or mannose-binding molecules have been shown to be associated with infertility, and the balance is crucial first for capacitation, and second for sperm-egg binding (Hershlag et al., 1998; Gamzu et al., 2009; Olejnik et al., 2015). Early work suggested that the

level of mannose receptor sites on the sperm head was dependent on the acrosome reaction, and the presence of cholesterol in the sperm plasma membrane. The proposed model suggests that mannose receptor ligands are initially protected by cholesterol in the membrane (Benoff et al., 1993 a,b,c). The removal of cholesterol from the membrane is a characteristic step of the late stages of capacitation, allowing for membrane permeability and the acrosome reaction to take place (Jin and Yang, 2017). Mannose receptor availability may contribute by allowing for acrosomal exocytosis and zona pellucida and sperm binding (Benoff et al., 1993 a,b,c).Mannose-binding molecules on the sperm recognize mannosyl residues on the zona pellucida and facilitate the binding of sperm and egg, by acting like a receptor-ligand interaction (Yoshida-Komiya et al., 1999). When examining our data, this requires further exploration as both lipid regulation transcripts and mannose-binding transcripts were disrupted, suggesting more than one disruption in these regulatory processes. This has not yet been studied in the context of aging, but it might help to explain decreased sperm fertilizing abilities with aging.

The final major class of dysregulated transcripts was those involved in epigenetics. Specifically, there was an increase in the expression of *Folr1* and *Folr2*, which are both interesting in the context of one-carbon metabolism and DNA methylation. Folate status has been studied extensively in the context of reproduction and epigenetics; the predominant find was that dietary folate supplementation does not alter sperm DNA methylation (Chan et al., 2017; Schisterman et al., 2020; Jenkins et al., 2022). It has been proposed that sperm Folr1 may have a role in maintaining cellular folate, and thus be implicated in DNA replication and establishment of methylation patterns in the embryo (Holm and Hansen, 2020).

5.4 DNA methylation with aging

Germline DNA methylation presents one mechanism of epigenetic inheritance. Hypermethylation results in very tightly condensed chromatin that is largely inaccessible to transcription machinery thereby blocking transcription. Hypomethylation results in more open chromatin and potentially increased gene expression in these regions (Trasler, 2009). Based on the global increase in gene expression observed in Chapter 3, we hypothesized that there may be

an accompanying hypomethylation at these gene regions. Overwhelmingly though, we observed hypermethylation across the genome. Correlating the gene expression changes observed with direct analysis of methylated cytosines within the gene regions would explain this story in greater depth. In an attempt to address this, we used the Idiographica software used in Chapter 4, to map the differentially expressed genes (DEGs) from Chapter 3, with the differentially methylated regions (DMRs) in Chapter 4 (Fig. 5-1). Specifically examining round spermatids, we do see an overlap in the hypomethylated DMRs and DEGs with increased expression. This suggests that though the majority of the DNA methylation observed is hypermethylation, the hypomethylation may have functional significance in gene regulation. To continue this analysis, we will examine specific DEGs and proximal and distal methylation sites.

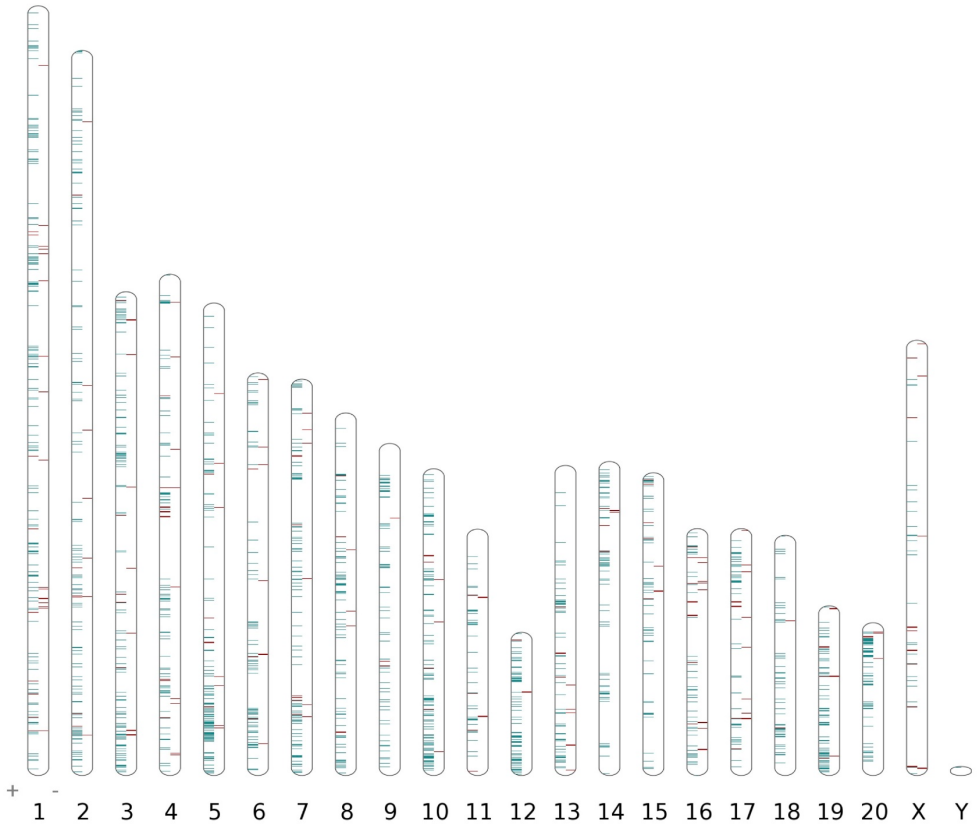


Figure 5-1. Round Spermatid Chromosome Mapping of Differential Gene Expression and Differential DNA Methylation with aging. DMRs shown in blue, DEGs shown in red.

It may be the case as well, that DNA methylation of individual genes is not altered with aging but there is altered DNA methylation of regulatory elements. Within the RNA-sequencing dataset we observed that there were common upstream regulators of many transcripts with increased expression. Though these regulators were not found to be significant within the RNA-seq analysis, it may be possible that a modest shift in DNA methylation of regulatory regions may significantly alter expression of the downstream targets. Thus, examining the cis-regulatory elements, transcription factor binding sites, and higher order chromatin organization through WGBS is also a goal of this objective. Two proteins of particular interest are CCCTC-binding factor (CTCF) and its testis specific paralog BORIS. CTCF/BORIS are DNA binding proteins, that govern both the formation of DNA loops/3D genome organization, and transcription at many sites throughout the genome (Hernández-Hernández et al., 2016; Sleutels et al., 2012; Ma et al., 2018). If CTCF binding sites are differentially methylated as a consequence of age, this may have an impact on the 3D organization of the genome, the varying interactions of chromosomes, and potentially gene transcription.

Sperm DNA methylation defects have been reported as a product of age in both rodents and men. The DNA methylation pattern observed is so strongly correlated that it can be used to predict the age of a man, with very little error (Horvath, 2013; Jenkins et al., 2018; Cao et al., 2020). The trend observed is for hypermethylation in intergenic or distal gene regions, and hypomethylation found proximal to gene regions (Cao et al., 2020). Our results fit within this context, as the majority of DNA methylation is hypermethylation in the intergenic regions of the rat genome in both round spermatids and spermatozoa. What remains to be elucidated from these data, is the functional significance of the intergenic or non-gene region DMRs. It has been proposed that some intergenic sites have roles in fertility and embryo development. Specifically, the expression of LINE-1 elements is implicated in fertilization and chromatin reorganization in the embryo. One study suggesting that LINE-1 hypermethylation, and the subsequent decrease in expression impedes chromatin accessibility in the early embryo (Jachowicz et al., 2017). In both round spermatids and sperm, we saw that LINE-1 elements developed hypermethylation with aging, suggesting this may be one potential cause of pathologies such as increased spontaneous abortion rate.

DNA methylation may in part control telomere length or telomere dynamics. Given the evidence that DNA methylation of the subtelomere may have regulatory roles in telomere length maintenance, it would be interesting to examine DNA methylation within the telomeric and subtelomeric regions of spermatids and sperm (Schoeftner and Blasco, 2009). Additional experiments to examine sperm from the caput of the epididymis for the telomeric/subtelomeric regions may be interesting in the future as well. This may help to explain the observation of sperm telomeric length increase between the caput and cauda of the epididymis.

There is evidence that hypomethylation of transposable elements, found in repeat regions of the genome, may lead to reproductive decline and transgenerational effects (Rey et al., 2016; Karahan et al., 2021). Transposable elements in the germline are of particular interest as they are classically highly methylated, thereby preventing their transcription and potential insertion into the genome (Molaro et al., 2011). If these regions have altered methylation as a result of aging, it may result in the insertion of jumping genes that have otherwise been repressed. These regions also represent as much as half of the mammalian genome, potentially providing a major source of genetic perturbation with age (Lander et al., 2001).

The major story that remains to be explored is differential DNA methylation inherent to the process of spermiogenesis and sperm maturation. Preliminary analysis of DNA methylation when comparing sperm and round spermatids suggests that with aging, there is aberrant DNA methylation. We observed less than 2000 DMRs between spermatids and sperm in the young rat, however in the aged saw over 8000 DMRs. In the aged rat, we also observed that a large proportion of these were hypomethylated DMRs. This needs further exploration, to understand the genomic location of DMRs, their size, and potential role or function. The analyses completed when comparing spermatids and sperm from young or aged rats will be replicated in the context of spermatogenesis.

5.5 Future Directions of Study

The common theme throughout this thesis is the effect of age on germ cell DNA, its modifications and expression, the next logical step in the pursuit of understanding how aging may affect germ cell DNA, is putting all of these pieces together and examining chromatin

organization within the nucleus. We know that sperm DNA is packaged differently within the nucleus which allows for small cell size, streamlined motility, and added DNA protection. This packaging is through protamines toroids, with histone-containing regions, and linker regions (Oliva, 2006). These linking regions anchor the DNA to the nuclear matrix, and provide functionality in the embryo when the paternal genome is repackaged and activated. Based on the nuclear matrix model of DNA packaging in sperm, it has also been shown that if protamines are experimentally removed what remains are DNA 'loops' tethered to the matrix (Kramer and Krawetz, 1996; Gawecka et al., 2013). Within the field of andrology though, what remains to be determined is whether the DNA 'loops' associated with the nuclear matrix confer broad genomic significance.

In recent years, cancer cell lines have been studied to examine the levels of organization of DNA. Chromosome territories, can first be broken down into compartments, followed by topologically associated domains (TADs), and finally chromatin loops (Fraser et al., 2015). Limited studies have been done using chromatin conformation capture (3C) methods in male germ cells, often due to the difficulty in performing these experiments. Initial studies were done comparing mouse fibroblasts or embryonic stem cells and sperm, and suggested that these cell types had similar spatial DNA organization (Jung et al., 2017; Battulin et al., 2015). Studies on sperm also report that there are increases in interaction frequency across the genome after spermiogenesis, which fits with our understanding of DNA compaction during this process (Alavattam et al., 2019; Ke et al., 2017). Interestingly, these studies also suggested that within the A compartment, typically transcriptionally active DNA, there were more unmethylated CpGs (Ke et al., 2017). Wang et al. (2019) reported that the during spermatogenesis DNA organization is fluid, and does not follow the classic somatic cell genome organization. There are defined TADs in spermatogonia that are lost during the early stages of spermatogenesis and seemingly re-established in the mature sperm. This suggests that during spermatogenesis the replicating, dividing, and transcriptionally active DNA is much more fluid around the nucleus. They support this claim with data to suggest that the transcriptionally active genes do not fall into the classical A compartment, and suggest that a new model is necessary when describing genome organization during spermatogenesis. This is an interesting finding, as Gaysinskaya et al. (2018) reported a transient decrease in methylation during the spermatocyte stage in mice.

What is necessary moving forward is a synthesis of what is known, and a collation of the data within each species. In this thesis, we report all gene expression in round spermatids in control and aged Brown Norway rats, and the WGBS methylation profile for round spermatids and mature spermatozoa. The next logical step in this progression would be using a Hi-C based method to examine genome organization in the BN rat, and to assess the interplay of gene expression, DNA methylation, and organization. Understanding sperm genome organization, and the interplay with epigenetics and genetics, would help us to understand better the events after fertilization. It then follows that this could be applied to any system, including infertility, drug treatment, toxicant exposure, and aging. It would be fascinating to understand how this elegant process may be perturbed, and the outcomes it would have on other critical elements of spermatogenesis and sperm quality. In a mouse model, the Corces group (Jung et al., 2022) recently combined methods for chromatin accessibility, DNA methylation, transcription factor binding, RNA-sequencing, and the transgenerational inheritance of Bisphenol A (BPA) induced obesity. Similarly, a study could be designed to combine the analysis of gene expression, DNA methylation, chromatin accessibility (ATAC-seq), and chromatin interaction frequencies within the framework of aging.

A major consideration when planning these studies however, is the inherently compact nature of sperm DNA. While it may sound trivial to use these methods in a sperm model, that is not the reality. Hi-C sequencing requires the use of restriction-enzymes, which have poor access to sperm DNA given the protamine packaging. Moritz and Hammoud (2022) are also quick to identify the numerous challenges with Hi-C based methodology in sperm. A critique of many studies using Hi-C, has been the reliability of these data and if the interactions are instead just artifacts of opening the DNA. Advancement and novel methods, both at the bench and with bioinformatics, are required before these methods are robust in studies using sperm.

5.6 Clinical Implications

The study of advanced paternal age is not new, and its effects on reproduction, fertility and offspring health are increasingly well characterized within the literature. At the level of the germ

cells in men, we know that with aging there are disruptions in sperm quality, motility, and morphology (Beguería et al., 2014). Paternal age then has implications for a couple's overall fecundity, with negative effects on spontaneous abortion rate and time to pregnancy (Sharma et al., 2015; Bray et al., 2006). When successful pregnancy is achieved, there are reported pregnancy complications such as an increase in the frequency of preterm births and preeclampsia with advanced paternal age. Finally, at the level of the children born to older fathers there are increased incidences of numerous genetic and neurodevelopmental disorders (Toriello and Meck, 2008). The risk of children from fathers over 40 developing achondroplasia increases by approximately 8x (Tiemann-Boege et al., 2002), and the risk of developing autism increases by 5.75x (Reichenberg et al., 2006). In fathers over 50, the risk of developing schizophrenia is 3 times as high for their children (Malaspina et al., 2001).

These epidemiological studies have been shared with clinicians and have been used to inform clinician databases, such as UpToDate, and guidelines from various societies including the American College of Medical Genetics (Harris, 2021; Anderson et al., 2002). These information sources share the risks associated with paternity in those greater than 40 years old, which is the generally accepted threshold for advanced paternal age, including by The Society of Obstetrics and Gynecology of Canada (Liu and Case, 2011). The American Society for Reproductive Medicine suggests that sperm donors be no older than 40 to prevent age related fertility issues (Toriello and Meck, 2008). Though the risks of advanced paternity are recognized, there exist no firmly set restrictions on assisted reproduction for men over 40 (Jennings et al., 2017).

Recently, these population-based studies have been followed by various studies examining the mechanisms underpinning advanced paternal age alterations, such as those presented in this thesis. We have begun to characterize the genomic and epigenomic alterations that may lead to these consequences in the offspring. These studies support the need for set restrictions, as we better understand the effects of advanced paternal age.

As men are able to produce sperm until very late in life, the focus for clinical implications should be through patient education. There should be equal preconception health care for mothers and fathers, as this would benefit men, their partners, and their offspring

(O'Brien et al., 2018). Many men are unaware of how their fertility may be impacted by age, lifestyle, environment, diet, exercise, even the type of underwear they prefer. According to Garfield (2018), the father's preconception health plan should include a general reproductive health plan, health assessment, health promotion and psychosocial interventions. Men's fertility and sperm quality with advancing age could be discussed with men when they are young allowing them to bank their sperm if necessary. Indeed, Frey et al., (2008, 2012) have examined the content of preconception healthcare for men, and identified that while presently lacking, if these opportunities were made available, men would take fertility advice from primary care providers.

5.7 Final Conclusions

Concerns over the effects of advanced paternal age and the effects on fertility and offspring health have provided the justification for the studies presented in this thesis. We used a genomic and epigenomic approach to understand what may be happening to germ cell DNA, its expression, and modifications, in a Brown Norway rat model for reproductive aging. We found that telomere length shortens in the sperm of BN rats with age, in contrast to the effects seen in man, and that the epididymis may have a functional role in regulating telomere length that is affected by aging. We also found that there are many dysregulated genes with aging in spermatids, attributed to pathways including inflammation, general fertility, germ-Sertoli cell interactions, and epigenetics. The dysregulated transcription observed, can be correlated with the final study of DNA methylation with aging in spermatids and spermatozoa. In this final objective, we show that DNA methylation in germ cells is perturbed with aging. There is abundant hypermethylation in intergenic regions; however, there may be a regulatory role of hypomethylation in transcription of genes altered due to aging.

Together, the objectives of this thesis provide novel insight into the genetic and epigenetic landscape of germ cells from aged mammals. They present the first of their kind in many respects, primarily by examining the well-established rodent model for reproductive aging, and examining post-meiotic round spermatids. These studies provide the building blocks by

which we may examine the interaction of epigenetic and genetic sources of paternal age effects in fertility and offspring health. The studies presented in this thesis also provide additional evidence towards the clinical regulation of paternity with advancing age.

5.8 References

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5.9 Original Contributions

1. Measured telomere length across spermatogenesis in the Sprague-Dawley and Brown Norway rat. Other studies have examined telomere length only in mixed testicular germ cells, or mature sperm from the cauda of the epididymis, and used a mouse model.

2. Measured telomere length changes after transit through the epididymis, observing an apparent increase in telomere length between the caput and cauda segments. No study has reported this.

3. Reported a decrease in sperm telomere length due to aging in the Brown Norway rat. Other studies have examined only mice and men.

4. Reported an age dependent loss of telomere lengthening after transit through the epididymis. No study has examined this in any context.

5. Profiled mRNA expression from round spermatids of Brown Norway rats in young and aged contexts using RNA-sequencing. Other studies have used microarray analysis, qPCR methods for individual mRNAs, or have examined alternate cell types such as pachytene spermatocytes.

6. Reported an increase in overall gene expression due to aging in round spermatids. This had never been examined with RNA-sequencing.

7. Classified the reproductive function of differential mRNA expression due to aging, based on the existing literature for various reproductive functions. Other studies solely use Gene Ontology Analysis, Ingenuity Pathway Analysis, and Kyoto Encyclopedia of Genes and Genomes Pathway based analyses.

8. Reported for the first time, a potential alteration in numerous signalling molecules from round spermatids to Sertoli cells as a consequence of aging. Other studies have examined independent molecules, and primarily focus on Sertoli cell aging.

9. Reported for the first time an increase in folate receptor transcripts within round spermatids due to aging.

10. Conducted whole genome bisulfite sequencing (WGBS) of round spermatids and sperm from young and aged Brown Norway rats. To date, no other study has used WGBS to examine aging in the rat germline. Further, no study has examined the DNA methylome using WGBS of round spermatids as a consequence of age in rodents.

11. Reported global hypermethylation as a consequence of age in round spermatids and sperm from BN rats.

12. Correlated the hypomethylation due to aging in round spermatids with the observed differential gene expression.

13. Examined dynamic DNA methylation as a result of aging in germ cells. No other study has examined the methylation level specifically.

14. Reported LINE-1 hypermethylation in round spermatids and spermatozoa as a consequence of age in the BN rat.

15. Examined the effect of aging on processes of spermiogenesis and sperm maturation through WGBS DNA methylation. No other study in rat has examined the transition between cell types in this manner.

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