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Fei Sun *Editors*

Molecular Mechanisms in Spermatogenesis

Second Edition

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Series Editors

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Preface

Since the publication of the First Edition of *Molecular Mechanisms in Spermatogenesis* by Springer in 2008, significant advances have been made in the studies of spermatogenesis, most notably in the use of bioinformatics tools to analyze findings collected from big dataset studies pertinent to human spermatogenesis. Some of these datasets came about due to advancements in sequencing of RNAs (including small regulating RNAs) and DNAs, including the use of scRNA-Seq, scATAC-Seq, whole-exome sequencing, whole-genome bisulfite sequencing, transcriptome profiling, and 3D genome structure analysis of chromatin. These techniques have helped identify novel infertility-causing variants and genes in humans as well as the epigenetic modification of DNAs. Nonetheless, much work is still needed to bring these bioinformatic findings to lab and clinical studies to benefit infertile men, especially those with unexplained etiology, such as NOA (non-obstructive azoospermia).

The goal of this Second Edition is to assemble a group of senior investigators, many of whom collaborated with me to publish our First Edition in 2008. The Second Edition includes a total of 15 chapters compared to the 14 chapters found in the First Edition. I am also delighted to have Dr. Fei Sun who joined me in editing this volume to bring in some fresh ideas in organizing various chapters. In this edition, Drs. Xiao (Chap. 1), Dufour (Chap. 2), Han (Chap. 3), Lui (Chap. 5), Ge (Chap. 6), Cheng (Chap. 7), Walker (Chap. 9), and Berruti (Chap. 11) provide some much needed updates and insights in their respective areas of spermatogenesis research and expertise. These authors have presented findings from their own latest research from their laboratories as well as those belonging to other investigators of different fields. Some of these include data from different animal models and insights found in the disciplines of molecular biology, biochemistry, immunology, and pharmacology.

Dr. Cheng (Chaps. 4, 8 and 12) detail findings of different aspects of rodent and human spermatogenesis using bioinformatics approaches from his laboratory. Meanwhile, Drs. Goldstein (Chap. 13) and Lee (Chap. 14) provide clinician-based updates on human spermatogenesis. Additionally, Dr. Mathur (Chap. 10) provides an account on the use of molecular modeling to study spermatogenesis while

Dr. Cheng (Chap. 15) utilizes traditional Chinese medicine to demonstrate how herbal medicines used to treat some diseases are toxicants that can also cause testis injury that leads to male infertility.

On this note, we wish to thank our contributors for authoring their respective chapters, especially in light of some significant and uncontrollable hurdles in the past year. The COVID-19 pandemic, and the corresponding lockdowns in different cities, unexpectedly disrupted the production of many of the chapters in this edition as some of the authors could not return to their respective laboratories or offices for an extended period of time. As such, there were time gaps in the submissions of some chapters that span as much as 2 years. Yet many of our contributors were willing to update their submissions before this volume went to production so that the latest updates based on recently published reports could be included. We are also indebted to our colleagues at the Publisher Office, including Ms. Haritha Shrivarshini, Ms. Kala Palanisamy and Ms. Alison Ball, for their patience, help, and enthusiasm during the publication of this work. We also want to thank Ms. Dana Bigelow at the Publisher Office who gave us the opportunity to publish this work through Springer and have it included in the Advances in Experimental Medicine and Biology series.

I also want to thank some of our colleagues. I wish first to recognize the late Dr. Wayne Bardin, who introduced me to the concept of spermatogenesis and testis biology when I was a postdoc in his laboratory at the Population Council in New York City in the early 1980s. He worked tirelessly to teach me the biology of spermatogenesis, which included innumerable discussions—that sometimes stretched late into the evening—on different aspects of spermatogenesis. I am also indebted to Professor Bruno Silvestrini, who introduced me to the use of different animal models to study spermatogenesis as well as, in particular, the development and the use of adjudin for our studies on spermatogenesis. We are also indebted to many colleagues who studied in our laboratories who also contributed many refreshing ideas to our studies on spermatogenesis over the past 12 years following the publication of our First Edition.

Hangzhou, Zhejiang, China

C. Yan Cheng
Fei Sun

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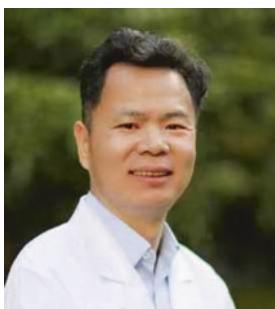
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About the Editors



C. Yan Cheng is a Distinguished Professor in Reproductive Medicine at Zhejiang University School of Medicine, Hangzhou, Zhejiang, China. He was a Senior Scientist and the Head of the Mary M. Wohlford Laboratory for Male Contraceptive Research at the Population Council's Center for Biomedical Research, inside the campus of the Rockefeller University in New York City, for four decades. Dr. Cheng was the recipient of numerous grants from National Institutes of Health, the CONRAD Program, Rockefeller Foundation, American Lupus Foundation, the Noopolis Foundation, and others, including pharmaceutical industry, for decades. He and his colleagues, including graduate students and postdocs, have made numerous discoveries in the field of reproductive biology, in particular the discoveries of several biologically active peptides released from structural proteins in the testis that regulate spermatogenesis. These autocrine and paracrine peptides create a local regulatory network that regulates and coordinates cellular events across the seminiferous epithelium to support the epithelial cycle of spermatogenesis, such as the release of sperm at spermiation and remodeling of the blood-testis barrier (BTB), that take place simultaneously across the epithelium at Stage VIII of the epithelial cycle. His research group also pioneers the study of cell polarity and planar cell polarity (PCP) proteins to support spermatogenesis. He has also pioneered in the study of adjuvin and related compounds for male contraception, including the use of toxicant and pharmaceutical models to study the biology of spermatogenesis and male infertility. His current studies focus on the

biology of human spermatogenesis. Dr. Cheng published over 500 scientific papers in leading journals including *Nature Medicine*, *Nature Communications*, *Nature Reviews Endocrinology*, *Physiological Reviews*, *Pharmacological Reviews*, *Endocrine Reviews*, *PNAS*, *FASEB Journal*, *Endocrinology*, *Journal of Clinical Endocrinology and Metabolism*, *Fertility and Sterility*, *Human Molecular Genetics*, *Cell Death and Disease*, and others. He also served as the Editor-in-Chief of the journal *Spermatogenesis*, and served on the Editorial Board of the journal *Endocrinology*, *Asian Journal of Andrology*, and *Andrology*. He also edited three books, and he is the inventor of numerous U.S. patents including a patent on adjudin.



Fei Sun is a Distinguished Professor of Zhejiang University in Reproductive Medicine, and a Chief Physician in Andrology. He is also the vice-President of Sir Run Run Shaw Hospital, Zhejiang University School of Medicine. Dr. Sun is the recipient of the prestigious National Fund for Distinguished Young Scholars, the National 973 Project, the National Key Research and Development Program of China, and the Hundred Talent Program of the Chinese Academy of Sciences. He serves as the Associate Editors for *Molecular Human Reproduction*, *Reproductive Biology and Endocrinology*, and *Asian Journal of Andrology*. His research focuses on clinical and basic research of male infertility, especially on genetic and epigenetic regulation of spermatogenesis. He has published over 90 peer-reviewed research articles, including *Am J Hum Genet* and *Hum Mol Genet*, and 14 of these studies were selected as the cover illustration .

The Seminiferous Epithelial Cycle of Spermatogenesis: Role of Non-receptor Tyrosine Kinases



Xiang Xiao, Dongwang Zheng, Fei Liang, Shibo Ying, Peibei Sun,
Jianmin Yu, and Ya Ni

Introduction

After cancer and cardiovascular disease, infertility has become the third most serious health problem in terms of its prevalence and impact on family quality of life, with more than half of male causes. Studies have found that infertile men are at higher risk of developing cancer, diabetes, and heart disease [1–3]. Data has shown that in the past 50 years, the average sperm counts of men in Europe and the United States have decreased by 30–60% [4, 5]. Despite the severe situation, the diagnosis and treatment of male infertility have always been a worldwide problem. One important reason is that spermatogenesis taking place within the seminiferous tubule of the testis is a complicated process, and little is known about its underlying mechanisms. Therefore, identifying potential molecular targets and elucidating their roles and signaling pathways during spermatogenesis will help to understand the pathogenesis of spermatogenic disorders, and provide a basis for developing specific and sensitive methods for clinical treatment of male infertility.

Tyrosine kinases are key mediators in cellular functions, including survival, proliferation, differentiation, apoptosis, cell adhesion, and motility. They specifically phosphorylate tyrosine residues in proteins to regulate their activity, subcellular localization, and interactions between proteins, so that organisms can respond to extracellular and intracellular physiological stimuli. There are two classes of

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tyrosine kinases, the receptor tyrosine kinases (RTKs) and non-receptor tyrosine kinases (NRTKs). RTKs function as cell surface receptors, relaying extracellular signal to receivers in the intracellular space, such as NRTKs. The cytosolic NRTKs work as intermediates, which process and propagate the signal further to downstream transduction effectors [6–8]. The hyperactivity, overexpression and mislocation of tyrosine kinases are often associated with many pathological conditions such as cancers, immunological disorders, inflammatory and infectious diseases. Thus, blockade of tyrosine kinases has been a therapeutic modality to control their activities and improve human health [9–11]. Similarly, they are good targets for identification of potential causes of male infertility.

During the past decades, studies have been conducted on several families of tyrosine kinases regarding their roles in the mammalian testis and germ cell development, such as the TAM (TYRO3, AXL and MERTK) family of RTKs, known for their importance in maintaining the testicular immune homeostasis [12]. TAM receptors have been shown to support phagocytic activity in Sertoli cells to regulate male fertility. In addition, their deficiency is associated with increased permeability of the blood-testis barrier (BTB) [12, 13]. The NRTKs, including SFKs (SRC family kinases), FAK (Focal adhesion kinase), FES/FPS (feline sarcoma/Fujinami poultry sarcoma) and FER (FES-related, sharing similar structural features with FES/FPS), have also been implicated in signaling to cytoskeletal regulators and/or germ cell transport during spermatogenesis [14–17]. For example, FER and its testis-specific truncated variant FERT coexpressed by rat testis, were shown to contribute to F-actin remodeling in the acroplaxome (an acrosome-associated cytoskeletal plate) during spermatid head shaping, as well as in germ cell adhesion to Sertoli cells [16, 18]. FAK, SFKs, and their corresponding tyrosine-phosphorylated forms were also observed to regulate Sertoli cell BTB by recrafting actin- and microtubule (MT)-based cytoskeletons [15, 17, 19]. These examples illustrate that tyrosine kinases are important regulators of spermatogenesis to facilitate the cytoskeletal changes and germ cell transport across the seminiferous epithelium.

In this review, we will focus on NRTKs, e.g. their roles in coordination of the spermatid transport across the seminiferous epithelium and the passage of preleptotene/leptotene spermatocytes across the BTB. These processes involve substantial turnover of the cell junctions and the underlying cytoskeletons. We will first briefly review the specialized cell junctions between Sertoli-germ cells and Sertoli-Sertoli cells in the testis, namely apical ectoplasmic specialization (ES) and BTB, respectively. We will then discuss the membrane association and subcellular localization of NRTKs regarding their structural and functional properties, as well as their possible roles in spermatogenesis based on findings from both the mammalian testis and other cell settings as references, with an emphasis on endocytic trafficking-mediated junction dynamics (For comprehensive reviews regarding details of NRTKs in the testis, see [17, 20, 21]). We conclude with a discussion of challenges when studying parallel tyrosine kinase pathways in the testis and highlight their differential roles that are currently being explored.

Junctional and Cytoskeletal Dynamics in the Testis

Mammalian spermatogenesis is a highly organized process of cell differentiation and maturation that transforms diploid spermatogonial stem cells into haploid spermatozoa that can fertilize the ovum in the fallopian tubes of female reproductive tract [22–24]. The smooth progress of spermatogenesis is guaranteed by the structural and functional support of specialized cell junctions between Sertoli-germ cells or Sertoli-Sertoli cells (Fig. 1). First, germ cells are maintained in a metabolically quiescent and non-motile state, lacking filopodia or lamellipodia found in other motile cells such as fibroblasts, keratocytes, or neutrophils. Their life activities, including nourishment and translocation, depend entirely on Sertoli cells that extend from the basement membrane to the luminal surface of the seminiferous epithelium [25]. Germ cells at earlier stages of development are connected to Sertoli cells by actin-based gap junction (GJ) and intermediate filament-based desmosome, which are replaced by apical ES (a testis-specific actin-rich adherens junction) arising at step 8 spermatid–Sertoli cell interface in Stage VIII tubules in rodent testis. Once it appears, apical ES is the only anchoring device present at the site until spermiation [26–28]. Secondly, near the basement membrane, intercellular junctions between adjacent Sertoli cells constitute the BTB, which divides the seminiferous epithelium into a basal and an adluminal compartment. The BTB is different from most other tissue barriers (e.g. blood-brain barrier or blood-retina barrier) since in it tight junction (TJ) coexists and jointly functions with basal ES, GJ, and desmosome, to form a unique adluminal environment for meiosis and spermiogenesis. Preleptotene spermatocytes moving into the leptotene phase initiate the passage across the BTB from the basal to the adluminal compartment, with the aid from Sertoli cells in Stages VIII–IX tubules [21, 29].

Timely transport of spermatids by Sertoli cells across the adluminal compartment is one of the crucial cellular events to maintain and regulate spermatogenesis, whose smooth progress warrants spermiation [30]. Externally, apical ES connects spermatids to Sertoli cells, and BTB created a “shelter” to protect the developing spermatids; internally, these cell junctions interact with the cytoskeleton to convey changes in the cellular environment. The robust cytoskeleton system in Sertoli cells is rich in filamentous actin (F-actin), microtubules, intermediate and septin filaments [31], which intertwines to facilitate spermatid translocation and spermiation. For example, both F-actin and microtubule contribute to the transport of spermatids and intracellular organelles (e.g. endosome-based vesicles, residual bodies, and phagosomes) across the seminiferous epithelium [15, 31, 32]. However, how the cytoskeletal networks interact with each other to confer plasticity and dynamics during the seminiferous epithelium cycle remains unclear.

Studies have shown that NRTKs are important for Sertoli cell function, particularly in spermatid adhesion and BTB dynamics. Better studied NRTKs such as SRC and FAK are apical ES and BTB constituents. They may play a role in endocytic trafficking of ES/BTB components by changing the phosphorylation status of the proteins, to facilitate the apical ES/BTB assembly and/or disassembly [15, 17, 33].

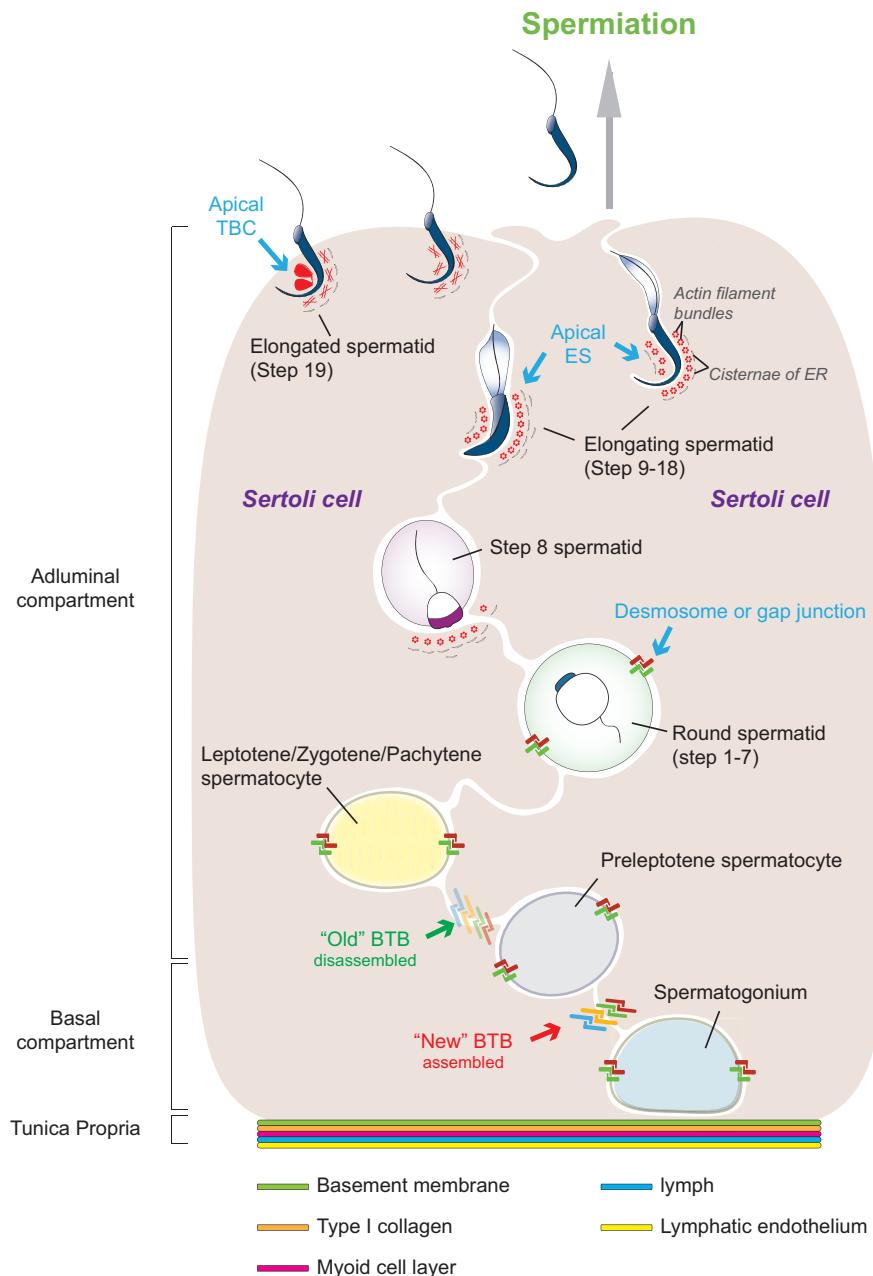


Fig. 1 A schematic diagram illustrating different types of cell junctions present in the rat seminiferous tubules. Sertoli cells extend from the basement membrane to the luminal surface of the seminiferous epithelium, and the intercellular junctions between adjacent Sertoli cells establish the blood-testis barrier (BTB). BTB is constituted by coexisting tight junction (TJ), basal ectoplasmic specialization (ES), gap junction (GJ) and desmosome, which periodically restructures (“new”

But the regulatory molecules and mechanism(s) behind these events, e.g. the upstream receptor(s) or downstream effectors, are still largely unrevealed, plus the fact that the signaling network of NRTKs is interfluent and complicated, what is known is only the tip of the iceberg. In the following section, we will briefly review the biology and regulation of NRTK families.

NRTKs: Membrane Targeting and Dynamic Localization

There are at least ten subfamilies of NRTKs identified based on their structural variability, including ABL, SRC, BRK/FRK, CSK, TEC/BTK, FAK, JAK, FER/FES, SYK/ZAP70, and ACK family of kinases. Among them, NRTKs from BRK/FRK, TEC/BTK, SYK/ZAP70 and ACK families have been shown to have oncogenic properties. ABL, SRC, and FER/FES family kinases are cellular forms of the corresponding retroviral oncoproteins, with their activity tightly controlled in cells. The other NRTKs such as FAK can form complexes with various oncogenic proteins, while JAK is involved in oncogenic signaling such as JAK-STAT pathway. Therefore, NRTKs had received a great deal of attention but most of the research focused on tumorigenesis [34, 35]. In mammals, more than one member from the same NRTK family has been observed to be expressed in the same tissue or cell. Single NRTK knockouts are generally viable and fertile, and do not display any overt abnormalities (Table 1). NRTKs from different subfamilies also show overlap in substrate specificity. These findings imply the possibility of functional redundancy within the same NRTK family, as well as among NRTKs from different families. On the other hand, when combined with mutations in more than one member of a family, such as in double and triple knockouts, phenotypes are normally quite distinct and profound [67–69]. The above examples probably illustrate the important biological roles of NRTKs, that a back-up mechanism is advantageous. It is also likely that the effect of some NRTK became evident only upon inactivation of another NRTK, pointing to the parallel pathways connecting one NRTK to another [70].

NRTKs are cytoplasmic proteins, lacking receptor structural features such as a ligand-binding ectodomain and a membrane-spanning segments. But they closely associate with cell membranes, functioning in membrane-proximal signaling events,

Fig. 1 (continued) BTB assembled before “old” BTB disassembled to accommodate the passage of spermatocyte from the basal to the adluminal compartment. Germ cells at earlier stages of development are connected to Sertoli cells by GJ and desmosome, which are replaced by apical ES arising at step 8 spermatid–Sertoli cell interface. The apical ES is typified by the actin filament bundles sandwiched in between cisternae of endoplasmic reticulum (ER) and the parallel plasma membrane of the spermatid and the Sertoli cell. Prior to spermiation, apical tubulobulbar complexes (TBCs) forming at the apical ES are involved in the junction disassembly at the Sertoli-spermatid interface to eliminate excess spermatid cytoplasm and prepare mature spermatids for release into the tubule lumen

Table 1 Phenotypes in NRTK knockout mice/embryos^a

Knockout(s)	Viability	Fertility	Phenotype(s)	Reference(s)
<i>abl</i> ^{-/-}	Increased perinatal mortality	Reduced fertility	Runtedness, foreshortened crania	[36–38]
			Abnormal thymus, spleen, head, and eye development	
			T and B cell lymphopenia, and defects in lymphocyte development	
			Osteoporotic and defects in osteoblast maturation	
<i>arg</i> ^{-/-}	Viable	Fertile	Developed normally	[39]
			Runtedness	
			Defects in neuronal function	
<i>abl</i> ^{-/-} <i>arg</i> ^{-/-}	Embryonic lethality (die before 11 dpc)		Defects in neurulation	[39]
<i>brk</i> ^{-/-}	Viable	Fertile	A deregulated balance between proliferation and differentiation in the intestinal epithelium; increased Akt activity and nuclear β-catenin in intestines; decreased nuclear localization of the Akt substrate FoxO1 in villus epithelial cells	[40]
<i>frk</i> ^{-/-}	Viable	Fertile	Similar growth rates to wild-types	[41]
			Increase in expression of SFK genes	
			Subtle changes in thyroid hormone regulated gene expression	
			Significant decreases in the circulating levels of T3	
<i>srms</i> ^{-/-}	Viable	Fertile	Displayed no apparent phenotype	[42]
<i>csk</i> ^{-/-}	Embryonic lethality (died by E11.5)		Defects in the neural tube	[43, 44]
			Increase in activity of SFKs	
<i>chk</i> ^{-/-}	Viable	Fertile	Apparently healthy	[45]
<i>tec</i> ^{-/-}	Viable	Fertile	No gross developmental defects	[46]
<i>btk</i> ^{-/-}	Viable	Fertile	Defective B cell development and function	[47, 48]
<i>Itk</i> ^{-/-}	Viable	Fertile	Reduced cytotoxic T-lymphocyte (CTL) responses	[49]
<i>bmx</i> ^{-/-}	Viable	Fertile	Had a normal life span without an obvious phenotype	[50]
<i>fak</i> ^{-/-}	Embryonic lethality (die by E8.5)		Defect of mesoderm development	[51, 52]
			Cells from these embryos had reduced mobility in vitro	
			Increased number of focal adhesions in FAK-deficient cells	
<i>pyk2</i> ^{-/-}	Viable	Fertile	No overt impairment in development or behavior	[53]
			Impaired macrophage morphology and migration	
<i>jak1</i> ^{-/-}	Perinatal lethality		Runted at birth, fail to nurse	[54, 55]
			Defects in lymphoid development	

(continued)

Table 1 (continued)

Knockout(s)	Viability	Fertility	Phenotype(s)	Reference(s)
<i>jak2</i> ^{-/-}	Embryonic lethality (die at 12.5 dpc)		No erythropoiesis	[56, 57]
<i>jak3</i> ^{-/-}	Viable	Fertile	Severe combined immune deficiency (SCID)	[54, 58]
<i>tyk2</i> ^{-/-}	Viable	Fertile	Displayed multiple immunological defects	[59]
<i>fer/fert</i> ^{-/-}	Viable	Fertile	Reduced Cortactin Phosphorylation	[60]
<i>fes</i> ^{-/-}	Viable	Fertile	Display slightly reduced numbers of myeloid cells More sensitive to lipopolysaccharide (LPS)	[61]
<i>fer</i> ^{-/-} <i>fes</i> ^{-/-}	Viable	Reduced fertility	Defects in hematopoiesis	[62]
<i>syk</i> ^{-/-}	Perinatal lethality		Severe hemorrhaging	[63, 64]
<i>zap70</i> ^{-/-}	Viable	Fertile	immunodeficient	[65]
<i>tnk1</i> ^{-/-}	Viable	Fertile	Developed spontaneous tumors and displayed hyperactivated Ras	[66]

dpc day post coitum

^aThis table is not intended to be exhaustive. But it illustrates the importance of NRTKs in fertility, immunity and development. It is noted that, some of the knockouts are kinase knockouts created by deleting the catalytic domain, which may not reflect NRTK functions outside of the kinase domain. Please refer to Reference [67] for phenotypes in SFK knockout mice/embryos which are not included in this table

such as cell-cell and cell-matrix interactions [71]. Many NRTKs bear highly conserved protein modules of three functional domains: the Src homology 3 (SH3), SH2, and tyrosine kinase (TK) domain (Fig. 2), in which SH3 and SH2 are protein-protein interacting motifs, binds proline-rich sequences and phosphotyrosine (p-Tyr) residues, respectively. Therefore, SH3 and SH2 domains may contribute to adaptor/scaffolding functions independent from the kinase activity. The regions flanking the SH3–SH2–TK cassette are diverse, harboring distinct N- or C-terminal sequences or functional domains. NRTKs lacking the core structure of SH3–SH2–TK contain otherwise subfamily-specific domains used for protein-protein interactions and regulation of enzymatic activity (Fig. 3). Since there is a rich body of literature describing the details of NRTK structure, function, and regulation [68, 72–78], here we focus on the membrane association and dynamic subcellular localization of NRTKs, which may shed light on studies about junctional and cytoskeletal dynamics during spermatogenesis.

Cell membrane composed of lipids (e.g. phospholipids, glycolipids, and cholesterol) and proteins is an active area of receiving and amplifying signals, where NRTKs carry out necessary functions to sustain life. Translocation to plasma membrane is a critical step in activation of many NRTKs such as SRC, BTK, TEC, FER/FES, and ACK, whereas inactivation and termination of signaling may occur in the cytoplasm and/or nucleus [68, 79–82]. Studies have found that the structural

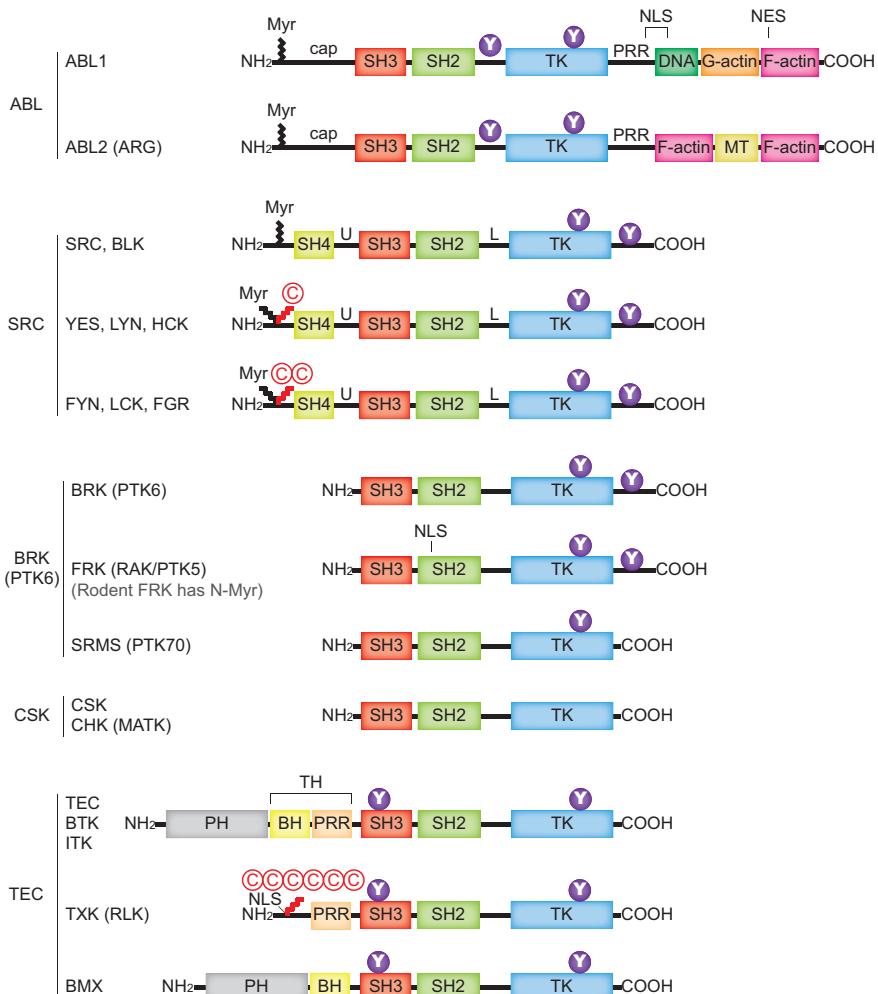


Fig. 2 Linear diagrams illustrating the domain architecture of different non-receptor tyrosine kinase (NRTK) subfamilies harboring an SH3 (Src homology 3)-SH2-TK (tyrosine kinase) domain cassette. Y, Tyr; Myr, myristoylation; cap, 'cap' region; PRR, proline-rich region; NLS, nuclear localization signal; NES, nuclear export signal; DNA/G-actin/F-actin/MT, DNA/G-actin/F-actin/microtubule binding domain; U, unique domain; L, polyproline linker; C, palmitoylated cysteine residue; PH, pleckstrin homology domain; BH, BTK homology motif; TH, Tec homology domain

characteristics are basis for membrane targeting and cellular localization of NRTKs, which in turn determines their activation and context-specific functions. For instance, ABL and SRC have posttranslational N-myristoylation but lack second acylation such as palmitoylation for stable membrane anchoring [83–86].

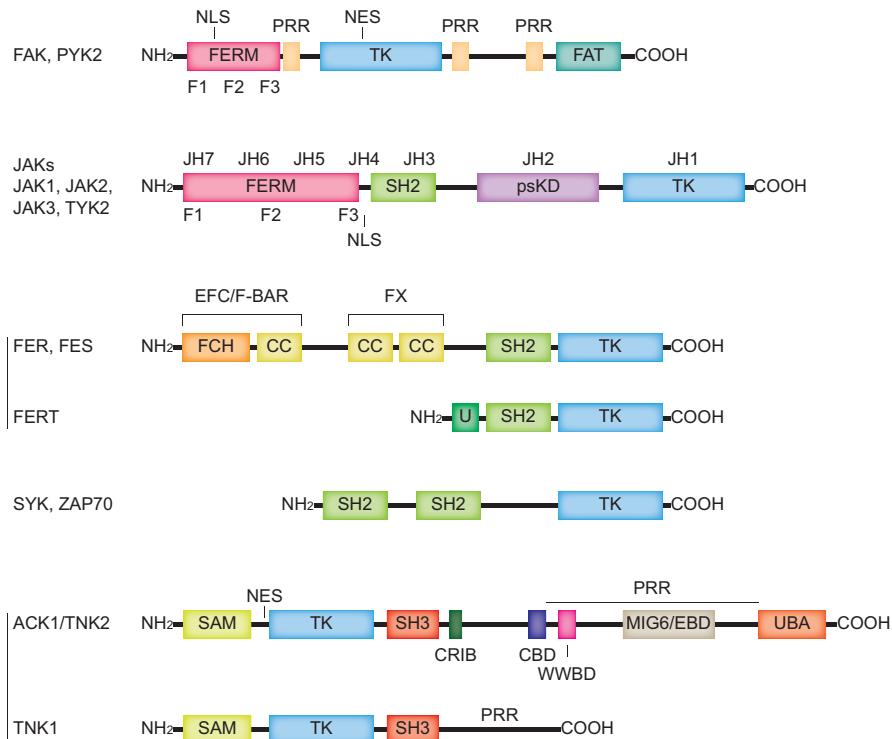


Fig. 3 Linear diagrams illustrating the domain architecture of different non-receptor tyrosine kinase (NRTK) subfamilies without an SH3 (Src homology 3)-SH2-TK (tyrosine kinase) domain cassette. NLS, nuclear localization signal; NES, nuclear export signal; PRR, proline-rich region; FERM, four-point-one, ezrin, radixin, moesin domain which contain three lobes, F1, F2, and F3; FAT, focal adhesion targeting domain; JH, JAK homology domain; psKD, pseudokinase domain, a tyrosine kinase-like domain without catalytic activity; FCH, FPS/FES/FER/CIP4 homology domain; CC, coiled-coil motif; EFC, extended FCH domain; F-BAR, FCH homology-Bin/Amphiphysin/Rvs domain; FX, F-BAR extension domain; U, FERT-unique sequence of 44 amino acid residues; SAM, sterile α motif; CRIB, Cdc42/Rac interactive binding motif; CBD, clathrin-binding domain; WWBD, WW-binding domain; MIG6, MIG6 homology domain; EBD, epidermal growth factor receptor (EGFR)-binding domain; UBA, ubiquitin association domain

ABL has been shown to shuttle between the nucleus and the cytoplasm since it accommodates short-peptide signal sequences, i.e., three nuclear localization signals (NLS) for its direct import into the nucleus, and one nuclear export signal (NES) for export from the cell nucleus to the cytoplasm [87–90]. SRC is attached to the membrane through a polybasic cluster of amino acids in its N-terminal region, and rapidly exchanged between the perinuclear region and the plasma membrane [79, 83–86, 91]. It is noted that, except SRC and BLK that only possess N-myristoylation, all SFKs interact with membrane through a dual acylation

mechanism (myristoylated plus palmitoylated) (Fig. 2), and the reversibility of palmitoylation promotes dynamic relocation between different intracellular compartments [83–86]. SFKs have been described to reside in a variety of cellular compartments, such as nucleus, early endosomes, recycling endosomes, late endosomes, lysosomes, Golgi apparatus, secretory granules or phagosomes, suggesting different substrates and/or cellular functions on these segregated signaling platforms [83–86, 92, 93]. NRTKs without lipid modification for membrane attachment utilize membrane interacting domains such as phospholipid-binding domains, including pleckstrin homology (PH), four-point-one, ezrin, radixin, moesin (FERM), FER/CIP4 homology-Bin/Amphiphysin/Rvs (F-BAR), and sterile α motif (SAM) domains (Figs. 2 and 3). The predominantly cytoplasmic TEC/BTK kinases contain a PH domain at the N-terminus (with the exclusion of TXK which contains a string of palmitoylated cysteine residues instead of PH domain for membrane targeting), which binds to phosphorylated phosphatidylinositol lipids and thus mediates the recruitment of TEC/BTK to the membrane. Upon activation by translocation to the plasma membrane and phosphorylation by SFKs, BTK was observed to interact with caveolae [68, 80, 94]. FAK and JAK kinases contain FERM domain, which binds to acidic phospholipids to activate the kinases at the plasma membrane [95]. Nevertheless, FERM–SH2 module in JAK and the FERM domain interaction with membrane receptors may greatly contribute to JAK membrane localization as well [96]. Similarly, the membrane-dependent activation of FER/FES is regulated by the binding of the N-terminal F-BAR and F-BAR extension (FX) domains to signaling phospholipids such as phosphatidic acid (PA) and phosphatidylinositol 4,5-bisphosphate (PIP2) at the membranes [81]. The same mechanism applies to the SAM domain as in ACK family kinases [97]. For those NRTKs lacking N-terminal acylation signals or phospholipid-binding domains for membrane association, such as BRK/FRK, CSK and SYK/ZAP70 (Figs. 2 and 3), they have more flexible intracellular localization and can be found in multiple cellular compartments. BRK has been reported to phosphorylate β -catenin, AKT, FAK and paxillin at the plasma membrane [98–100], while FRK is localized to the juxtanuclear region resembling Golgi and γ -tubulin centrosomal compartments [41]. SFK (e.g. Lck and/or Fyn)-initiated phosphorylation and binding to the receptors containing immunoreceptor tyrosine-based activation motif (ITAM) which serve as a docking site, appear to be crucial parameters in SYK/ZAP70 membrane engagement [101]. Intriguingly, besides ABL, quite a few NRTKs have been detected in nucleus or displaying nucleocytoplasmic shuttling, such as FRK, TXK, FAK, PYK2, JAK1, JAK2, and ACK kinases that harbor NES and/or NLS required for nucleus transport [80, 82, 97, 98, 102, 103], as well as SRC, BRK, BTK, FERT, SYK, and ZAP70 without canonical nuclear targeting signals [16, 68, 79, 98, 99, 104], implying that they may travel freely and efficiently transduce signals between different subcellular locales.

NRTKs and Endocytosis During Spermatogenesis

An adult male can produce up to 50–70 million and 300 million sperm daily in rats and humans, respectively [105, 106], indicating substantial cellular activity in the seminiferous tubules, including turnover of cell junctions. The apical ES and BTB restructure periodically to support spermatogenesis, such as undergoing junction assembly and disassembly. For instance, at Stages VIII of the seminiferous epithelial cycle in rats, the following coordinated events occur synchronically: (1) apical ES close to the luminal edge at the interface of step 19 spermatid and Sertoli cell needs to be disassembled to release mature spermatid, which results in spermiation (Fig. 4); (2) new apical ES has to be assembled at step 8 spermatid–Sertoli cell interface, replacing gap junction and desmosome (Fig. 1); (3) BTB restructures, which involves “new” BTB assembly and “old” BTB disassembly, to facilitate the spermatocyte transport across the BTB without disrupting the apical immune privilege (Fig. 4). Adhesion protein complexes at the apical ES (N-cadherin/β-catenin, α₆β₁-integrin/laminin-α₃β₃γ₃, nectin-2/-3/afadin, CAR/β-catenin, and JAM-B/JAM-C, etc.), basal ES (N-cadherin/β-catenin and nectin-2/afadin, etc.), TJ (occludin/ZO-1, claudin 11/ZO-1, CAR/ZO-1, CAR/β-catenin, JAM-A/ZO-1, and JAM-B/ZO-1, etc.), GJ (connexin 43/plakophilin-2, etc.), and desmosome (desmoglein-2/desmocollin-2, etc.) [25, 107] are in a constant state of association and dissociation to accommodate the cell junction restructuring. In other words, the constituent integral membrane proteins have to be removed from the sites of Sertoli cell-spermatid or Sertoli-Sertoli cell contact to facilitate the disassembly of the apical ES and the “old” BTB, to allow spermiation and the transport of spermatocyte across the BTB. Furthermore, these proteins moved away from the “old” apical ES and BTB sites have to be reused, at least in part, for the assembly of new apical ES and BTB because it is physiologically unfeasible for de novo synthesis of all the required proteins [21]. This enormous task is achieved by endocytosis of ES/BTB proteins, and subsequent intracellular trafficking after protein internalization, including protein degradation, recycling and transcytosis, which is also called post-endocytic sorting (Fig. 4). So that with levels and localizations changing dynamically at the cell-cell contact and intracellular compartments, the cell adhesion proteins can be circulated rapidly, enabling the timely restructuring of the apical ES and BTB [108, 109]. Studies have shown that both cytokines [e.g. TGF-β (Transforming growth factor β), TNF-α (Tumor necrosis factor α) and interleukins] and androgens (e.g. testosterone) increase the endocytosis of integral membrane proteins, with TGF-β promoting endosome-mediated protein degradation for old junction disassembly, while testosterone accelerating the recycling of proteins back to the plasma membrane for new junction reassembly [108–110].

SFK kinases SRC and YES, expressed by both Sertoli and germ cells in adult rat testis, are recently shown to be the integrated part of BTB and apical ES [17] (Fig. 4). They were found to play differential roles in endocytic vesicle-mediated protein trafficking, including protein endocytosis, transcytosis and/or recycling, as well as protein degradation [111]. Specifically, SRC/YES was knocked down by

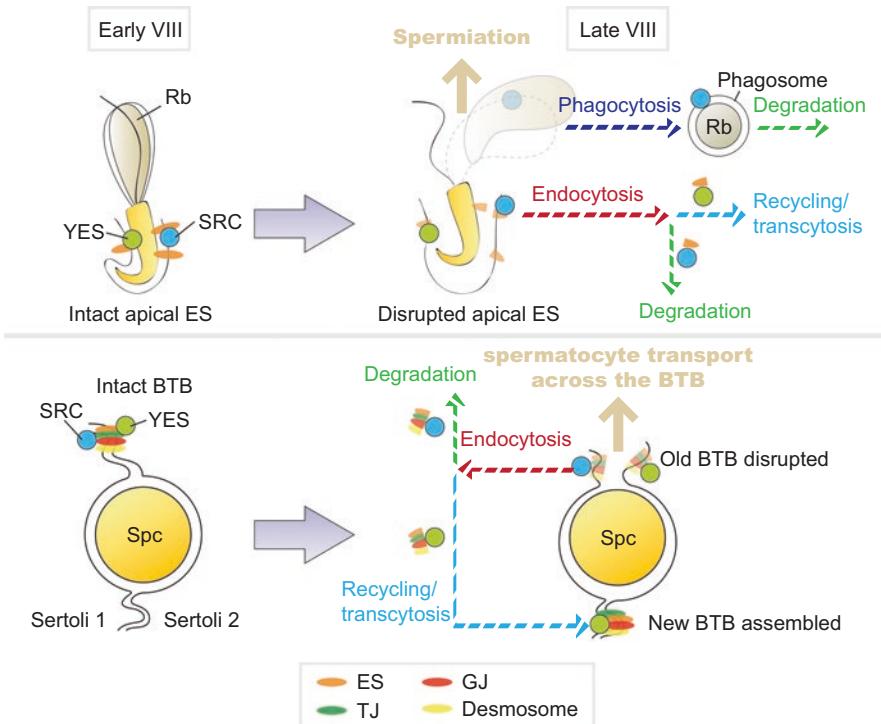


Fig. 4 Schematic diagrams illustrating non-receptor tyrosine kinases (NRTKs) such as SRC and YES differentially participating in endocytic vesicle-mediated protein trafficking to facilitate the restructuring of the apical ectoplasmic specialization (ES) and blood-testis barrier (BTB) to support spermiation and spermatocyte transport across the BTB. The diagram in the upper half shows at early Stage VIII, the apical ES at the interface of step 19 spermatid and Sertoli cell needs to be disassembled to release mature spermatid, which results in spermiation at late Stage VIII. SRC and YES direct endocytosed apical ES proteins to the pathways of degradation and recycling/transcytosis respectively. SRC may also regulate Sertoli cell phagocytosis and lysosomal degradation of the residual bodies (Rb) shed from spermatids. The diagram in the lower half shows similar junctional restructuring events mediated by SRC and YES happening at the BTB site to facilitate the transport of spermatocyte (Spc). *TJ* tight junction, *GJ* gap junction

RNAi in an in vitro rat Sertoli cell system mimicking the BTB in vivo [112], cell surface proteins (i.e. integral membrane proteins at the Sertoli cell BTB in vitro) were labeled by using an irreversible biotinylation reagent Sulfo-NHS-Biotin [113], and the rate of protein internalization, disappearance of internalized proteins, and protein recycling to plasma membrane was measured respectively by semi-quantification of the remaining biotinylated proteins after removal/quenching of biotins on biotinylated proteins that failed to internalize in experiment settings [111]. These biochemical approaches demonstrated that SRC and YES disfavored the retention of internalized biotinylated proteins in cell cytosol with one important

difference: SRC accelerated but YES decelerated protein endocytosis at the Sertoli cell BTB. Additionally, YES could direct endocytosed biotinylated BTB proteins to the pathways of transcytosis and/or recycling. Coupled with the degradation assay using another biotinylation reagent NHS-LC-Biotin to label both cell surface and intracellular proteins, SRC was shown to advance endosome-mediated protein degradation [111]. These observations make evident that SFK kinases may work in concert to regulate the BTB/apical ES restructuring via the endosomal protein trafficking pathways, e.g., cargo proteins can be recycled/transcytosed to form the new BTB/apical ES by YES, or degraded to dissolve the old BTB/apical ES by SRC. Consistent with this, YES expression decreased after TGF- β 3 treatment in Sertoli cells cultured in vitro to facilitate the BTB disassembly [114]. In rat testis, YES was initially found at the convex side of the spermatid head, presumably working with Eps8 (an actin barbed-end capping and bundling protein which can stabilize BTB/apical ES and physically interact with YES in rat testis) [115, 116], but not Arp3 (an actin barbed-end nucleation and branched polymerization protein which can destabilize BTB/apical ES) [117], to maintain the apical ES function [114, 116]. But at Stages VI to early VIII prior to spermiation, YES became visible at both the convex and the concave sides of the spermatid head [114], coincided with the appearance and development of the apical tubulobulbar complexes (apical TBCs), a giant endocytic structure forming at the apical ES, to recycle integral membrane proteins (when at the convex side of the spermatid head) or eliminate unwanted cellular debris from the spermatids (when at the concave side of the spermatid head) [118]. YES disappeared at stage late VIII at the apical ES when mature spermatids were released into the tubule lumen [114]. By contrast, SRC was reported to be likely involved in endosome-associated actin nucleation activity [91], which may function with Arp3 to facilitate spermiation and BTB restructuring. Another NRTK, FAK, being the functional partner and putative substrate of SRC/YES in the testis [114, 119], was also linked to endosome-mediated trafficking of E-cadherin [79]. Although additional data is needed to better understand the role of different NRTKs in endocytosis during spermatogenesis, these findings suggest a novel mechanism to support the timely dissolution and (re)assembly of BTB and apical ES.

In Stage VIII tubules, cytoplasmic waste and debris shed from mature spermatids before spermiation (e.g. residual bodies) also have to be removed through an endocytic process called phagocytosis (i.e. cell eating), which heads towards the final lysosomal degradation by Sertoli cells at Stages IX of the seminiferous epithelial cycle. Although phagocytosis and formation of the residual body are actin dependent, the transport of residual body-containing phagosome (a large intracellular vesicle of diameter >250 nm) from the apical to basal Sertoli cell subcellular compartments is a microtubule-dependent movement [120, 121]. Furthermore, during spermatogenesis, up to 75% of germ cells from various stages have been estimated to undergo apoptosis, which must be timely eliminated by Sertoli cell phagocytosis as well [26, 121–123]. However, there is only limited understanding of the clearance of apoptotic germ cells and residual bodies. One phosphorylated form of FAK, namely p-FAK-Y407, co-localized with both Arp3 and Eps8 to facilitate spermatid transport during spermiogenesis [20], are found abundantly expressed in structures

analogous to residual bodies/phagosomes in the seminiferous tubule [124]. In a study by using fluorescent microspheres that mimicked the apoptotic germ cells to add to the primary cultures of rat Sertoli cells, SRC knockdown was shown to significantly compromise Sertoli cell phagocytic activity [111], implicating that SRC may regulate phagocytosis and the subsequent lysosomal degradation. To collaborate with this, when inactivated from the plasma membrane to the cell cytosol, SRC was found at the microtubule-dependent perinuclear locations [92, 93].

Concluding Remarks

In this review, we have summarized findings in the field that support the involvement of NRTKs in endocytic vesicle-mediated protein trafficking events in apical ES/BTB reorganization to facilitate spermiation and spermatocyte transport across the BTB during spermatogenesis. NRTKs are versatile in various cellular processes, with their subcellular locations flexible. The increased difficulty of understanding NRTKs' role in spermatogenesis is their extraordinarily high expression in the mammalian testis with seemingly substantial functional overlap. It is conceivable that male fertility is so important that the testis has to make every endeavor to retain all the necessary machinery to ensure the smooth progress of spermatogenesis. So that NRTKs as the key regulator may be redundantly expressed at high levels to function under miscellaneous conditions, with the possibility that under normal circumstances one might never encounter such a situation, not to mention under controlled experimental conditions to make it come to the surface. NRTKs may well play divergent roles to support spermatogenesis which are yet to be uncovered.

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The Good, the Bad and the Ugly of Testicular Immune Regulation: A Delicate Balance Between Immune Function and Immune Privilege



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Introduction

The testis is the male reproductive organ responsible for testosterone/androgen production and spermatogenesis [1]. It consists of the seminiferous tubules separated by the interstitial tissue (Fig. 1a). The interstitium contains Leydig cells, macrophages, blood vessels, lymphocytes and fibroblasts, while the seminiferous epithelium is comprised of the Sertoli cells and germ cells and is surrounded by peritubular myoid cells (Fig. 1a). Testosterone produced by Leydig cells within the interstitial space is necessary for spermatogenesis and the development of male reproductive tissues and secondary sexual characteristics. In the testis, testosterone acts via classical and nonclassical activation of the androgen receptor pathways in Sertoli cells and is needed for progression through meiosis, maintenance of the blood testis barrier, attachment of elongating spermatids, and spermatozoa release [2, 3].

Spermatogenesis takes place within the seminiferous tubules and is the process of transforming the undifferentiated spermatogonia into highly specialized spermatozoa. Within the tubules, the developing germ cells are closely associated with the Sertoli cells and together they coordinate the progression of the germ cells through spermatogenesis, which is divided into three phases of development: mitosis

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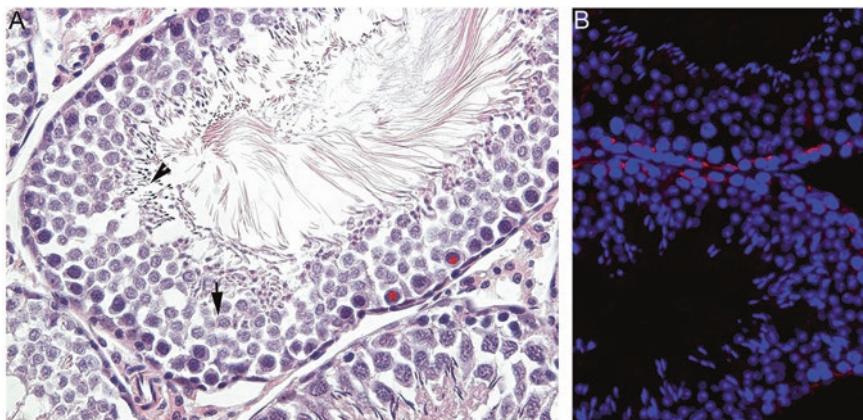


Fig. 1 Rodent testis morphology. Testes were collected from adult Lewis rats (**a**; Charles Rivers Laboratories, Wilmington, MA) or BALB/c mice (**b**; Charles Rivers Laboratories), fixed with Z-fix, paraffin embedded and sectioned. (**a**) Rat testis tissue section was stained with hematoxylin and eosin. (**b**) Mouse testis tissue section was immunostained for claudin 11 (red, **b**; 1:100 dilution; Santa Cruz Biotechnology, Inc., Dallas, TX) to detect tight junctions between Sertoli cells. Sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI; ThermoFisher Scientific, Waltham, MA) for cell nuclei (blue, **b**). Red asterisks (**a**) are over spermatocytes, arrow points to round spermatids and arrow head points to residual bodies. All animal experiments were performed in accordance with guidelines of the National Institutes of Health and Institute for Laboratory Animal Research Care and Use of Laboratory Animals, and Texas Tech University Health Sciences Center (TTUHSC) Institutional Animal Care and Use Committee approved protocols

(spermatogonial proliferation), meiosis (spermatocyte DNA recombination, reduction and division), and spermiogenesis (spermatid differentiation) [4]. Initially the spermatogonia must proliferate to replace the pool of stem cells and produce spermatocytes. This process occurs differently in rodents and primates. In rodents, the spermatogonial population is divided into two categories: undifferentiated (type A_{single}, A_{paired} and A_{aligned}) and differentiated (A1-A4, Intermediate and type B) [4, 5]. In primates and humans, the spermatogonia consist of three subtypes: type A_{dark} (reserve spermatogonial stem cells), type A_{pale} (active spermatogonial stem cells that divide to produce type B spermatogonia), and type B [4, 5]. In all mammals, the type B spermatogonia divide to produce preleptotene spermatocytes. Preleptotene spermatocytes enter prophase I of meiosis and as they progress through meiosis transform into leptotene, zygotene, pachytene and diplotene spermatocytes, which quickly finish meiosis I and form secondary spermatocytes. Secondary spermatocytes quickly complete the second meiotic division, meiosis II, to produce the haploid round spermatids [4, 5]. During the long meiotic prophase I several important processes occur including chromosome condensation, genetic recombination and passage through the blood testis barrier (BTB)/Sertoli cell barrier (SCB) (Fig. 1b) as they migrate from the basal to the adluminal compartment of the seminiferous epithelium [4].

Round spermatids differentiate into spermatozoa through the process of spermiogenesis [4]. During spermiogenesis the acrosomal cap is formed, and the shape of the nucleus changes and decreases in size while the chromatin is compacted. This is associated with cessation of gene transcription and replacement of histones by protamines. Additionally, the spermatids elongate and the tail is formed. Prior to release of the spermatozoa into the lumen the cytoplasm is condensed forming the residual bodies (Fig. 1a), which are phagocytosed by the Sertoli cells [6]. As the developing spermatocytes and spermatids progress through spermatogenesis, which first occurs at puberty, they express novel antigens that were not present when immune self-tolerance was established [7, 8]. Therefore, these germ cells express auto-antigens and are in danger of eliciting a detrimental immune response. However, due to the immune privileged environment within the testis these germ cells fail to evoke this response.

Within the testis there is a balance between immune privilege and immune function. Immunoregulatory cells induce tolerance resulting in protection of the germ cells while proinflammatory cytokines and innate immunity prevent infections and cancerous transformations. At the same time, pathogens can hijack immune privilege and persist within immune privileged sanctuary sites such as the testis. In this review, we will describe the delicate balance necessary to maintain spermatogenesis and provide immune protection to the auto-immunogenic germ cells, yet continue to provide an adequate immune response to protect against pathogens and tumors.

The Good: Testis Immune Privilege

Immune privilege sites are places in the body where detrimental inflammatory immune responses are not activated despite the presence of antigens to which tolerance has not been established. This immune privilege is necessary to protect the auto-antigen expressing germ cells [7, 8]. The requirement for immunological tolerance to the developing testicular germ cells was shown by studies of autoimmune orchitis where activation of an autoimmune response to the germ cells resulted in loss of spermatogenesis and fertility [9, 10]. For instance, within 7–10 days after mice were immunized with syngeneic testicular homogenates, IgG deposits were detected within the testes on preleptotene spermatocytes, which are located outside the BTB/SCB. The IgG deposition occurred 5–6 days before the onset of orchitis and transfer of activated CD4+ T cells, isolated from rodents with orchitis, successfully transferred the disease to normal syngeneic rodents with intact BTB/SCB [11].

Evidence that the testis is an immune privileged site was provided over two and a half centuries ago when it was observed that testes from roosters survived after allotransplantation into hens (reviewed in [12]). Further evidence comes from transplantation studies demonstrating the survival of foreign (allograft and xenograft) tissue grafts transplanted into the testis (reviewed in [12]). For example, allogeneic pituitary grafts, adrenal grafts, insulinomas, and skin grafts, and allogeneic or xeno-geneic pancreatic islet grafts and parathyroid grafts enjoyed prolonged graft

survival after transplantation into the testis compared to controls where the tissue was transplanted to a nonimmune privileged site. For the parathyroid tissue transplanted to the testis, all 14 parathyroidectomized Wistar rat recipients of parathyroid allografts exhibited normocalcemia for over 3 months post-transplantation and recipients of xenografts from guinea pigs (11 of 12) or rabbits (4 of 6) remained normocalcemic for over 25 days [13]. For the xenografts two recipients remained normocalcemic for more than 6 months. In contrast, all parathyroidectomized rat controls that either did not receive transplants (12 total) or were recipients of parathyroid allografts (14 total) or xenografts (six guinea pig xenografts and four rabbit xenografts) to the abdominal wall muscle remained hypocalcemic throughout the study [13].

Other evidence of testicular immune privilege is provided by the studies described below. Mice immunized with testicular homogenates can elicit an immune response to the developing germ cells that ultimately results in autoimmune orchitis [7, 8, 11]. However, under normal circumstances an immune response is not generated against these germ cells [14]. Instead it is difficult to generate models of autoimmune orchitis requiring both adjuvant and specific strains of mice. Moreover, collection of human testicular biopsies with a fine needle to obtain testis samples or spermatozoa for *in vitro* fertilization, can induce damage to the testis and yet no serious adverse effects of the procedure were detected [15].

Sertoli cells are closely associated with the germ cells within the seminiferous epithelium and have been implicated in immune protection of the germ cells. The importance of Sertoli cells in immune privilege has also been demonstrated by transplantation studies where Sertoli cells were able to survive when transplanted across immunological barriers as allografts or xenografts (Fig. 2a) and provide immune protection to co-grafted allogeneic or xenogeneic cells or tissue such as pancreatic islets (Fig. 2b), hepatocytes, skin grafts, adrenal chromaffin cells or neurons (reviewed in [16]). For instance, the first study describing the protection of foreign tissue co-grafted with Sertoli cells involved the transplantation of Sertoli cells and pancreatic islets as allografts underneath the kidney capsule of diabetic rats [17]. Seventy-five percent of the recipients enjoyed normal blood glucose levels throughout the study, with nine of the grafts surviving over 300 days (longest surviving over 475 days), while none of the controls (islets only, no Sertoli cells) achieved normoglycemia. Similar results were observed in both male and female graft recipients. However, this initial study required a 3-day course of the immunosuppressive drug cyclosporine. In a follow-up study, 100% of the recipients maintained normoglycemia for over 100 days without the use of immune suppression [18]. The improved graft survival was attributed to modifications in the isolation and culture of the Sertoli cells. Since publication of these studies, several others describing prolonged grafts survival of cells/tissues co-grafted with Sertoli cells with variable success have been reported (reviewed in [16]).

Of particular relevance to the testis and autoimmune orchitis are transplantation studies using models of type 1 diabetes mellitus (T1DM). T1DM is an autoimmune disease where the immune system attacks the islets within the person's own pancreas. Once the islets are destroyed the individual no longer produces insulin and is

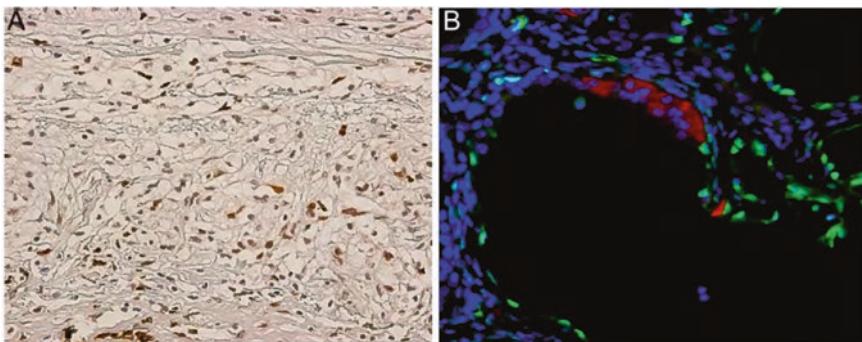


Fig. 2 Sertoli cells survive and protect co-transplanted islets after transplantation. **(a)** Male neonatal pigs (1–3 days old; Texas Tech University Research and Experimental Farm) were used as Sertoli cell donors. Eleven million neonatal pig Sertoli cells were transplanted underneath the kidney capsule of naïve adult Lewis rats as xenografts. Graft bearing kidneys were collected at Day 40 post-transplantation and tissue sections were immunostained for Wilms' tumor 1 (1:10 dilution, brown; Dako/Agilent Technologies, Santa Clara, CA) to identify Sertoli cells. **(b)** Mouse Sertoli cells were isolated from BALB/c mice (8–10 days old). Mouse islets were isolated from BALB/c mice (6–8 weeks old). Three million Sertoli cells were co-transplanted with 500 islets underneath the kidney capsule of diabetic C3H mice (6–8 weeks old; Jackson Laboratories, Ban Harbor, ME) as allografts. The graft bearing kidneys were collected from normoglycemic mice at Day 102 post-transplantation and immunostained for Wilms' tumor 1 (green) and insulin (1:1000 dilution, red, to identify islets; Dako/Agilent Technologies). Tissue sections were counterstained with hematoxylin (blue, **a**) or DAPI (blue, **b**) to detect cell nuclei

unable to maintain glucose homeostasis. Initially, Selawry et al., transplanted islet allografts or xenografts into the testes of diabetic BB/W rats (a model of TIDM) and found that the rats enjoyed prolonged normoglycemia of 65–441 days in 87% of the allograft recipients and 30–40 days in 78% of the xenograft recipients, although a short course of immune suppression (anti-lymphocyte serum or cyclosporine) was required [19, 20]. Later, the transplantation of Sertoli cells and islets into non-obese diabetic (NOD) mice (auto-immune mouse model of TIDM) resulted in normalization of blood glucose and prolonged islet graft survival for over 60 days in 40–60% of the transplanted mice [21, 22]. More recently, it was found that Sertoli cells can prevent and reverse diabetes in 88% and 81% of NOD mice, respectively [23]. This was attributed to regulatory T cells (Tregs), which will be discussed in more detail in the next section.

Mechanism(s) of Immune Privilege

The fundamental role of testicular immune privilege is to allow successful progression of spermatogenesis. The BTB/SCB, tight junctions between adjacent Sertoli cells together with the Sertoli cell body, develops around the same time the spermatocytes first appear. The BTB/SCB divides the seminiferous tubules into the

basal and the adluminal compartments (Fig. 1b). The basal compartment contains spermatogonia and preleptotene spermatocytes and the adluminal compartment contains the advanced germ cells. Previously, testis immune privilege was attributed to the BTB/SCB as it sequesters the majority of the auto-antigenic germ cells and prevents the entry of immune cells and antibodies into the adluminal compartment. Later several observations such as the presence of auto-antigens on preleptotene spermatocytes (present outside the BTB/SCB) and development of the BTB/SCB after completion of meiosis in non-mammalian vertebrates contradicts the role of the BTB/SCB as the sole reason for testicular immune privilege (extensively reviewed in [12, 24]). Additionally, even though the BTB/SCB is repeatedly broken down and reformed between each breeding cycle in seasonal breeders, repetitively exposing the meiotic spermatocytes, an immune response does not occur and fertility is maintained [25].

Recently Tung et al., found that not all antigens from meiotic and haploid germ cells are sequestered behind the BTB/SCB, instead some were detected within the interstitial space outside the BTB/SCB and were involved in maintaining tolerance [26]. Their study focused on two advanced germ cell antigens: lactate dehydrogenase 3 (LDH3) and zonadhesion (ZAN). Immune complexes formed outside the BTB/SCB were detected in mice immunized with LDH3 antibody but not with ZAN antibody suggesting that only ZAN is sequestered behind the BTB/SCB. Further it was demonstrated that non-sequestered meiotic germ cell antigens are exported in residual bodies to induce Treg dependent tolerance as depletion of Tregs resulted in enhanced LDH3 antibody response in the serum of the animals injected with testicular homogenate as compared to controls (non Treg depleted mice). The sequestered meiotic germ cell antigens such as ZAN are not tolerogenic and are protected from an immune response by the BTB/SCB [26].

Prolonged survival of allogeneic or xenogeneic tissues, such as skin fragments, pancreatic islets or parathyroid tissue transplanted into the testis interstitium, outside of the BTB/SCB, suggest that the whole testis is immune privileged. The testis interstitium has an anti-inflammatory environment containing regulatory immune cells (M2 macrophages and immature dendritic cells) (Fig. 3b). On the same note, testis interstitial fluid (IF) exhibits immunosuppressive activity as it inhibited the activation and/or proliferation of stimulated peripheral blood lymphocytes [27–29]. More recently, it was demonstrated that treatment of rat T cells with syngeneic testis IF resulted in a significant increase in CD4+CD25+Foxp3+ cells (CD4 Tregs) compared to T cells cultured without IF [30]. Similarly, treatment of rat monocytes with IF resulted in the polarization of granulocyte-macrophage colony-stimulating factor induced M1 macrophages toward M2 (regulatory) macrophages which secreted significantly higher levels of interleukin (IL)-10 and lower levels of tumor necrosis factor (TNF)- α after lipopolysaccharide (LPS) stimulation, and induced significantly higher numbers of CD4 Tregs when co-cultured with T cells [30]. Somatic cells (Sertoli cells, Leydig cells and peritubular myoid cells) and tolerogenic immune cells (mainly macrophages) in the testis contribute toward the testicular IF.

Sertoli cells express and/or secrete several immune modulatory factors that inhibit complement mediated cell lysis, immune cell proliferation and apoptosis

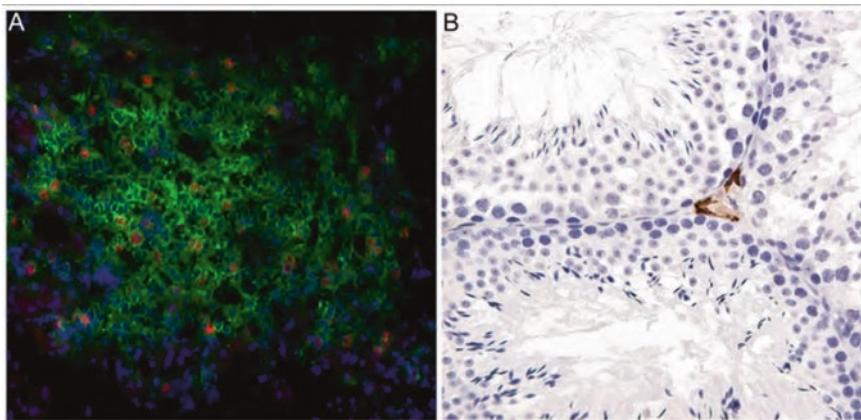


Fig. 3 Immune cells detected within the Sertoli cell grafts and mouse testis. **(a)** Four million primary Sertoli cells, isolated from 19 to 20 day old C57BL6x129 mice (Jackson Laboratories), were transplanted underneath the kidney capsule of naïve BALB/c mice (6–8 weeks old) as allografts. The graft bearing kidneys were collected at Day 20 post-transplantation and immunostained for CD4 (1:25 dilution, green; BD Biosciences, San Jose, CA) and foxp3 (1:400 dilution, red; Abcam, Cambridge, MA) to detect Tregs. **(b)** Mouse testis (collected from 6 to 8 weeks old) tissue section was immunostained for galectin-3 (brown, 1:200 dilution, BD Biosciences) to detect macrophages. Tissue sections were counterstained with DAPI (blue, **a**) or hematoxylin (blue, **b**) to detect cell nuclei

(for details see Table 1). Regulatory immune cell induction by Sertoli cell secreted factors has also been demonstrated (Table 1, Fig. 3a). Similarly, peritubular myoid cells express immunomodulatory factors and can induce species-specific immune tolerance as splenocytes from mice injected with rat peritubular myoid cells showed a reduced response to rat spleen cells (responder cells) as compared to the controls [40, 41]. Leydig cells secrete testosterone under the influence of pituitary luteinizing hormone. In the absence of testosterone, spermatogenesis halts at the meiosis stage [42]. In addition to its role in supporting spermatogenesis, testosterone also has immunomodulatory properties. For instance, induction of experimental autoimmune orchitis (EOA) in rats significantly decreased testosterone levels compared to controls [43]. Exogenous testosterone supplementation protected against the development of orchitis (17–33% developed orchitis), compared to EAO rats with no hormone intervention (80% developed orchitis). Testosterone supplementation also resulted in decreased testicular infiltration of macrophages (ED1/ED2) and CD4 T cells while the number of CD4 Tregs was significantly increased compared to EAO controls. Furthermore, *in vitro* treatment of naïve T cells with testosterone resulted in a significant increase in CD4 Tregs with suppressive activity [44, 45]. In addition to the adaptive immune response, the immunomodulatory effects of testosterone on innate immune cells have also been well documented (reviewed in [46]).

Macrophages regulate fetal testis vascularization and contribute to the spermatogonial niche in the adult testis [47, 48]. Testicular macrophages are closely associated with Leydig cells (Fig. 3b) and play an important role in Leydig cell development

Table 1 Key factors expressed or secreted by Sertoli cells for immune modulation

Sertoli cell factors	Function
Humoral Immune response	
Serine protease inhibitor (SERPIN)-G1	Inactivates C1 proteinases in the C1 complex, preventing formation of C3 convertase
C1 inhibitor (C1-inh)	Prevents spontaneous activation of the complement cascade by irreversibly binding and inactivating C1r and C1s proteases in the C1 complex
Decay Accelerating Factor (DAF, CD55)	Limits the amplification step of the complement cascade by preventing the formation of C3 convertase (C4b2b; classical pathway and C3bBb; alternative pathway)
Membrane Cofactor Protein (MCP, CD46)	
CD59	Prevents C9 binding and formation of membrane attack complex.
Clusterin	
Evidence (extensively reviewed in [24, 31]):	
1. Neonatal pig Sertoli cells survive when subjected to human antibody/complement mediated cell lysis <i>in vitro</i> . Additionally, neonatal pig Sertoli cells have the ability to inhibit both alternative and classical pathways of complement mediated cell lysis both <i>in vitro</i> and <i>in vivo</i> [32, 33].	
2. Neonatal pig Sertoli cells survive when transplanted as discordant xenografts into mice, rats and dogs [34, 35].	
Adaptive Immune response	
SERPIN-A3N	Granzyme B inhibitor. CD8 T cells and NK cells deliver cytolytic granules containing perforin, granzymes and granzylsin to the target cells. Granzyme B activates the caspase cascade thereby killing the target cell via apoptosis.
SERPIN-B9 (protease inhibitor-9)	Inhibitor of granzymes A and B. Also inhibits FAS-FAS ligand induced cell death.
Unidentified factor(s)	Inhibits B and T cell proliferation. Acts by suppressing IL-2 (required for lymphocyte proliferation) production and responsiveness of the lymphocytes to exogenous IL-2.
B7-H1	Ligand for programmed death-1 (negative regulatory receptor present on lymphocytes). Induces T cell anergy and expands Tregs.
TGF- β , indoleamine 2,3-dioxygenase (IDO), soluble form of JAGGED-1, galectin-1	Induces regulatory T cells and tolerogenic dendritic cells.

(continued)

Table 1 (continued)

Sertoli cell factors	Function
Evidence (extensively reviewed in [24, 31]):	
1.	Sertoli cells survive allo and xenotransplantation by inhibiting apoptosis and inducing regulatory T cells at the graft site ([36] and Dufour JM, unpublished data (2011)).
2.	Transplantation of syngeneic Sertoli cells and islets into the NOD mice significantly prolonged the islet graft survival. This protection was dependent on TGF- β as administration of anti-TGF- β antibody resulted in loss of transplanted islets [22].
3.	Transplantation of neonatal pig Sertoli cells into the NOD mice resulted in diabetes prevention and reversion in 88% and 81% of the animals, respectively. Induction of autoantigen-specific CD4 Tregs in Sertoli cells-treated NOD mice was attributed to these protective effects. TGF- β and IDO were required for generation and functionality of CD4 Tregs, respectively [23].
4.	Treatment of mouse Sertoli cells with IFN- γ resulted in upregulation of B7-H1 and MHC-II while the positive co-stimulatory molecules were not detected. Co-culture of these Sertoli cells with T cells resulted in inhibition of T cell proliferation and an increase in CD4 Tregs [37].
5.	Culturing of CD4 T cells in the presence of mouse Sertoli cell conditioned media (SCCM) induces the conversion of these CD4 T cells into functional CD4 Tregs. This study also demonstrated that JAGGED-1, present in the SCCM, induces CD4 Tregs by activating the Notch pathway. Induction of CD4 Tregs by Sertoli cells was TGF- β dependent [38].
6.	Co-culture of immature dendritic cells with Sertoli cells or SCCM downregulated the expression of MHC-II and co-stimulatory molecules (CD80, CD83 and CD86) on dendritic cells. Induction of tolerogenic dendritic cells was evident as these cells expressed high levels of anti-inflammatory cytokines such as IL-10 and TGF- β , inhibited proliferation of T cells and induced CD4 Tregs. Knock down of galectin-1 expression by Sertoli cells abrogated the induction of these tolerogenic dendritic cells [39].

and steroidogenesis (reviewed in [49]). The testicular macrophage phenotype is skewed toward regulatory M2. For example, stimulation of testicular macrophages with LPS or interferon (IFN)- γ resulted in a diminished inflammatory response as demonstrated by low production of pro-inflammatory cytokines or chemokines such as TNF- α , IL-6, IL-1 β , monocyte chemoattractant protein (MCP)-1, and inducible nitric oxide synthase, while the production of anti-inflammatory molecules such as IL-10 and suppressor of cytokine signaling 1 was upregulated as compared to conventional macrophages [50–52]. Under normal conditions, testicular macrophages express low levels of toll like receptor (TLR) signaling pathway genes, higher levels of negative regulators of TLR signaling pathways and have impaired ability to support T cell activation [52, 53]. They have the capability to inhibit the activation of the nuclear factor-kappa B signaling pathway after being exposed to inflammatory stimuli [53]. Additionally, co-culture of T cells with testicular macrophages results in increased generation of CD4 Tregs [30]. Thus, testicular macrophage response to inflammatory stimulation is dampened as compared to conventional macrophages suggesting the role of macrophages in maintaining testis immune privilege. Collectively, the above mentioned studies demonstrate that the testis immune privileged environment is actively maintained by somatic cells and M2 macrophages.

The Bad and the Ugly: The Testis as a Sanctuary Site for Pathogens and Cancer

Viruses

The immune system is designed to protect the body from bacterial and viral pathogens and cancer. However, as the recent outbreaks of Ebola and Zika viruses highlight, there is a potential downside to this immune privilege where the testis can act as a reservoir and allow the persistence of infectious agents [54]. At least 27 different viruses have been detected in human semen so far [55] and 12 viruses have been detected in human testes [55, 56] (Table 2). It is well known that human immunodeficiency virus (HIV) can be transmitted through sexual contact. While the testis is not the major site for HIV replication as resident testicular cells do not support HIV replication, the testis can act as a HIV sanctuary site and may protect infected immune cells in the testis from being targeted by antiretroviral therapy (ART) drugs [107]. This is supported by the fact that cells with integrated HIV DNA were detected in testes collected from 5 of 6 individuals with HIV on ART [107].

Ebola virus and Marburg virus are filoviruses in the family *Filoviridae* that cause severe hemorrhagic fever with high mortality rate. The first confirmed case describing the transmission of a filovirus through sexual contact was reported in 1967. In this case, a woman was infected with Marburg virus after exposure through sexual contact with her husband who had previously contracted the virus and whose semen was positive [108, 109]. Since the initial Marburg report, it was suspected that Ebola virus could be transmitted through sexual contact [110]. Recently this was confirmed in a report describing the sexual transmission from a male (whose blood was negative for Ebola virus) to a female (who had not previously been in contact with anyone infected with Ebola virus) in Liberia [111, 112]. After the sexual encounter the female patient contracted Ebola virus and died from the disease. Further testing of the male detected Ebola virus in the semen sample collected 20 days after the sexual encounter and 199 days after his estimated onset of disease. Sequencing results showed that the Ebola virus genome isolated from the semen sample was similar to the Ebola virus sequence from the female patient further confirming the role of sexual contact in virus transmission 6 months after the initial infection. This study provides strong evidence that the virus can hide in the testis even after it is cleared from the blood.

Several studies have examined the prevalence of Ebola virus in semen samples. A study from the recent outbreak in Sierra Leone reported that the semen samples of all nine males tested were positive for Ebola virus 2–3 months after the onset of illness, 65% of 40 males tested at 4–6 months were positive, and 26% of 43 males tested 7–9 months after the onset of illness were positive for the virus [113]. In these samples, the viral RNA copy numbers detected in the semen decreased with time. Of note, most studies simply test for the presence of viral RNA even though detection of viral RNA does not necessarily indicate the presence of infectious virus [114]. Another study reported detection of Ebola virus RNA and infectious virus up

Table 2 Summary of viruses either detected in or isolated from human semen or testis

Family	Virus	Abbrev.	Detected in human semen [55, 56]	Detected in human testis [56]	References
Adenoviridae	Adenoviruses	ADV	Y	Y	[57, 58]
Anelloviridae	Transfusion Transmitted virus	TTV	Y	Y ^a	[59, 60]
Arenaviridae	Lassa Fever virus	LASV	Y	NDF	[61]
Bunyaviridae	Rift Valley Fever virus	RVFV	Y	NDF	[62]
Filoviridae	Ebola virus	EBOV	Y	NDF	[63, 64]
Filoviridae	Marburg virus	MARV	Y	NDF	[65, 66]
Flaviviridae	Human Pegivirus (GB virus C)	GBV-C	Y	NDF	[67]
Flaviviridae	Hepatitis C virus	HCV	Y	NDF	[68, 69]
Flaviviridae	Zika virus	ZIKV	Y	NDF	[70, 71]
Hepadnaviridae	Hepatitis B virus	HBV	Y	Y ^a	[72–75]
Herpesviridae	Cytomegalovirus	CMV	Y	NDF	[76, 77]
Herpesviridae	Epstein Barr virus	EBV	Y	Y	[77–80]
Herpesviridae	Human Herpes viruses 6, 7 and 8	HHV-6/7/8	Y	NDF	[77, 79, 81–83]
Herpesviridae	Herpes Simplex viruses 1 and 2	HSV-1/2	Y	Y	[57, 58, 77, 79]
Herpesviridae	Varicella Zoster virus	VZV	Y	NDF	[77, 79]
Herpesviridae	Mumps virus		Y	Y	[84–88]
Papillomaviridae	Human Papillomavirus	HPV	Y	Y	[89, 90]
Parvoviridae	Adeno-Associated virus	AAV	Y	Y	[91, 92]
Parvoviridae	Parvovirus B19	B19V	NDF	Y	[93]
Picornaviridae	Coxsackie viruses A and B	CVA/B	NDF	Y	[94]
Polyomaviridae	BK virus	BKV	Y	NDF	[95]
Polyomaviridae	JC virus	JCV	Y	NDF	[95]
Polyomaviridae	Simian virus 40	SV40	Y	NDF	[96]
Retroviridae	Human Immunodeficiency virus	HIV	Y	Y	[97–102]
Retroviridae	Human T-cell Lymphoma virus	HTLV	Y	NDF	[103]
Retroviridae	Human Endogenous retrovirus	HERV	NDF	Y	[104]
Retroviridae	Simian Foamy virus	SFV	Y	NDF	[105]
Togaviridae	Chikungunya virus	CHIKV	Y	NDF	[106]

Y yes, NDF no data found

^aOne abstract found with positive results

to 290 days and 70 days after illness onset, respectively [115]. While another paper reported that Ebola virus was present in 9% of the semen samples tested from 429 survivors and 6% of these participants were positive 12 months after recovery [116]. The longest time between recovery and positive detection of Ebola virus in the semen was 565 days (18.8 months) [116]. Given the increased possible infection period, survivors are advised to practice safe sex for at least 12 months after recovery [117].

Zika virus is a member of the *Flaviviridae* family, genus *Flavivirus*, which includes Dengue virus, West Nile virus and Yellow Fever virus. These viruses are generally transmitted by mosquitoes. The recent outbreaks of Zika virus in 2015–2016 were associated with the unique finding of disease transmission via the sexual route not reported for other flaviviruses [118]. Transmission of the Zika virus through the semen from sexual contact has been confirmed in almost 50 cases in the United States [119]. Evidence shows that Zika virus can spread sexually by both asymptomatic and symptomatic males. In Zika virus endemic regions where the mosquito vector is present, the role of sexual transmission in disease spread may be difficult to predict; however, a recent report indicated 56.5% of Zika virus serum-positive males were also semen-positive for the virus for up to 108 days after infection [120]. Another study was able to detect the virus in the semen over 6 months after the initial infection [70]. These studies collectively suggest a likelihood of a much higher contribution of the sexual route in virus spread than what was previously thought and a longer infectious phase (3–6 months after initial infection) as compared to the transmission via mosquitoes (1–2 weeks after initial infection).

Wild type mouse models do not support robust infection of Zika virus, therefore recent studies have used immunocompromised mouse models to show the presence of Zika virus in the testes and epididymides even after the virus was cleared from blood and other body fluids [121–123]. Infection resulted in decreased testosterone levels and testicular damage including the loss of germ cells, disruption of seminiferous tubules and immune cell infiltration. The cellular targets of Zika virus are not clear as each study reported different Zika virus positive cells. Govero et al., indicated that spermatogonia, primary spermatocytes and Sertoli cells within the testis, and sperm in the testis and epididymis were infected in type I IFN deficient mice [121]. While Ma et al., reported that only spermatogonia and peritubular myoid cells were positive [122]. A study by Sheng et al., demonstrated that Sertoli cells, testicular macrophages and semen were positive, while Leydig cells and peritubular myoid cells were negative in AG6 mice deficient with IFN $\alpha/\beta/\gamma$ [123]. Yet another study used electron microscopy to show Zika virions associated with mouse spermatozoa in the testis and sperm in the epididymis [124]. In addition, recent nonhuman primate studies also provide evidence for the presence of Zika virus RNA in the testes for up to 28 days following infection with Zika virus [125, 126].

Since mouse studies are mainly conducted in type I IFN deficient mouse, it is still a question whether these abnormalities are also seen in human testes. The data on cellular targets and immune responses in human testes is limited largely due to unavailability of human tissue from infected men. However, recently our group has shown infection and intracellular replication of Zika virus in human Sertoli cells *in*

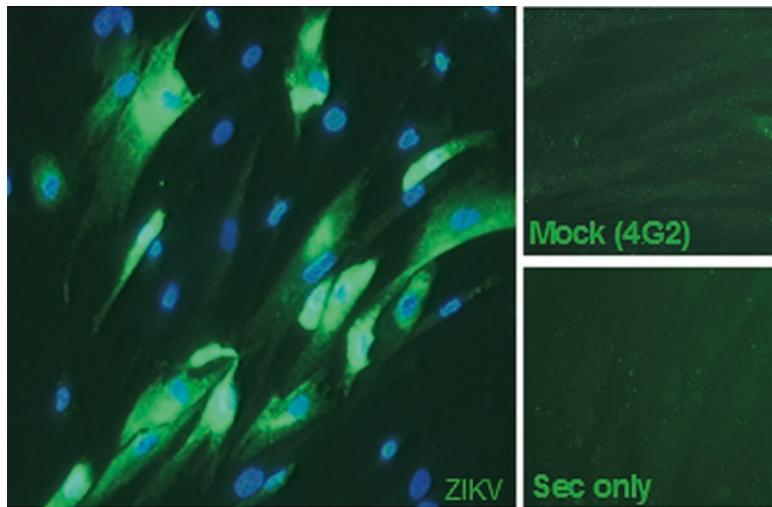


Fig. 4 Zika virus infection in human Sertoli cells. Human primary Sertoli cells were infected with Zika virus (ZIKV, Asian strain) at the multiplicity of infection (MOI)-1. (a) Representative image of ZIKV immunostaining in infected Sertoli cells at 48 h after infection. Cells were immunostained for envelope protein (green) using the 4G2 anti-flavivirus group antibody. Cell nuclei were stained with DAPI (blue). Cells incubated with secondary antibody only (Sec only)

in vitro (Fig. 4) [127]. Primary human Sertoli cells were able to support Zika virus infection for at least 9 days without cell death in spite of production of inflammatory cytokines and type I IFN [127]. Using an *in vitro* SCB model, where Sertoli cells were cultured on polyethylene terephthalate inserts, transendothelial electrical resistance and transmigration of Zika virus across the inserts were quantified following infection. We demonstrated that infection did not affect the barrier permeability although the virus was released on both the adluminal and basal sides of the SCB model. These results suggest that infection of Sertoli cells and transmigration of the progeny virus across the BTB/SCB can be one of the routes by which Zika virus can reach the lumen of the seminiferous tubules and this process does not require disruption of the barrier. Human macrophages were also infected with Zika virus resulting in the production of inflammatory cytokines. Incubation of Sertoli cells with media from infected macrophages resulted in increased BTB/SCB permeability. Interestingly, human Sertoli cells were also highly susceptible to infection with West Nile virus, but not Dengue virus *in vitro*. Lack of infection by Dengue virus was also observed in mice [121]. So far there is no epidemiological data to support the notion that West Nile virus can also establish persistence in humans but more careful studies are warranted to further validate this possibility.

These recent outbreaks have increased the interest in the testis as a viral sanctuary site where viruses could take advantage of the immune privileged mechanisms mentioned above. For example, viruses like Zika and Ebola modulate host antiviral responses and cellular proliferation pathways to favor their long-term survival.

One major question remaining is why, despite the strong antiviral response in Sertoli cells, does the virus still persist in the testis. A possible explanation might be that alterations in other negative regulators of innate immune pathways such as transforming growth factor- β and Tyro3, Axl and Mer (TAM) receptor signaling in Sertoli cells may allow infected cells to survive longer as compared to other cellular targets, thus contributing to viral persistence. Moreover, an increase in the TNF- α and IL-6 families of cytokines may have implications on other important testicular cell functions during virus infection, including steroidogenesis and leukocyte transmigration, and therefore further investigation in this area is also warranted. It should also be mentioned that in some instances, the presence of the HIV and Zika virus were still present in the semen after vasectomy indicating that persistence in other regions of the genitourinary tract other than the testis may also be responsible. Given the large diversity in the virus families detected in the testis and semen (Table 2), mechanisms of virus entry and immune evasion used by these viruses may also vary and require further study.

Bacteria

Most infections in the testis are the result of viral bloodborne pathogens. Nonviral infections within the testis are rare and are typically associated with progression of epididymitis into epididymo-orchitis as the inflammation spreads up the reproductive tract [128]. The type of bacteria causing the infection varies with age with the most common source of infection causing epididymitis in males 14–35 years of age being *Neisseria gonorrhoeae* (*N. gonorrhoeae*) and *Chlamydia trachomatis* (*C. trachomatis*) [128, 129], while it is *Escherichia coli* (*E. coli*) in males under 14 or over 35 [128, 129]. Other sources of infection include *Pseudomonas* spp. and *Ureaplasma urealyticum* [130]. For example, *C. trachomatis*, a microbe with extracellular infectious and non-infectious intracellular forms, has been detected in urethral or urine samples from 11% to 35% of men with epididymo-orchitis [131]. Acute epididymo-orchitis is associated with decreased sperm counts and decreased sperm motility [131], which is thought to be due to direct interactions of bacteria with sperm, obstruction of the epididymal duct or testicular necrosis [131, 132]. Interestingly, *C. trachomatis* was isolated from the testes from two patients who presented for infertility. *C. trachomatis* was also isolated from the epididymis from one of these patients. Both testes and epididymides were normal on physical exam, however one patient had a complete lack of germ cells and was diagnosed with Sertoli cell only syndrome following testicular biopsy [133, 134]. It was not determined if *C. trachomatis* was the cause of this syndrome. The other patient had pyospermia, abnormal sperm with elementary bodies of *C. trachomatis*, coiled sperm tails, morphologic alternations of the epididymis, and increased immature germ cells with arrested maturation [134]. These reports are not surprising considering that approximately 50% of infected men are asymptomatic (reviewed in [129]) suggesting that symptoms of inflammation, generated by an immune response, may not always be

present when *C. trachomatis* is in the testis. In animal studies, *C. trachomatis* has also been detected in the testes [131]. Pal et al., cultured *C. trachomatis* from the urethra, urinary bladder, epididymis, and testes of mice initially inoculated with *C. trachomatis* in the urethra, a model that mimicked a mechanism of human transmission [135]. However, pathological or morphological changes or *C. trachomatis* inclusions in the epididymides and testes were not seen [135]. This could be because the study time frame was not long enough for the bacteria to cause testicular damage in the murine model.

Normally, the presence of bacteria in human testes is not confirmed, as biopsies are not recommended due to the risk of systemic spread of the pathogen. However, in some cases of testicular abscesses, bacterial species have been isolated from human testes. Testicular abscess is a rare complication of untreated or advanced epididymo-orchitis [136]. *Salmonella enteritidis*, for instance, has been cultured from pus drained from testicular abscesses from immunocompromised patients and one postoperative patient [137, 138]. In six other cases, *Actinomyces neuii* (*A. neuii*), *Strep pneumoniae* (bilateral infection), *Pseudomonas aeruginosa* (*P. aeruginosa*), and *E. coli* were detected from testicular abscesses in patients presenting with fever, a red swollen testicle or scrotum, and a hard testicular lump or mass [136, 139–141]. Orchidectomies were performed on one patient with *A. neuii* and three patients with *P. aeruginosa* infections. Testicular necrosis, fibrosis, and immune cell infiltrate was present in all three cases of *P. aeruginosa* infections [141]. Initially, most of these patients were unsuccessfully treated for epididymitis, but following isolation of the infectious agent and determination of its susceptibility to antibiotics, more appropriate antibiotic treatment was given to clear the infection.

Infections are generally cleared after treatment with antibiotics, however negative effects on semen parameters can persist. Rustz et al., found azoospermia and oligospermia in 40% of patients who were originally treated for epididymitis [142] and Osegebe et al., found that 2 years after fertile men were treated for *N. gonorrhoea*, only 40% of them produced adequate semen and the rest had testicular damage [143]. More research is needed to further understand the effects of treatment on semen parameters, male fertility, and more specifically the human testis. *Staphylococcus aureus* and *E. coli* were cultured from the testes of broiler breeders (roosters). One animal had intratubular orchitis with degenerate spermatozoa and the other intratubular epididymo-orchitis with necrotic lesions in 80% of the seminiferous tubules [144]. A recent study using a rat epididymo-orchitis model demonstrated the presence of *E. coli* in the interstitium of the testis that resulted in impaired spermatogenesis via damage to Sertoli cells and germ cell necrosis [145]. This could have important implications in humans as Sertoli cells are critical for spermatogenesis and male fertility. Therefore, more research is crucial to fully understand the mechanisms and cells targeted by bacterial infections in the testis.

Overall, bacterial infections of the testis are rare. Patients are normally treated when inflammation in the male reproductive tract is present prior to severe infection i.e. testicular abscess. Events such as chronic inflammation with or without the presence of viable bacteria, multiple re-infections, lack of detection of infectious agents,

and failure of antimicrobial treatment may lead to more severe infections and potential problems with male fertility [129, 146]. The mechanisms and pathology underlying bacterial infections of the testes need further elucidation in order to develop improved detection methods, not requiring a testicular biopsy, and antibiotic treatment.

Cancer

Given the immune privileged status of the testis you might expect an increased risk of testicular cancer. However, the overall rate of testicular cancer is low, accounting for approximately 1% of all male cancers globally [147, 148], although it is the most commonly diagnosed malignancy in younger men (ages 15–39) [148]. The testis cancer incidence rate (age-standardized) is higher in developed countries compared to developing countries, 5.2 versus 0.7 per 100,000, respectively [148–150]. Interestingly, first-generation immigrants have a risk of testicular cancer that mimics the risk in their home countries while environmental origin plays an additional role in increasing the risk in second-generation immigrants [150]. The World Health Organization classifies testis tumors into the following categories: (1) germ cell tumors, (2) sex cord-stromal tumors, (3) tumors containing both germ cells and sex cord-stromal elements, (4) miscellaneous tumors of the testis, (5) hematolymphoid tumors and (6) tumors of the collecting duct and rete testis [151]. Of all the categories, germ cell tumors account for 95% of the tumors arising in testis (Fig. 5b, c) [147]. Improved treatment of testicular cancer has increased survival from a 5-year survival rate of 80% in 1975 to 96.9% in 2009 [152].

Although, the immune privilege status of the testis does not increase the incidence of testicular cancer compared to cancer rates at other sites, the testis can serve as a reservoir for relapse of cancers such as acute lymphoblastic leukemia (ALL) and Markel cell carcinoma (MCC). The incidence of MCC relapse in testis is rare [153]. For ALL, testes and the central nervous system (another immune privileged organ) are considered the two most frequent extramedullary (outside the bone marrow) sites for relapse [154, 155]. In the early-mid 1980s, the incidence of isolated testis relapse of ALL was 5–17% which then decreased to 3–4% in the late 1980s to early 1990s (extensively reviewed in [154]). Although lessons learned from past experience along with advances in surgical technique, radiation therapy and chemotherapy has decreased the current incidence of ALL relapse to the testis to 2–5% [156, 157], it is still a challenging issue for developing countries with limited resources [157]. For example, retrospective case analysis of male children with ALL in India from January 1990 to December 2006 revealed a high incidence of isolated testicular relapse (15.3%, 17 out of 111) [157].

The exact reason for testis to be a frequent site for ALL relapse is not known. Some investigators believe that unequal distribution of the chemotherapeutic drug into the testis and low temperature of the testis resulting in reduced cytotoxicity of the drugs might be the culprits [154, 158]. The ability of methotrexate to pass from

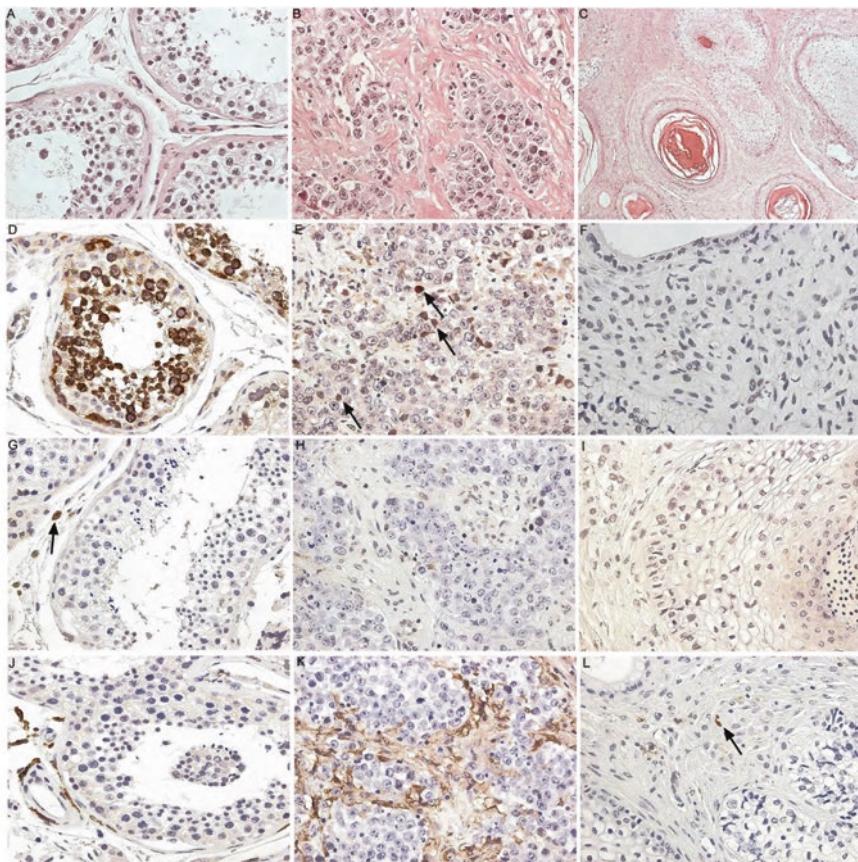


Fig. 5 Human testicular cancer. Non-cancerous (**a, d, g, j**) and germ cell tumor (**b, c, e, f, h, i, k, l**) testis tissues were collected. *Non-cancerous testicular tissue:* Orchidectomy was performed on a 51-year old patient with chronic right testicular/scrotal pain (**a, d, g, j**). Testicular germ cell tumor consisted of seminoma (**b, e, h, k**) and non-seminoma (**c, f, i, l**) samples. *Seminoma:* The 36-year old patient was diagnosed with classic seminoma, Leydig cell hyperplasia and intratubular germ cell tumor (**b, e, h, k**). *Non-seminoma:* The 29-year old patient was diagnosed with non-seminomatous mixed germ cell testicular cancer. Hematoxylin and eosin staining of non-cancerous (**a**), seminoma (**b**) and non-seminoma (**c**) testicular tissue sections was performed. Testicular tissue sections were immunostained with DDX4 (germ cell marker, brown, 1:100 dilution, **d–f**; Abcam), CD3 (T cell marker, brown, 1:25 dilution, **g–i**; Cell Marque, Rocklin, CA) and CD14 (monocyte/macrophage marker, brown, 1:10 dilution, **j–l**; Cell Marque). Cell nuclei were counterstained with hematoxylin (blue, **d–l**). Germ cells were detected in non-cancerous (**d**), and seminoma (**e**, arrows) but not in the non-seminoma (**f**) tissue. T cells were detected mainly in the non-cancerous (**g**, arrow) tissue sample while macrophages were detected in all the tissues (**j–l**) tested. However, higher macrophage infiltration was detected in seminoma (**k**) as compared to non-cancerous (**j**) and non-seminoma (**l**, arrow) tissue samples. This study was approved by the Institutional Review Board of TTUHSC. Informed consent was obtained from the patients

the blood into the rat testes demonstrated that methotrexate levels were two- to fourfold lower in the testicular IF and 18- to 15-fold lower in the seminiferous tubules when compared to the serum levels [158]. Additionally, the testis as an immune privileged site generates a perfect environment for tumor cell survival by providing anti-inflammatory factors and regulatory immune cells.

Activation of Innate and Adaptive Immunity

Despite testis immune privilege, activation of the innate and adaptive immune responses has been attributed to the low incidence of non-viral infections and cancer in the testis [159]. Innate immunity, the first line of defense against infections, involves recognition of pathogen-associated molecular patterns (found on bacteria, virus, fungus and protozoa) by pattern recognition receptors such as TLRs, retinoic acid-inducible gene I (RIG-I) like receptors (RLRs) and intracellular nucleotide-binding oligomerization domain (NOD) receptors or NOD-like receptors. Several studies have demonstrated that testicular cells express the majority of these pattern recognition receptors and have the capability to initiate inflammation, thereby clearing pathogens and protecting the host [160–162]. For instance, mumps virus infection significantly increased the expression of chemokines (MCP-1 and C-X-C motif chemokine ligand-10), pro-inflammatory cytokines (TNF- α and IL-6) and type 1 IFN- α by Sertoli cells and Leydig cells. TLR and RIG-I signaling was attributed to the activation of the innate immune response as silencing of these receptors in Sertoli cells and Leydig cells resulted in reduced expression of chemokines, pro-inflammatory cytokines and type 1 IFN- α after mumps virus infection as compared to controls [163].

Testicular germ cell tumors, specifically seminomas (Fig. 5b, e, h, k), are mainly infiltrated by CD8 T cells and macrophages (Fig. 5h, k) [164–167]. Other immune cells such as B cells, CD4 T cells and NK cells are detected infrequently in seminomas [164, 166]. Extensive immune cell infiltrate is rarely observed in non-seminoma testicular germ cell tumors (NSGCTs; Fig. 5c, i, l). For instance, analysis of patient samples ($n = 40$ total, $n = 20$ seminomas and $n = 20$ NSGCTs) revealed that cytotoxic T cell infiltration (CD8 T cells) was significantly increased in the seminomas as compared to the NSGCTs [167]. The number of CD8 T cells expressing cytotoxic markers such as T-cell-restricted intracellular antigen-1 and granzyme B were significantly higher in seminomas than in NSGCTs. The increased number of granzyme B+ lymphocytes also strongly correlated with the tumor cell apoptotic index in the seminomas suggesting the presence of activated cytotoxic T cells [167].

On the same note, peripheral blood mononuclear cells were obtained from individuals ($n = 45$) diagnosed with testicular cancer in order to investigate the prevalence, magnitude and phenotype of cancer/testis antigen (such as melanoma associated antigen (MAGE)-A1, MAGE-A3 and MAGE-A4) specific T cells [168]. Patients with seminomas and mixed germ cell tumors developed a T cell response

against several MAGE-A family proteins (MAGE-A1 (29%), MAGE-A3 (34%) and MAGE-A4 (29%)) whereas patients with NSGCTs only exhibited a T cell response against MAGE-A3 (30%). The immune response to MAGE-A3 in patients with NSGCT was only detected when the tumor was metastasized out of the testis. Analysis of the effector functions of MAGE-A-specific T cells, after stimulation with overlapping immunogenic peptides, revealed the presence of IFN- γ^- TNF- α^+ and IFN- γ^+ TNF- α^+ CD4 and CD8 T cells. The frequency of MAGE-A-specific CD8 T cells was sixfold higher than MAGE-A-specific CD4 T cells. The frequency of MAGE-A-specific T cells significantly decreased following orchidectomy suggesting that the continuous presence of cancer/testis antigens is required to maintain the cytotoxic T cell immune response [168]. Although, in this study the presence of these MAGE-A-specific T cells at the tumor site was not analyzed due to limited availability of fresh tumor tissue, another study demonstrating the presence of MAGE-A3 specific cytotoxic CD8 T cells in a patient with seminoma [169] supports that these T cells have the capability to infiltrate testicular tumors. Overall, in case of testicular seminomas, activation of cytotoxic immune cells suggests the loss of testis immune privilege thereby resulting in a good prognosis.

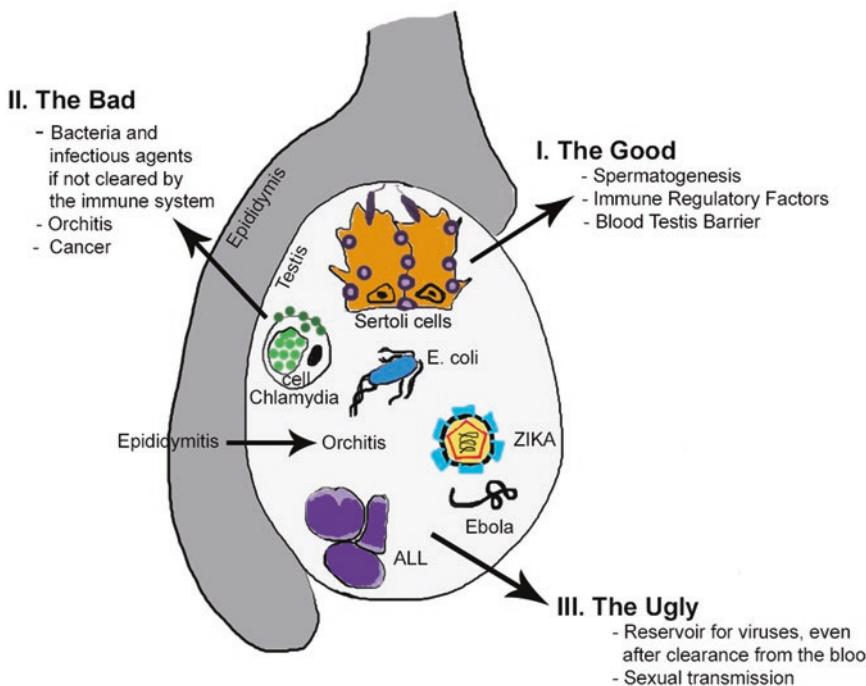


Fig. 6 Summary picture. A cartoon picture depicting the good, the bad and the ugly of testis immune privilege

Summary

In conclusion, a delicate balance between immune privilege and immune function persists in the testis (Fig. 6). The “good” of immune privilege provides an anti-inflammatory environment with regulatory immune cells that allows completion of spermatogenesis and protection of the auto-immunogenic germ cells. However, as is also true for other immune privileged sites, like the brain or eye, immune privilege can be “bad” as bacterial and viral pathogens and cancers can hijack this immune privilege and remain in the testis as a sanctuary site. Recent outbreaks of Zika and Ebola virus highlight the “ugly” of this situation as these viruses can be sexually transmitted and use the testis as a reservoir for months after they have been cleared from the bloodstream.

This draws attention to the need for increased study as there is still a major gap in our understanding of testis-pathogen interactions and cancer relapse within the testis. Moreover, use of the testis as a sanctuary site is problematic because treatments that clear the pathogens from the blood may not cross the BTB/SCB, which provides additional protection to the pathogen. Therefore, careful delineation of pathways by which viruses and cancer cells utilize testis immune privilege to their advantage are warranted to ultimately design therapeutic strategies to prevent or clear testicular infections and cancer.

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Immunologic Environment of the Testis



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Introduction

The mammalian testis possesses a specialized immunologic environment due to its immunoprivileged status (preventing an autoimmune response to male germ cells), and its effective local innate defense against microbial infections [1]. Allo- and auto-antigens can thereby be tolerated without evoking immune rejection in the testis because of its immune privilege. Spermatogenesis is completed after puberty, a long time after the establishment of immune self-tolerance during the fetal and neonatal periods; and yet male germ cells produce a large number of autoantigens that are regarded as non-self with respect to the immune system and can therefore induce immune responses. However, these autoantigens are immunologically tolerated in the testis because it carries an immunoprivileged status under physiologic conditions. The mechanisms underlying this immune privilege in the testis have been intensively investigated and comprehensively reviewed [2–4]. The present chapter briefly summarizes the major achievements in the area of testicular immune privilege.

While the testis is an immunoprivileged organ, major microbial pathogens including viruses, bacteria, and parasite, may infect the testis via the circulation and ascending urogenital tracts. To overcome this immunoprivileged status, the testis

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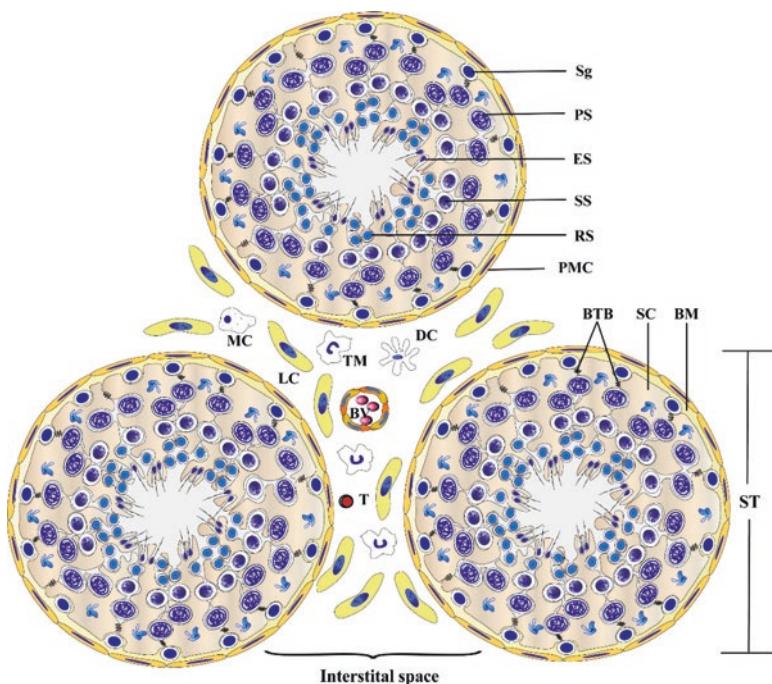


Fig. 1 Schematic histology of the murine testis: The testis is composed of two distinct compartments: The seminiferous (ST) and interstitial space. The ST is surrounded by peritubular myoid cells (PMC), and consists of developing germ cells, including spermatogonia (Sg), primary spermatocytes (PS), secondary spermatocytes (SS), round spermatids (RS), and elongated spermatids (ES), germ cells are embraced by columnar Sertoli cells extending from the basal membrane to the lumen of the ST. The blood-testis barrier (BTB) is formed by the junctions between neighboring Sertoli cells (SC) in close proximity to the basal membrane (BM). The interstitial spaces are located outside of the ST and contain a majority of Leydig cells and testicular macrophages (TM), as well as minor immune cells, including T lymphocytes (T), dendritic cells (DC), mast cells (MC). The blood vessel (BC) is located in the interstitial spaces

adopts an efficient local innate defense system against microbial infections. Various leukocytes reside in the intestinal spaces of the testis (Fig. 1), with macrophages representing approximately 20% of interstitial cells or 80% of the total leukocytes in rat testis. It is been believed that testicular macrophages maintain the front line of innate defense against invading microbes. In addition to leukocytes, the majority of the tissue-specific cells including Leydig, Sertoli, and male germ cell, is also equipped with innate defense mechanisms that may participate in testicular defense against microbial infections. Importantly, Sertoli and male germ cells encounter microbes from the ascending genital tract, and immune cells are not found in the seminiferous tubules. Therefore, the innate defense capabilities of Sertoli and male germ cells would be expected to play critical roles in counteracting invading microbes within the seminiferous tubules. Pattern recognition receptor (PRR)-initiated innate immune responses in testicular cells have been recently investigated.

This chapter will describe signaling of PRR-initiated innate immune responses in major testicular cell types.

Testicular immune status must be precisely controlled to maintain immune homeostasis that is essential for spermatogenesis. However, immune homeostasis in the testis may be disrupted by microbial infection or noninfectious factors, such as chemical toxicants, physical trauma, or testicular cancer, resulting in infectious and autoimmune orchitis, an important etiologic factor in male infertility [5]. The pathomechanism governing autoimmune orchitis have been intensively investigated using experimental autoimmune orchitis (EOA) models in rodents [6]. However, although the pathogenesis of infectious orchitis is likely caused by both direct microbial infection and indirect systemic immune responses, this remains to be clarified. This chapter will also describe immunologic disorders in the testis and their effects on male fertility.

Immune Privilege in the Testis

Immune privilege represents a special immunologic status of several organs in mammals, including the pregnant uterus, anterior chamber of the eye, brain, and testis; where the immune responses to allo- and auto-antigens are remarkably reduced to protect these vital organs from detrimental immune responses [7]. However, mechanisms underlying immune privilege in these organs are not identical. The testis possesses a unique immunoprivileged environment based on its histologic structure, local immunoregulation and peripheral immune tolerance.

Features of Immune Privilege in the Testis

The phenomenon of testicular immune privilege was observed as early as 1767 when John Hunter transplanted a cock testis into the belly of a hen [8]. Tissue transplantation studies in the 1970s demonstrated that various xeno- and allografts including skin, parathyroid, and islet cells can survive for long periods in the testis without immunosuppressive drug treatments. Moreover, the transplantation of xeno- and allogenic spermatogonial stem cells into the germ cell-depleted mouse testis can restore spermatogenesis [9]. Although male germ cells produce a large number of antigens that can induce immune response in extra-testicular sites, these auto-antigens are tolerated in the testis under physiologic conditions. These observations indicate that the systemic immune response in the testis is reduced, and underlying mechanisms have been intensively investigated.

Early studies focused on the role of testicular structure in maintaining immunoprivileged status. However, the physical structure of the testis was not found to be critical to testicular immune privilege. The scrotal temperature is ~5 °C below core body temperature in humans, which was initially believed to contribute to testicular

immune privilege because immune response is attenuated in cold-blooded animals [10]. However, the parathyroid allografts in cryptorchid testes survive longer than those placed subcutaneously in the ear, suggesting that temperature is not a critical factor involved in testicular immune privilege [11]. Testicular immune privilege was also once proposed to contribute to deficient lymphatic drainage in the testis, but this contention was disproved when an afferent lymphatic vessel between the testis and kidney lymph node was found [11]. Many types of immune cells reside in the interstitial spaces of the testis under physiologic conditions. The sequestration of most germ cells in the abluminal compartments of the seminiferous epithelium from the interstitial immune cells at the blood-testis barrier (BTB) contributes to testicular immune privilege. The BTB is formed by adjacent Sertoli cells near the basal membrane and divides the seminiferous epithelium into abluminal and basal compartments. Although the BTB separates the late stages of germ cells, spermatogonia and pre-leptotene spermatocytes that are located at the basal compartments outside the BTB produce immunogenic antigens [12]; and allografts in the interstitial spaces also enjoy immune privilege. Therefore, the BTB cannot be fully responsible for testicular immune privilege. The same is true for the physical structure of the testis, which should not be considered for immunoprivileged status; rather, there must be other mechanisms involved in the maintenance of immune privilege. Substantial evidence supports the hypothesis that the local immunosuppressive milieu, negative immunoregulatory systems and systemic immune tolerance also play important roles in maintaining the immunoprivileged status of the testis [3].

Testicular Immunosuppressive Milieu

A dense system of endocrine and paracrine networks is produced by testicular cells, and numerous immunoregulatory molecules comprise an immunosuppressive milieu for the maintenance of testicular immune privilege (Fig. 2). The testis is the only organ that produces testosterone via its Leydig cells, and this steroid is critical for normal spermatogenesis. Testosterone inhibits autoimmune response as testosterone administration suppresses autoimmune diseases in women [13], and a reduction in testosterone levels in the testis promotes the rejection of intra-testicular allografts, suggesting that testosterone favors a state of immune privilege. Testosterone also inhibits experimental autoimmune orchitis (EAO) in the rat [14]. Significantly, testosterone cannot act directly on immune cells, as immune cells do not express androgen receptors (AR). In contrast, Sertoli cells express AR, and therefore, testosterone should contribute to testicular immune privilege by regulating Sertoli cell functions. Previous studies have shown that mutation in the AR in mouse Sertoli cells disrupted BTB permeability and impaired testicular immune privilege [15]. Considering that the BTB is not fully responsible for testicular immune privilege, testosterone may play roles in the testicular immune environment via immunoregulatory factors produced by the Sertoli cells. The mechanisms by which testosterone regulates immune environment in the testis via Sertoli cells remains to be clarified.

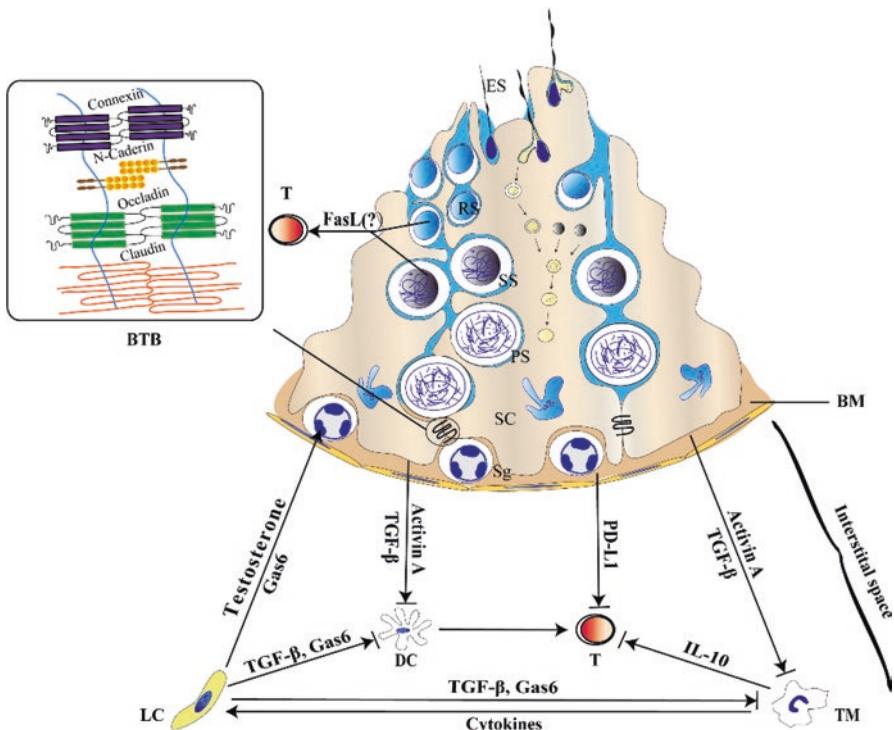


Fig. 2 Schematic of immunosuppressive milieu favoring immune privilege in the testis. Sertoli cells (SC) and Leydig cells (LC) secrete various immunosuppressive molecules, including Activin A, transforming growth factor beta (TGF- β), programmed death ligand-1 (PDL-1), testosterone, growth arrest-specific gene 6 (Gas6). Testicular macrophages (TM) secrete interleukin-10 (IL-10). This density of immunosuppressive networks inhibits T cell activation in the interstitial spaces. Notably, germ cells express Fas ligand (FasL), and whether FasL induces T cell apoptosis within the seminiferous tubules remain unclear

In addition to testosterone, the testis produces several potent anti-inflammatory cytokines that contribute to its immunoprivileged status (Fig. 2). Transforming growth factor β 1 (TGF- β 1) is predominantly produced by Sertoli cells [16], and it inhibits immune response, prolonging the survival of islet grafts in the testis [17]. Since TGF- β 1 is expressed in various testicular cell types, TGF- β 1 might regulate immune status by acting on testicular cells [18]. Activin A is a homologue of TGF- β 1 and it is also principally predominantly produced by Sertoli cells. Activin A regulates spermatogenesis under physiologic conditions, and is an anti-inflammatory factor that inhibits inflammation and lymphocyte activation. Activin A additionally inhibits pro-inflammatory cytokine production by testicular cells, thereby favoring immune-privilege status. Another potent anti-inflammatory factor in the testis is interleukin 10 (IL-10), which is primarily produced by testicular macrophages. The overexpression of IL-10 in mice inhibits EAO induction [19].

Negative Immunoregulatory System

Several immunoinhibitory systems have been found in the testis that contribute to immune privilege. The Fas ligand (FasL)/Fas system is a critical negative immunoregulatory mechanism, in which FasL induces apoptosis of lymphocytes through the activation of Fas (receptor) found on activated lymphocytes [20]. An early study showed that FasL is abundantly expressed in Sertoli cells and plays a critical role in maintaining testicular immune privilege [21]. However, a later study demonstrated that neutralizing antibodies against FasL did not affect the survival of beta islet cells in diabetic mice after co-transplantation with Sertoli cells [22]. In fact, Sertoli cells apparently do not express FasL, whereas male germ cells show high expression [23], and the function of FasL located on germ cells remains elusive. The programmed death ligand-1/programmed death receptor-1 (PD-L1/PD-1) system also induces T cell apoptosis, thus inhibiting immune response [24]. PD-L1 is highly expressed in male germ cells, which contributes to immunoprivileged status in the mouse testis [25]. These observations suggest that male germ cells may be implicated in the regulation of testicular immune privilege, but this contention requires clarification.

Tyro3, Axl and Mer (TAM) receptor tyrosine kinases and their common ligand, growth arrest-specific gene 6 (Gas6) and protein S, negatively regulate immune response [26]. Recent studies showed that the TAM/Gas6 system is involved in the regulation of testicular immune privilege [27], with TAM receptors being abundantly expressed in Leydig cells, and Gas6 exclusively expressed in Leydig cells [28]. TAM triple knockout ($\text{TAM}^{-/-}$) male mice are infertile and progressively develop autoimmune orchitis [29]. The TAM/Gas6 system plays a role in maintaining testicular homeostasis by facilitating the clearance of male germ cell antigens and inhibiting local innate immune responses. TAM signaling also facilitates phagocytosis of apoptotic germ cells by Sertoli cells [30]. The removal of germ cell antigens by Sertoli cells might thereby prevent autoantigen-induced inflammatory responses in the testis, as damaged male germ cells induce inflammatory cytokine production by Sertoli cells [31]. In addition, the TAM/Gas6 system inhibits the innate immune response to pathogen stimulation in Sertoli and Leydig cells [32, 33]. In accordance with these observations in the testis, the TAM/Gas6 system also negatively regulates systemic immunity and prevents autoimmune diseases [34].

Role of Leukocytes in Immune Privilege

Various leukocyte types are found in the interstitial spaces of the testis. These resident leukocytes play roles in regulating spermatogenesis and testicular immune privilege under physiologic conditions. Macrophages represent a major population of leukocytes in the murine testis [35], and interact intimately with Leydig cells and regulate steroidogenesis. Testicular macrophages also regulate Sertoli cell function

and germ cell development by secreting cytokines. Although macrophages belong to the overall group of innate immune cells, testis-resident macrophages exhibit a relatively slight innate immune response and highly immune-suppressive properties compared with the macrophages located in other tissues. Testicular macrophages in rats are less sensitive in response to pathogen stimulation and constitutively produce anti-inflammatory cytokines that favor an immunoprivileged environment [36]. However, circulating macrophages significantly infiltrate the testis and impair male fertility under inflammatory conditions [37].

Several types of lymphocytes reside in the testis [38]. Intriguingly, only T lymphocytes but not B cells are found in the testis, which may prevent humoral immune response to male germ cell autoantigens in orchitis [39]. Minor regulatory T cells (Tregs) can be found in the rat testis, and Tregs are significantly increased during EAO induction. Notably, CD4⁺CD25⁺ Tregs inhibit the proliferation of effector T cells in response to testicular antigen stimulation and prolong the survival of islet allografts in the testis, suggesting that Tregs suppress immune response there [40].

Dendritic cells (DCs) are antigen-presenting cells that link innate and adaptive immunity. Only a few DCs are found in normal rat testis, but DCs can be markedly increased during EAO induction [41]. DCs in the testis display immature phenotypes in their commitment to immunoprivileged status under physiologic conditions [42]. However, DCs from testicular draining lymph nodes of EAO induce the proliferation of effector T cells, suggesting that DCs might participate in autoimmune orchitis [43]. Therefore, DCs are expected to be involved in pathophysiologic conditions, but this area is in need of further investigation.

Peripheral Immune Tolerance to Male Germ Cell Antigens

Testicular immune privilege is not consistent among different species. In fact, prolonged graft survival in the testis has been convincingly demonstrated in small laboratory animals, including guinea pigs, rats, and mice. However, the same studies conducted in larger species, such as sheep and monkeys, were less successful [44]. Moreover, the susceptibility to EAO induction varies greatly even among different mouse strains [45]. These observations certainly suggest that there is peripheral immune tolerance to germ cell antigen in different species, and mechanisms underlying peripheral immune tolerance to male germ cell autoantigens have been recently investigated. Axl and Mer tyrosine kinase receptors are involved in peripheral immune tolerance to male germ cell antigens, as mice lacking Axl and Mer were susceptible to EAO induction [46]. In contrast, Toll-like receptor (TLR) 2 and TLR4 mediate immune responses to germ cell antigens, as mice lacking TLR2 and TLR4 are resistant to EAO induction [47]. These observations indicate that peripheral immunoregulatory mechanisms are involved in testicular immune privilege.

Innate Defense Against Microbial Infections in the Testis

Although the testis is an immunoprivileged organ, numerous microbial pathogens may infect this site via the circulating blood and ascending male genitourinary tracts. To overcome this immune privilege and mount an effective response against invading microbes, the testis adopts the local innate defense system. In addition to testicular immune cells, which are believed to create a frontline defense against microbes, major testicular tissue-specific cells including Leydig, Sertoli, and germ cells also possess anti-microbial defense capacities. These testicular cells then play important roles in testicular defense against microbial infection.

Interferons and Antiviral Protein

The testicular innate antiviral defense system was initially revealed in a series of studies on the expression of interferons (IFNs) and antiviral proteins in the testis. IFN- α and IFN- γ can be induced by the Sendai virus in most testicular cells of rats, including Sertoli cells, peritubular myoid cells, and male germ cells [48]; and IFN- α and IFN- γ induce the expression of several antiviral proteins in these cell types [49]. Moreover, Leydig cells and macrophages in the interstitial spaces constitutively express IFN- α and IFN- γ , and antiviral proteins can also be induced in these testicular cells [50, 51]. The aforementioned studies have revealed that most types of testicular cells manifest antiviral capabilities. Notably, human Leydig cells display relatively weak antiviral activities compared to their rat counterparts, suggesting that antiviral capabilities may be weaker in the human testis than in the murine testis [52]. This observation corresponds with the fact that a broad spectrum of viruses may impair testicular function in humans. However, natural viral infection-related testicular dysfunction has not been observed in murine species, suggesting that human and murine testis possess different anti-viral activities. Understanding the mechanisms underlying innate defense against viral infections in murine and human testes might ultimately provide novel clues into developing a therapeutic strategy for infectious male infertility.

Antimicrobial Defensins

Defensins are potent antimicrobial peptides that can be produced by several organs, exhibiting activity against a broad spectrum of microorganisms, including bacteria, fungi, and viruses. Defensins rapidly kill microorganisms by permeating the membranes of microbes and impairing microbial metabolism [53]. α - and β -Defensins have now been isolated from various mammalian species. Neutrophils produce α -defensins, whereas β -defensins are principally produced by the epithelial cells in the skin and genitourinary tract [54]. The male reproductive tracts of rats, mice, and humans including the testis and epididymis express various defensins [55].

The β -defensins are relatively abundant in the mouse epididymis relative to testis, and the roles of β -defensins in the epididymis have therefore been intensively investigated [56]. When the expression of defensins was analyzed in a testicular cell-specific manner [55], investigators demonstrated that compared to the interstitial cells, the seminiferous tubule cells (particularly Sertoli and peritubular myoid cells), express the majority of defensins, suggesting that the seminiferous tubules are well equipped to combat microbial infections. In addition, male germ cells also specifically express certain defensins depending upon developmental stage. The expression of defensins in the seminiferous epithelial cells likely contributes to antimicrobial defense within the seminiferous tubule. However, direct evidence that defensins counter invade microbes in the testis is still absent.

Pattern Recognition Receptors (PRRs)

The innate immune response is the first line of bodily defense against microbial infection. PRRs initiate the innate immune response after recognition of conserved molecular patterns of microorganisms, namely, pathogen-associated molecular patterns (PAMPs) [57]. PRRs belong to a large family of receptors that recognize PAMPs. Several families of PRRs, including TLRs, retinoic acid-inducible gene I (RIG-I)-like receptor (RLRs), and cytosolic DNA sensors have been identified (Fig. 3). There are currently 13 TLRs that have been characterized in mammals, and they belong to a subset of transmembrane proteins localized on the plasma and endosomal membranes. TLRs recognize a large spectrum of PAMPs and trigger innate immune responses. Most of the TLRs initiate the myeloid differentiation protein 88 (MyD88)-dependent pathway, except for TLR3 and TLR4. TLR3 initiates innate immune responses using a Toll/IL-1R domain-containing adaptor that induces IFN- β (TRIF), whereas TLR4 initiates both MyD88- and TRIF-dependent pathways. The MyD88 pathway induces the expression of numerous pro-inflammatory cytokines and chemokines through the activation of nuclear factor kappa B (NF- κ B) and mitogen-activated protein kinases (MAPKs). The activation of TRIF-dependent signaling leads to the production of pro-inflammatory cytokines and type 1 IFNs (IFN α and IFN β) though the activation of NF- κ B and IFN regulatory factors 3 (IRF3).

RLRs are cytosolic receptors that recognize viral double-stranded RNA (dsRNA), and they are produced by many types of viruses during replication, thereby initiating innate antiviral responses [58]. RLRs can also be activated by synthetic dsRNA analog, polyinosinic-polycytidylic acid [Poly (I:C)]. The RLRs contain three members: RIG-I, melanoma-associated protein 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2). LGP2 does not mediate an innate immune response because it lacks the domain essential for signaling; in contrast; however, RIG-I and MDA5 initiate innate antiviral responses. After the recognition of viral dsRNA, RIG-I and MDA5 recruit an adaptor protein, interferon promoter stimulator 1 (IPS-1) that is located on the outer membrane of mitochondria and peroxisomes, thereby activating downstream signaling pathways. The IPS-1-dependant pathway

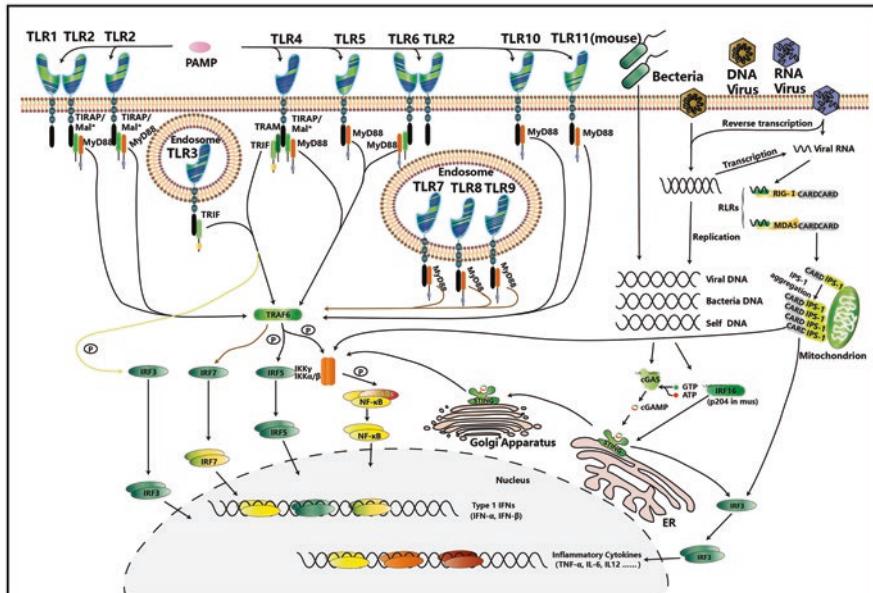


Fig. 3 PRR signaling pathways. Toll-like receptors (TLR) are localized on plasma or endosomal membranes and initiate the MyD88-dependent pathway, exception for TLR3 and TLR4. TLR3 initiates the TRIF-dependent pathway, whereas TLR4 initiates both MyD88- and TRIF-dependent pathways. MyD88-dependent pathway induces the expression of various pro-inflammatory cytokines, including TNF- α , IL-1 β , IL-6, and MCP-1, through NF- κ B activation; and TRIF-dependent pathway induces the expression of the pro-inflammatory cytokines and type 1 interferons (IFN- α and IFN- β) through the activation of NF- κ B and IRF3. RIG-I and MDA5 are two RNA sensors, which can be activated by dsRNA that can be derived from RNA viral genome or DNA virus transcription, thus initiating IPS-1-dependent pathway to induce the expression of pro-inflammatory cytokine and type 1 IFNs through NF- κ B and IRF3 activation. Cytosolic DNA sensors can sense viral and bacterial DNA, thereby initiating STING-dependent pathway to induce the expression of IFN- α and IFN- β through the activation of IRF3. dsRNA double-stranded RNA, ER endoplasmic reticulum, IKK IkB kinase, IPS-1 IFN- β promoter stimulator-1, IRF3 interferon regulatory factor 3, MCP-1 monocyte chemotactic protein-1, MDA5 melanoma differentiation-associated protein 5, MyD88 myeloid differentiation protein 88, PAMP pathogen-associated molecule pattern, RIG-I retinoic acid-inducible gene I, STING stimulator of IFN gene, cGAS cyclic GMP-AMP synthase, TLR toll-like receptor, TRAF tumor necrosis factor receptor-associated factor, TRIF toll/IL-1R domain-containing adaptor inducing IFN- β

activates NF- κ B and IRF3, and subsequently induces the expression of pro-inflammatory cytokines and type 1 IFNs, respectively.

Several intracellular DNA sensors, including absent in melanoma 2 (AIM2), IFN- γ inducible protein 16 (IFI16, namely p204 in mouse), and polymerase III (Pol III), have been identified. Notably, cyclic-di-GMP-AMP synthase (cGAS) was recently defined as a primary DNA sensor [59]. Cytosolic DNA sensors recognize DNA and initiate innate immune response. Stimulator of IFN genes (STING, located in the endoplasmic reticulum) is an adaptor protein that binds to DNA sensors, initiating signaling pathways to induce the expression of pro-inflammatory cytokines and type I IFNs through the activation of NF- κ B and IRF-3 (Fig. 3).

TLRs in Sertoli Cells

A role for PRRs in the testis was initially recognized by examining the expression and activation of TLRs in mouse Sertoli cells, demonstrating that TLR2 and TLR4 were expressed in these cells; and both of them can be activated by their respective ligands [60]. The expression of additional TLR members in the rat testis was then demonstrated by a subsequent study [61]. Of the TLR members, TLR2-TLR6 initiated innate immune responses in mouse Sertoli cells and induced the expression of various inflammatory cytokines after stimulation by their respective ligands [62]. The expression and activation of TLRs in Sertoli cells indicated that these cells possess innate defense capabilities against microbial infections. The testis is an immunoprivileged organ where the systemic immune response is weak; in particular, immune components are absent from the seminiferous tubules within the BTB. However, the innate immune properties of Sertoli cells would be important in defense against microbial infections within the seminiferous tubules. In addition to TLRs, various cytosolic DNA and RNA sensors are also expressed in Sertoli cells, suggesting that Sertoli cells are well equipped with an innate antiviral system. Recent studies showed that mumps virus induces immune response through the activation of TLRs and RIG-I in mouse Sertoli cells [63], and this response limits viral replication [64]. The antiviral roles of Sertoli cells should invite further investigation.

PRRs in Leydig Cells

The roles of PRRs in Leydig cells are currently being intensively investigated. Mouse Leydig cells abundantly express TLR2, TLR3 and TLR4, and both TLR3 and TLR4 in Leydig cells can be activated by their ligands [33]. The activation of TLRs in Leydig cells induces the expression of various pro-inflammatory cytokines and type 1 IFNs, suggesting that mouse Leydig cells possess innate defense against bacterial and viral infections. Moreover, TLR3 and TLR4 activation suppresses testosterone in Leydig cells via pro-inflammatory cytokine production, suggesting that innate immune responses in the testis may perturb steroidogenesis.

In addition to TLRs, other PRRs have been investigated in mouse Leydig cells. For example, both RIG-I and MDA5 are constitutively expressed in Leydig cells [65]. Poly (I:C) triggers RIG-I and MDA5 signaling in Leydig cells, which induces the production of pro-inflammatory cytokines and type 1 IFNs. Moreover, poly (I:C) also upregulates various antiviral proteins, including IFN-stimulating gene 15 (ISG15), 2'5'-oligoadenylate synthetase (OAS1), and myxovirus resistance 1 (MX1) in Leydig cells. ISG15, OAS1, and MX1 amplify antiviral signaling, degrade viral RNA, and block viral gene transcription, respectively, thereby limiting viral replication within infected cells [66]. Notably, poly (I:C)-triggered RIG-I and MDA5 signaling in Leydig cells inhibits testosterone synthesis, suggesting that RLR-initiated antiviral responses perturb testicular function [65].

RNA sensor signaling has also been identified in mouse Leydig cells [67], as p204 and STING are constitutively expressed in Leydig cells. p204 activation induces the expression of type 1 IFNs and antiviral proteins via a STING-dependent pathway, but does not significantly induce the production of pro-inflammatory cytokines in Leydig cells. In contrast to RNA sensor signaling in Leydig cells, p204 activation does not inhibit testosterone synthesis.

In accordance with these results, viral orchitis is usually caused by RNA viral infection, such as with mumps virus and human immunodeficiency virus (HIV). In contrast, DNA viruses rarely induce orchitis [68]. Therefore, the manipulation of DNA signaling might constitute an ideal approach against viral infection in the testis and thereby protect testicular function.

PRRs in Male Germ Cells

Male germ cells represent >90% of testicular cells during adulthood. These germ cells can be infected by viruses via the circulation and by bacteria from the ascending genital tract. Therefore, innate immune responses of these male cells should be important in the testicular defense against microbial infections, and studies of PRR-initiated innate immune responses in male germ cells have been undertaken.

Spermatogonia and primary spermatocytes express TLR3, and poly (I:C) induces the production of pro-inflammatory cytokines and type 1 IFNs through the activation of TLR3 in these cells [69]. The antiviral proteins are also expressed in spermatogonia and spermatocytes in response to poly (I:C) stimulation. As spermatogonia and primary spermatocytes are both located outside the BTB, TLR3-initiated anti-viral responses in germ cells should contribute to the defense of viral infections arising in the blood circulation.

In mice, TLR11 is a functional sensor in *Toxoplasma gondii* (*T. gondii*) and uropathogenic *Escherichia coli* (UPEC), two pathogens that can infect the testis and impair spermatogenesis. TLR11 is abundantly expressed in mouse spermatids and both *T. gondii* and UPEC induce the expression of MCP-1, IL-12 and IFN- γ in spermatids through TLR11 signaling [70]. UPEC also induces the production of TNF- α , IL-6 and IL-1 β in these germ cells. Notably, functional TLR11 is absent in humans, although UPEC is a major pathogen that infects the testis and impairs spermatogenesis in humans.

Although TLR3 and TLR11 can initiate innate immune responses in male mouse cells, the response is relatively small compared to that in somatic cells *in vitro*. However, male germ cells exhibit efficient defense capabilities against microbial infections, suggesting that these cells may possess other antimicrobial mechanisms. Defensin production by germ cells may also play a role in the defense against bacteria [55]. Moreover, male mouse germ cells possess active autophagy, and the autophagy inhibits the replication of mumps virus in germ cells [64]. The mechanisms underlying germ cell defense against microbial infection is worthy of further investigation.

TLR-Initiated Immune Responses in Testicular Macrophages (TMs)

Macrophages build the first line of defense against invading microbes in the body. Most PRR members are expressed in macrophages and initiate innate immune response, and TMs represent a majority of immune cells in the testis. However, TMs exhibit a relatively slight innate immune response upon stimulation compared to macrophages in other tissues, and this favors an immunoprivileged status. The mechanisms underlying the attenuated innate immune responses in TM have been recently investigated.

TMs express low levels of TLR signaling-related genes, whereas negative regulators of TLR signaling are highly expressed in TMs compared with peritoneal macrophages [71]. Accordingly, LPS faintly induces the expression of pro-inflammatory cytokines, including IL-6 and TNF- α in TMs. Moreover, NF- κ B signaling pathway is blocked by the prevention of I κ B degradation in TMs after LPS stimulation or UPEC challenge [72]. In contrast, TMs produce a high level of the anti-inflammatory cytokine IL-10 under basal conditions and LPS stimulation, indicating that TMs exhibit immunosuppressive properties favoring the immunoprivileged environment in the testis.

Orchitis

Although the mammalian testis maintains an immunoprivileged environment and an effective innate defense system, some stimuli (e.g., microbial infections, chemical toxins, physical trauma, and testicular tumors) can disrupt the immune homeostasis of the testis, leading to orchitis and impaired male fertility [73].

Infectious Orchitis

A number of microorganisms can infect the testis and induce orchitis. *Neisseria gonorrhoeae* and *Chlamydia trachomatis* are the most common sexually transmitted microbes among the bacteria, and frequently cause orchitis and epididymitis in men [74]. Increased leukocyte infiltration and pro-inflammatory cytokine production are characteristic of the testis in rats after a bacterial challenge, and this increase can perturb testicular functions. Bacterial infections in the testis are usually susceptible to antibiotic treatments, but they can result in infertility if not treated in a timely fashion [75].

A broad spectrum of viruses can infect the testis. Several viral types primarily induce orchitis at high rates, and impair fertility in humans [68], with the mumps

virus (MuV) being the most well-known [76]. MuV infection inhibits testosterone production and results in the degeneration of germ cells [77]. While pre-pubertal males usually recover from MuV-induced orchitis, MuV viral infection in adult men correlates with a high incidence of infertility [78]. Regarding its mechanism of action, MuV induces innate immune responses in testicular cells through the activation of TLR2 [63]. Moreover, MDA5 and IPS-1 mediate inflammatory cytokine production in testicular cells, suggesting that RNA sensor signaling plays a role in MuV orchitis. Notably, different types of testicular cells show distinct innate immune responses to MuV infection. While IFN- β inhibited MuV replication in LC, macrophages, and SC, male germ cells blocked MuV replication via autophagy [64]. Human immunodeficiency virus (HIV) infection frequently leads to orchitis and impairs testicular function [79, 80], and recent studies have demonstrated that ZiKa virus damages testicular function and leads to male infertility in mice [81, 82]. Coxsackie virus, influenza, and dengue viruses are also frequently associated with orchitis [83]. Importantly, these are RNA viruses, suggesting that RNA viruses predominantly result in orchitis. However, it must be noted that many other viruses including RNA and DNA viruses can impair male fertility without evident orchitis [84]. The mechanisms by which viral infections induce orchitis and impair male fertility require further clarification.

Autoimmune Orchitis

Although the mammalian testis is an immunoprivileged organ that protects immunogenic germ cells, physical trauma, microbial infections, cryptorchidism, and tumors may disrupt this immunoprivileged status and induce autoimmune response against germ cell antigens, resulting in autoimmune orchitis [85]. Autoimmune orchitis is characterized by inflammatory cell infiltration, apoptosis, and sloughing of germ cells in the testis, and by the presence of specific anti-sperm antibodies in the serum, both of which lead to aspermatogenesis and infertility. Autoimmune orchitis is classified into two types, including primary isolated autoimmune orchitis and secondary autoimmune orchitis; and grouped together with systemic autoimmune diseases, such as systemic lupus erythematosus and chronic rheumatoid arthritis.

The mechanisms underlying the pathogenesis of autoimmune orchitis have been investigated using EAO models in rodents [85], and we now know that CD4 T cells play a central role in the pathogenesis of EAO [86]. Massive infiltration of macrophages occurs in EAO, and these cells produce large amounts of pro-inflammatory cytokines such as TNF- α and IL-6, both of which induce germ cell apoptosis [87, 88]. In addition to immune cells, most tissue-specific cells also secrete a large number of pro-inflammatory cytokines through the activation of PRRs in response to endogenous and microbial stimuli. The roles of testicular cell-produced cytokines in the pathogenesis of autoimmune orchitis require further investigation.

Conclusions

As an organ with a special immune status, the mammalian testis adopts two immunologic features to fulfil its functions. The immunoprivileged environment is essential for the protection of germ cells from untoward immune responses and the local innate immune system is critical for the testicular defense against microbial infection. Although testicular immunoregulation has been investigated for more than a century, the underlying mechanisms are not fully understood. Further investigations into the mechanisms underlying the special immune environment in the testis can aid in the development of preventive and therapeutic approaches for immunologic infertility. Recent achievements in such testicular defense mechanisms are currently stimulating studies in this field. Importantly, several issues must be prioritized for future research endeavors. (1) Specific germ cell antigens that induce autoimmune responses require identification, and this should be helpful in the development of a diagnostic approach with respect to local testicular autoimmunity. (2) Most testicular cells produce significant amount of pro-inflammatory cytokines in response to endogenous and infectious stimuli. Therefore, the role of testicular cell-produced cytokines in local inflammation and subsequent impairment of testicular functions are in need of further elucidation. (3) Microbial infection may impair testicular function and male fertility but whether microbes act directly on the testis or indirectly via systemic inflammatory mediators has yet to be determined. (4) Immunoregulation in the testis has been largely studied using murine models, which are unfortunately not identical to the human testis. A clarification of the immune environment within the human testis is therefore urgently required. (5) Since a broad spectrum of viruses may infect the testis, virally induced damage to testicular function can vary greatly depending upon the viral type; and underlying mechanisms require clarification. (6) Roles for tissue-specific cells in regulating the testicular immune environment and defense against microbial infections in humans should also be a focus of future studies. Further illumination of the aforementioned issues should provide novel insights into the mechanisms underlying immunoregulation in the testis, as well as infectious and immunologic cause of male infertility.

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Unraveling the Regulation of Cancer/Testis Antigens in Tumorigenesis Through an Analysis of Normal Germ Cell Development in Rodents



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Introduction

During the past two decades, much effort has been made to enhance anti-tumor immunological responses in patients by tumor antigen-specific immunization, which targets cancer cells [1, 2]. Among the different types of tumor antigens, cancer/testis (CT) antigens are promising therapeutic targets for the development of cancer vaccines due to their unique characteristics. Under normal physiological conditions, CT antigens are predominantly expressed in germline cells, but also in spermatocytes, and even in elongating/elongated spermatids. Since the testis is an immune privileged organ and also an immune privileged site, indicating that foreign tissue grafts into the testis can survive longer, or indefinitely, without being rejected; or the testis also resists rejection when its tissue is grafted into a non-privileged site [3, 4]. CT antigens residing in germ cells do not provoke immune response, and thus avoiding their eradication by the immune system. Interestingly, CT antigens can be aberrantly expressed in multiple cancers, which is suggestive of an aberrant reactivation of the silenced gametogenic program in somatic cells during tumorigenesis [5, 6]. Thus, CT antigens hold great promise for highly specific and safe immunotherapy. The first CT antigen MAGE1 (melanoma-associated antigen 1) was

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discovered more than two decades ago through autologous typing [7]. Since then, more than 200 CT antigens have been identified [8]. Some of these CT antigens have been tested as tumor vaccines in clinical trials. Unfortunately, most clinical studies involving CT antigens have so far been unsuccessful [9–11], partly due to the fact that there is a lack of systematic understanding of the expression pattern of CT antigens across different cancer types, as well as the molecular mechanisms underlying their aberrant expression and oncogenic functions in cancer cells.

Previous studies have reported shared characteristics between germ cells and cancer cells (for a review, see [6]). For instance, CT antigens that are involved in germ cell implantation and migration during gametogenesis can regulate cancer cell invasion and metastasis [6, 12]. Therefore, it is likely that similar mechanisms underlie the regulation of CT antigens during gametogenesis and their ectopic expression during tumorigenesis. Thus, understanding the regulation of CT antigens during gametogenesis may provide valuable insights into their regulation in the context of cancers. Recently, one research group has carried out a comprehensive analysis using publicly available databases to systematically identify CT antigens, which has significantly expanded the repertoire of CT antigens [13]. Furthermore, the past few years have witnessed a significant surge in publicly available next generation sequencing datasets both from the fields of reproduction and cancer. In this review, we analyzed these datasets and focused our attention on the regulation of CT antigens in the contexts of gametogenesis and tumorigenesis. We found that many aberrantly expressed CT antigens in cancer cells associated with super-enhancers, and some of these associations were conserved in normal testes and cancers. We also reported that methyltransferase DNMT1 is a potential regulator of the expression of CT antigens, and identified CT antigen and transcription factor TAF7L as an important regulator of the expression of a subset of CT antigens. This information may be useful in the identification of new targets among CT antigens for the treatment of cancer. This review also provides a credible approach of using big datasets deposited in public domain for meaningful analysis by investigators in the field.

Approaches Used for Analysis

1. **Data sources.** Raw datasets from published studies were retrieved from the Gene Expression Omnibus (GEO) (<https://www.ncbi.nlm.nih.gov/geo/>). A complete list of the GEO accession numbers used in this report is provided in Table 1. Gene expression and DNA methylation data (Illumina 450k platform) from the TCGA Database were retrieved via the UCSC Xena Browser (<http://xena.ucsc.edu/>). Cancer samples lacking expression of target CT antigens were removed from the analysis. Gene expression data from normal human tissues were obtained from the GTEx Database via the UCSC Genome Browser (<https://genome.ucsc.edu/>). Promoter information was obtained from the Eukaryotic Promoter Database (<http://epd.vital-it.ch/index.php>). CpG island information was acquired from the UCSC Genome Browser. The MiPanda Michigan Portal for the Analysis of NGS Data (<http://www.mipanda.org/>) and the Firehose

Table 1 List of GEO accession numbers used in this report

Data name	GEO accession number	Data type
Expression profiling of mouse PGCs from DNMT1 KO model	GSE74938	Bulk RNA-Seq
Expression profiling of human PGCs	GSE63818	Single cell RNA-Seq
Chromatin IP against multiple factors and histone modifications in multiple human cancer cell lines	GSE36354	ChIP-Seq
Chromatin IP against H3K27ac in normal testis	GSE75734	ChIP-Seq
Chromatin IP against TAF7L and POLII in mouse testis; expression profiling of mouse testis from TAF7L KO model	GSE50807	ChIP-Seq; Bulk RNA-Seq
Expression profiling of mouse PGCs	GSE41908	Bulk RNA-Seq
Expression profiling of purified mouse spermatogonia, spermatocytes, spermatids and spermatozoa	GSE43717	Bulk RNA-Seq

Browser (<https://gdac.broadinstitute.org/>) were also used to search and validate the data during the preparation of this report.

- RNA-Seq analysis.** RNA-Seq analysis, bulk RNA-Seq or single cell (sc) RNA-Seq, was performed based on the ‘new tuxedo’ pipeline [14]. In brief, raw reads were aligned to the mouse reference genome mm10 or human genome hg38 using the HISAT2 Program (version 2.1.0) [15] with default parameters. BAM files were then sorted and converted into SAM files using SAM Tools (version 1.4) (<http://www.htslib.org/>). The expressed genes and transcripts were assembled and quantified using the StringTie Program (version 1.3.3) [16]. **Differential gene expression** analysis was performed using the R/Bioconductor package Ballgown (version 2.10.0) [17]. Genes with FPKM = 0 (no fragments per kilobase of transcript per million mapped reads) were removed from the analysis. The multiple testing errors were corrected using the false discovery rate (FDR). Genes with a ≥ 2 -fold difference in expression and a FDR $< 5\%$ were considered differentially expressed. For studies in which processed data were available, the results were cross-referenced with those from in-house analysis, and the analyzed data from the original studies were only used if the results matched.
- ChIP-Seq analysis.** To build version mm10 of the mouse genome or version hg38 of the human genome with default parameters, ChIP-Seq datasets were aligned using the Bowtie 2 Program (version 2.3.2) [18] BAM files were sorted and converted into SAM files using SAM Tools. Peak calling was performed in MACS2 (version 2.1.1) [19] to identify regions of ChIP-Seq enrichment over the background. A *p*-value threshold of enrichment of 0.05 was implemented. ChIP peak annotation was performed using the R/Bioconductor package ChIPseeker (version 1.14.1) [20]. ChIP peak coverage was visualized within the Integrative Genomics Viewer Desktop Application (version 2.4) [21, 22]. For studies in which processed data were available, the results were cross-referenced with those from in-house analysis, and the analyzed data from the original studies were only used if the results matched. SEs (Super-Enhancers) were identified using the ROSE algorithm (version 0.1) with default parameters [23, 24]. The annotations of SE-associated genes based on the proximity to SEs in normal

mouse testes were kindly provided by Dr. Alena Shkumatava from Institut Curie, PSL Research University.

- 4. Functional annotations and statistical analysis.** Gene ontology analysis was performed using DAVID Online Tools (<http://david.abcc.ncifcrf.gov/>). The top-enriched clusters were identified by automated functional annotation clustering with default parameters. Statistical analysis between two groups was performed using a non-parametric *t*-test. In all cases, $p < 0.05$ was considered significant. Spearman's rank correlation coefficient (ρ) was calculated as R (version 3.4.3) (<https://www.r-project.org/>).

A Subset of CT Antigens Belongs to the Core Fitness Gene Family

We obtained the complete list of CT antigens systematically identified by a recent study in which 1019 testis-specific transcripts expressed in at least 1% of the samples from any of the 19 cancer types were examined [13]. We first asked if any of these CT antigens are human core fitness genes. The 1580 core fitness genes were defined as genes whose disruption decreased cell growth and proliferation in three or more of the five human cell lines (one normal immortalized cell line and four cancer cell lines) tested as revealed by CRISPR screens [25]. We compared the list of CT antigens with the list of human core fitness genes and identified 28 CT antigens belonging to the core fitness gene family (2.7% of the CT antigens and 1.8% of the core fitness genes) (Fig. 1a; Table 2). GO term enrichment analysis suggested that these 28 CT antigens were enriched in important cellular processes critical for cell survival such as cell division, cell cycle control and DNA replication (Fig. 1b). Therefore, these CT antigens may serve as potential targets for the treatment of cancer.

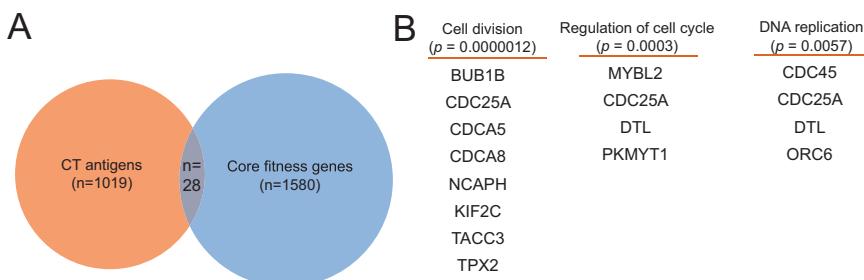


Fig. 1 A subset of CT antigens belongs to the human core fitness gene family. (a) Venn diagram shows that 28 CT antigens belonged to the core fitness gene family whose perturbation may have decreased cell growth and proliferation. (b) These 28 CT antigens were enriched in cellular processes such as cell division, cell cycle control and DNA replication

Table 2 CT antigens that belong to the family of core fitness genes

Gene names
PLK1
CT62
SGOL1
CDC25A
DDX20
BUB1B
RAD51
OIP5
MYBL2
CDC45
TPX2
ORC6
TTK
PKMYT1
TRIP13
HJURP
DMRTC2
CASC5
CDCA5
DTL
NUF2
KIF2C
CDCA8
KIF18A
NCAPH
TOP2A
PLK4
TACC3

Multiple CT Antigens Are Under the Control of Super-Enhancers (SEs)

To gain insight into the regulation of CT antigens, we examined their association with SEs. SEs are large clusters of enhancers that are occupied by master transcription factors. They associate with key genes and are critical for cell identity [23]. Furthermore, SEs are hyperactive chromatin regions characterized by high levels of histone H3 lysine 27 acetylation (H3K27ac) that distinguish them from shorter, less active typical enhancer regions [23, 26]. SEs have been found at key oncogenic drivers in multiple cancer cells [24]. It has also been reported that cancer cells can acquire SEs at oncogenic drivers during the process of tumorigenesis as revealed by a comparison of SEs in cancer cells with corresponding normal cells [27]. Thus, SEs may be helpful for identifying key oncogenes in specific cancers. We accessed

the datasets for the lists of genes that associated with SEs in a multiple myeloma (MM) cell line, a glioblastoma multiforme (GBM) cell line and a small cell lung cancer (SCLC) cell line [24, 27] and looked for CT antigens in each gene list (Fig. 2). We identified 17, 15 and 19 CT antigens in MM, GBM and SCLC, respectively (Fig. 2a). Each cancer cell type contained a set of CT antigens that was largely different from the other types with only a few overlaps (four between MM and SCLC, two between MM and GBM, two between GBM and SCLC) (Fig. 2a). These results were consistent with earlier observations that SEs are usually cell-type-specific and play key roles in the control of cell identity [23]. Nonetheless, two SE-associated CT antigens, namely, TPX2 and KIF2C, were aberrantly expressed in the three cancer cell lines. The SEs associating with these two CT antigens were found at similar chromatin positions across the three cancer cell types, although the lengths of the SEs varied (Fig. 2b, d). TPX2 is a spindle-associated microtubule (MT)-binding protein [28]. It is required for MT assembly to facilitate the formation of the spindle during mitosis in Hela cells [29] and meiosis in *Xenopus* eggs [30, 31]. To understand the role of TPX2 during gametogenesis, we accessed and analyzed RNA sequencing (RNA-Seq) datasets from human primordial germ cells (PGCs) [32], mouse PGCs [33, 34] and adult mouse germ cells at different developmental stages [35]. High expression of TPX2 was found throughout PGC development in both the human and the mouse, which is characterized by active cell division (Fig. 3a–c). High expression of TPX2 was also observed at the transition of adult mouse spermatocytes to round spermatids during which meiosis I/II takes place (Fig. 3a, d). This expression pattern of TPX2 during gametogenesis coincides with its reported functions. Therefore, it is plausible that the acquisition of SEs drives the aberrant expression of TPX2 in cancer cells, which facilitates cell division via mitosis. KIF2C belongs to the kinesin-13 family, which induces MT depolymerization to regulate chromosome movement and segregation during mitosis [36, 37]. It has been reported that KIF2C is overexpressed in colorectal carcinoma as well as several types of pancreatic, gastric, breast and head and neck cancers [38]. KIF2C is also overexpressed in gliomas, and it has been proposed to be a potential prognostic marker for glioma [39]. Thus, the acquisition of SEs may underlie the ectopic expression of KIF2C in different cancer types. Of interest, both TPX2 and KIF2C belong to the human core fitness gene family (Table 2). Taken collectively, our observations suggest that both TPX2 and KIF2C are potential targets for the treatment of cancer.

To further understand the association between SEs and CT antigens, we examined SE-associated CT antigens in normal testes. Due to a lack of ChIP-Seq (chromatin immunoprecipitation followed by high-throughput sequencing) data on H3K27ac in human testes, we obtained and analyzed the H3K27ac ChIP-Seq dataset from mouse adult testes [26]. Among all SE-associated genes, 164 CT antigens were identified, which accounted for 16.1% of the total number of CT antigens and 3.1% of all SE-associated genes in adult mouse testes (Fig. 2c). We then compared the list of SE-associated CT antigens in normal mouse testes with the list of SE-associated CT antigens identified in MM, GBM and SCLC cell lines (Fig. 2a). Ten SE-associated CT antigens were found in both lists. For instance, SYNGR4 belongs

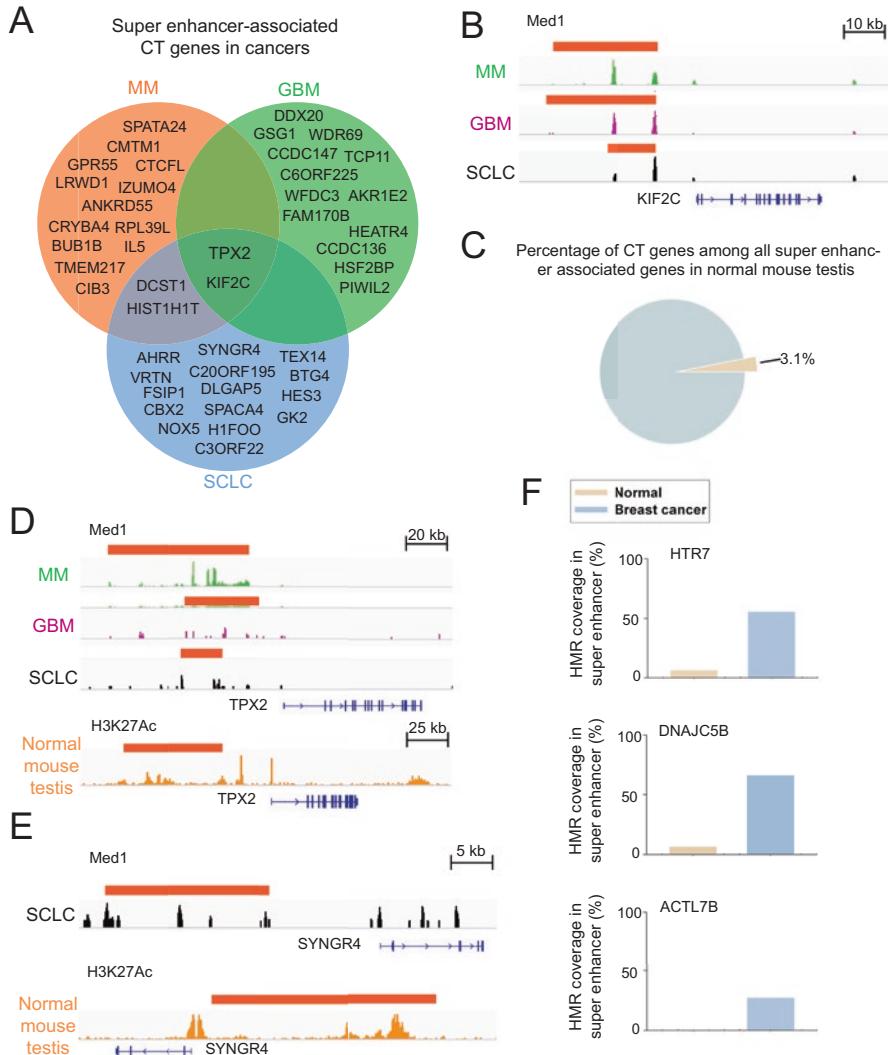


Fig. 2 A subset of CT antigens is under the control of super-enhancers (SEs). (a) Venn diagram shows SE-associated CT antigens in multiple myeloma (MM), glioblastoma (GBM) and small cell lung cancer (SCLC) cell lines, respectively. TPX2 and KIF2C are the two common CT antigens found in the three cancers. (b) CT antigen KIF2C associates with a SE (red rectangle) in MM, GBM and SCLC, respectively, as indicated by the chromatin immunoprecipitation (ChIP) peak coverage of Med1. (c) Pie chart shows that SE-associated CT antigens account for 3.1% of all SE-associated genes in normal mouse testes. (d) CT antigen TPX2 associates with a SE (red rectangle) in MM, GBM, SCLC and normal mouse testes, respectively, as indicated by ChIP peak coverage of Med1 and H3K27Ac. (e) CT antigen SYNGR4 associates with a SE (red rectangle) in both SCLC and normal mouse testes as indicated by ChIP peak coverage of Med1 and H3K27Ac. (f) Bar graphs show that the hypomethylated region (HMR) coverage of SE-associated CT antigens HTR7, DNAJC5B and ACTL7B, respectively, increases in breast cancer compared with normal tissues

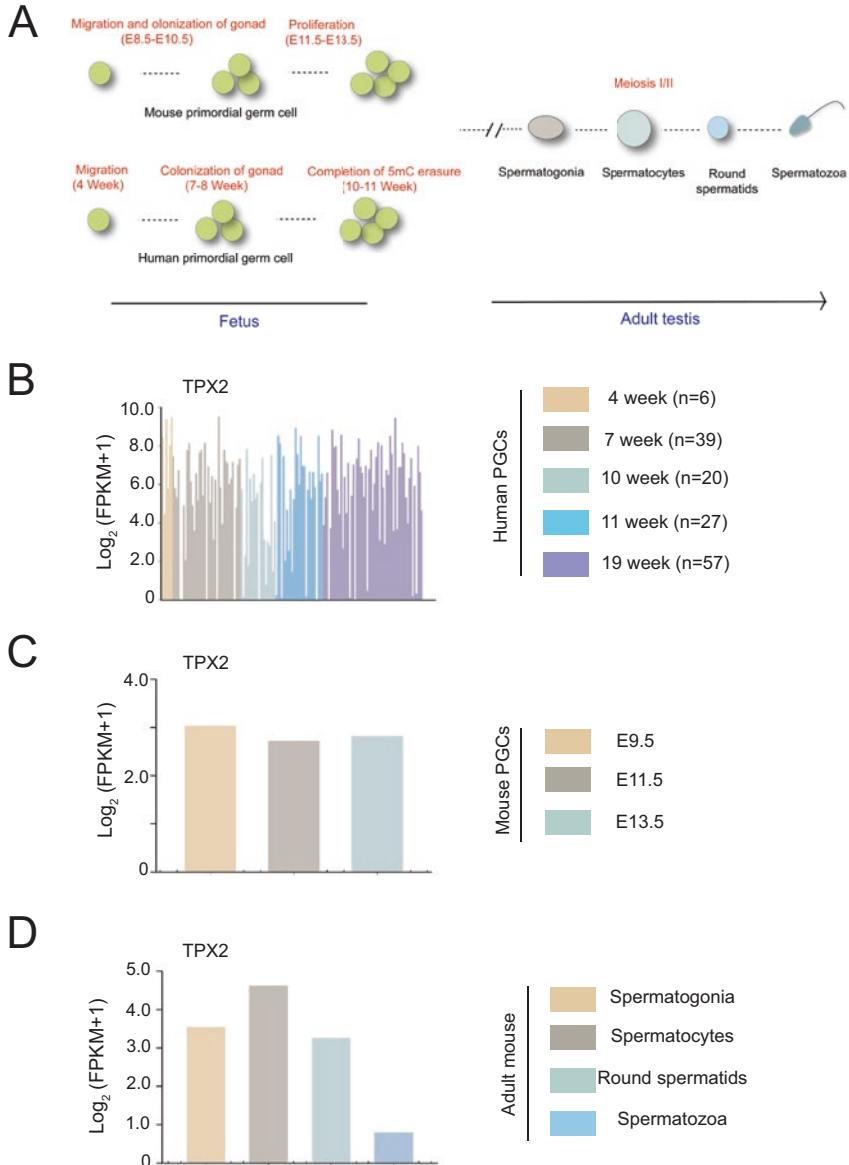


Fig. 3 CT antigen TPX2 is expressed throughout male germ cell development. **(a)** Schematic drawing shows the entire process of male germ cell development from early primordial germ cells (PGCs) to mature spermatozoa both in the mouse and human. Key biological events are highlighted in red. **(b)** Bar graph shows the expression of CT antigen TPX2 in human PGCs. FPKM, fragments per kilobase of transcript per million mapped reads. **(c)** Bar graph shows the expression of CT antigen TPX2 in mouse PGCs. **(d)** Bar graph shows the expression of CT antigen TPX2 in mouse developing male germ cells in testes

to the synaptogyrin family and functions in pulmonary carcinogenesis and tumor progression [40, 41]. SYNGR4 associated with a SE in both normal mouse testes and a SCLC cell line at similar locations (Fig. 2e). We also found that TPX2 (but not KIF2C) associated with a SE in normal mouse testes, and this TPX2-associated SE shared a similar chromatin location with that in MM, GBM and SCLC cell lines (Fig. 2d). Taken collectively, our findings show that the associations with SEs in a certain subset of CT antigens are conserved from normal testes to various cancers, suggesting that a conserved mechanism underlies the regulation of these CT antigens between gametogenesis and tumorigenesis.

It was noted that only a small fraction of the associations (22.2% in our case) between the CT antigens and SEs were conserved from normal testes to cancers. This may have been due to the fact that mouse testes instead of human testes were used for the analysis. However, it may also have been due to the fact that there are other mechanisms responsible for the acquisition of SEs for CT antigens during tumorigenesis. The association of DNA methylation with enhancer elements has been proposed to influence enhancer activity [42, 43]. Thus, we speculated that an alteration in DNA methylation in SEs might contribute to the ectopic expression of certain CT antigens during tumorigenesis. To test this hypothesis, we obtained data from a recent study in which genome-scale DNA methylation analysis of normal tissues and multiple tumors were performed and the DNA methylation landscape across SEs among different normal tissue-matched tumors was reported [44]. We focused on breast cancers and found that three CT antigens (HTR7, DNAJC5B, ACTL7B) associating with SEs exhibited marked hypomethylation compared with normal tissues in which the occupancy of the hypomethylated regions (HMRs) increased dramatically in SE regions in the breast cancer samples (Fig. 2f). These results suggest that aberrant DNA methylation in SEs may be another contributing factor to the ectopic expression of CT antigens in cancers.

DNA Methyltransferase DNMT1 Is Involved in the Regulation of CT Antigen Expression

The mission to identify the link between DNA methylation in SEs and CT antigen expression in different cancer types prompted us to further investigate the relationship between DNA methylation and CT antigen expression. The status of DNA methylation in selective genomic regions can be instructive for gene expression [43, 45]. In normal testes, aging-associated alterations in DNA methylation during spermatogenesis have been reported to have broad effects on gene expression and cell signaling in offspring [46]. During embryonic development, early PGCs undergo epigenetic reprogramming where their genomes display very low levels of global DNA methylation [47]. However, certain genomic regions, including CpG islands (regions where CpG is present at significantly higher levels than is typical for the genome as a whole) on the X chromosome and germline-specific genes, are

protected from the global loss of methylation, and they can only become demethylated upon gonad colonization of PGCs [48]. This protective effect has been shown to be mediated by methyltransferase DNMT1, because DNMT1 is the only DNA methyltransferase consistently expressed throughout the development of PGCs [49] (Fig. 4) and a DNMT1 knockout (KO) resulted in the up-regulation of post-migratory germ cell-specific genes [50]. Furthermore, a conditional loss of DNMT1 in PGCs leads to precocious differentiation in male germ cells by invoking cell lineage-specific gene expression [51]. Thus, we speculated that DNMT1 may also regulate the expression of specific CT antigens during PGC development. To test this hypothesis, we analyzed RNA-Seq data obtained from both wild type and DNMT1 KO male PGCs at E13.5 [51]. We found 141 differentially expressed genes with a fold change ≥ 2 . Among them, we identified 12 CT antigens that were differentially expressed in PGCs from the DNMT1 KO (Fig. 5a, b). Interestingly, all 12 differentially expressed CT antigens were up-regulated (Fig. 5a), which accounted for 11% of the total number of up-regulated genes (Fig. 5c). These results indicated that DNMT1 can suppress the expression of these CT antigens by maintaining DNA methylation during early PGC development. To confirm this, we looked at the methylation coverage of the promoter regions of these CT antigens in PGCs at E9.5 [48, 51]. Eight out of 12 CT antigens were found to have high methylation occupancy at their promoter regions ($>20\%$) in PGCs at E9.5 (Fig. 5d), suggesting that the expression of these CT antigens may be suppressed by DNMT1 at early stages of PGC development.

We then asked if a similar regulatory mechanism exists between DNMT1 and CT antigens in cancers. To answer this question, we focused on one of the eight CT antigens shown in Fig. 5d, TEX101, because it has been shown to play crucial roles in both spermatogenesis and tumorigenesis. TEX101 KO male mice were infertile due to failed uterotubal junction migration of their sperm [52, 53]. Its aberrant elevated expression has been reported in basal cell carcinoma [54], breast cancer [55, 56] and head and neck squamous cell carcinoma [57]. We first probed the expression levels of both TEX101 and DNMT1 across 54 breast cancer cell lines [58]. Indeed, TEX101 was overexpressed in specific breast cancer cell lines (Fig. 5e). An inverse correlation (Spearman's correlation, which applied to all correlation analyses in this report) was found between the expression levels of TEX101 and DNMT1 in these breast cancer cell lines (Fig. 5f), consistent with our observations from the PGC analysis. We also acquired gene expression data on TEX101 and DNMT1 from The Cancer Genome Atlas (TCGA) database. Interestingly, an inverse correlation between the expression of TEX101 and DNMT1 was also observed in head and neck cancer samples (Fig. 5g). Taken collectively, this information suggests that DNMT1 may negatively regulate the expression of these CT antigens in different cancer types.

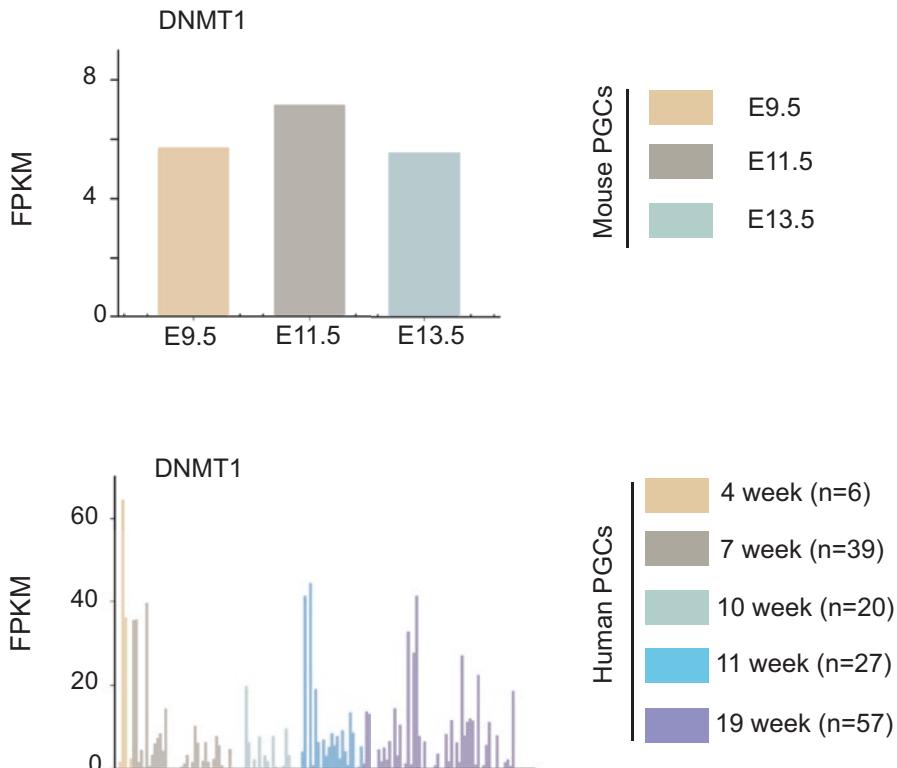


Fig. 4 DNA methyltransferase DNMT1 is expressed throughout primordial germ cell (PGC) development. Bar graphs show the expression of DNMT1 in both mouse and human PGCs

The Expression of CT Antigen TAF7L Is Regulated by DNA Methylation in Cancers

We earlier showed that DNA methylation maintained by DNMT1 may control the expression of specific CT antigens during PGC development (Fig. 5b, d), and this may also hold true in cancer settings (Fig. 5e–g). To further test this idea in cancers, we focused on TAF7L, another CT antigen shown in Fig. 5d. TAF7L is a germ cell-specific transcription factor [59, 60]. Although TAF7L KO mice are fertile, their sperm exhibit abnormal morphology and significantly reduced motility [61]. Little is known about the role of TAF7L in cancers, although one study has reported a down-regulation of TAF7L expression in acute myeloid leukemia [62]. Two alternative promoter regions have been identified for TAF7L. The first promoter (promoter 1) was located at hg19 chrX: 100,548,043-100,548,102, whereas the second one (promoter 2) was located at hg19 chrX: 100,546,312-100,546,371 (Fig. 6a). A 5' CpG island was present at hg19 chrX: 100,546,064-100,546,550, associating

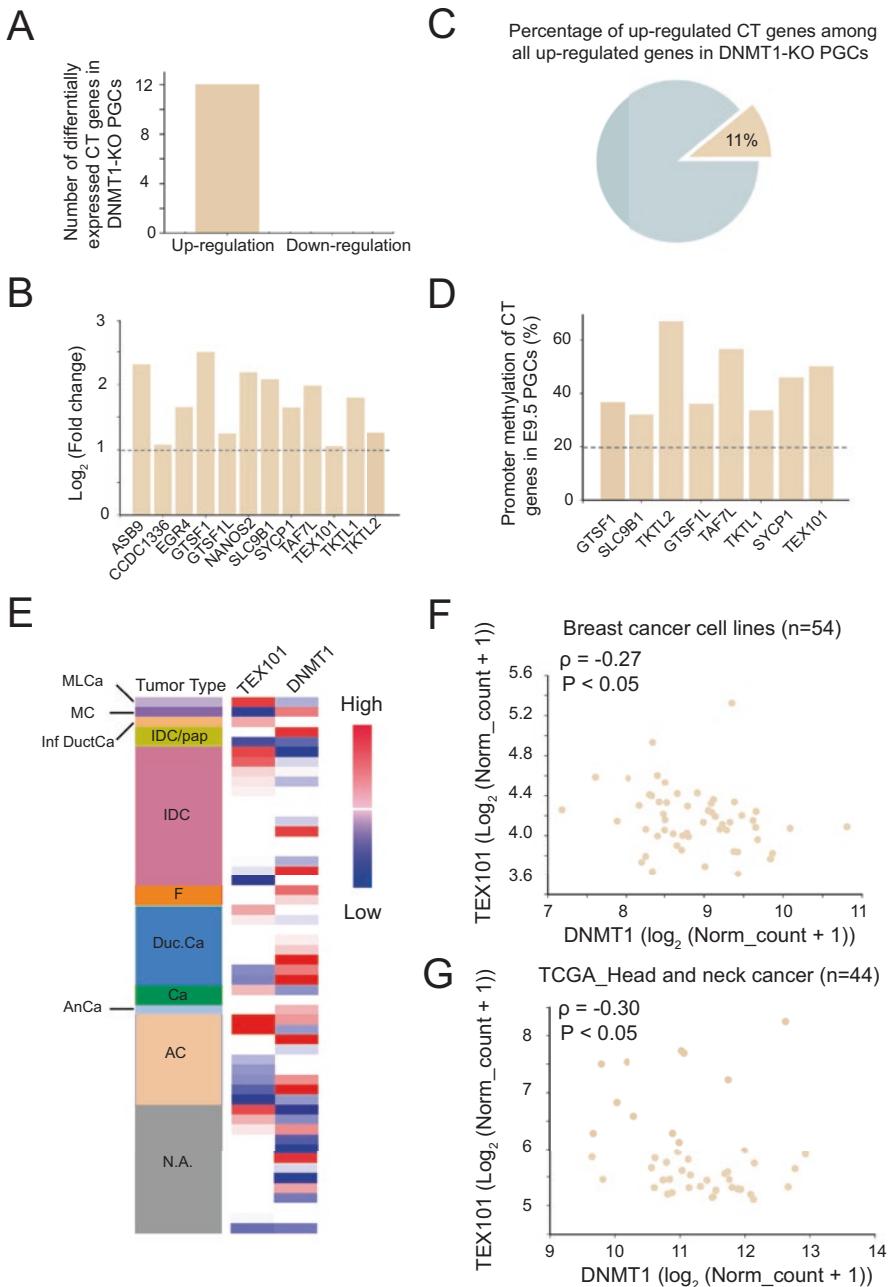


Fig. 5 The expression of a subset of CT antigens is under the control of DNA methyltransferase DNMT1. **(a)** Bar graph shows that 12 CT antigens were found to be significantly up-regulated upon conditional deletion of DNMT1 in mouse primordial germ cells (PGCs) at E13.5. No CT antigens were significantly down-regulated. **(b)** Bar graph shows that the 12 up-regulated CT

with promoter 2 of TAF7L (Fig. 6a). Furthermore, elevated expression of TAF7L was observed in thyroid cancers compared with normal tissues by analyzing TCGA datasets (Fig. 6b). We then set out to investigate if DNA methylation in the promoter regions of TAF7L or its associated CpG island affects its expression in thyroid cancer. We obtained DNA methylation profiles (Illumina 450k) of TAF7L in both normal and thyroid cancer tissues and coupled them with the TAF7L gene expression data (Fig. 6c). Interestingly, at probe cg13248004 (hg19 chrX: 100,548,042-100,548,044) near promoter 1 of TAF7L (Fig. 6c, green box), DNA methylation significantly decreased in cancer tissues compared with normal tissues (Fig. 6d). DNA demethylation at the same region was also observed in colon and rectal cancers (Fig. 6e, green box and Fig. 6f), suggesting that promoter 1 of TAF7L may undergo DNA demethylation during tumorigenesis. On the other hand, we observed an inverse correlation between the expression level of TAF7L and DNA methylation at probes cg08704539 (hg19 chrX: 100,546,355-100,546,357), cg01538344 (hg19 chrX: 100,546,290-100,546,292) and cg19386336 (hg19 chrX: 100,546,063-100,546,065) within the 5' CpG island in thyroid cancer tissues, respectively (Fig. 6c, blue box; Fig. 7). These findings indicate that DNA methylation at the promoter or CpG island plays a significant role in the ectopic expression of TAF7L in cancer.

TAF7L Regulates the Expression of Other CT Antigens

The fact that TAF7L is a transcription factor indicates that it can potentially regulate the expression of many other genes upon its activation during both gametogenesis and tumorigenesis. A recent study has shown that TAF7L targets promoters of many spermatogenesis-specific genes in testes [63]. We analyzed RNA-Seq data obtained from normal and TAF7L KO testes in this previous study and identified 267 differentially expressed CT antigens (fold change ≥ 2) upon TAF7L deletion (Fig. 8a-g). Among these differentially expressed CT antigens, 84 were up-regulated and 183 were down-regulated (Fig. 8a). We subsequently focused on the CT antigens that were down-regulated after TAF7L KO, because they accounted for the majority of the differentially expressed CT antigens. We filtered the 183 down-regulated CT

Fig. 5 (continued) antigens showed >2-fold change in their expression levels. (c) Pie chart shows that the 12 up-regulated CT antigens account for 11% of all up-regulated genes in PGCs at E13.5. (d) Bar graph shows that eight of the 12 up-regulated CT antigen contained promoter regions with high DNA methylation (more than 20%) in PGCs at E9.5. (e) Heat map shows the expression of CT antigen TEX101, together with that of DNMT1, in different breast cancer cell lines. MLCa, metastatic lobular carcinoma; MC, metaplastic carcinoma; Inf DuctCa, infiltrating ductal carcinoma; IDC/pap, ductal breast cancer/papillary; IDC, ductal breast cancer; F, fibrocystic disease; Duc.Ca, ductal carcinoma; Ca, carcinoma; AnCa, anaplastic carcinoma; AC, adenocarcinoma; N.A., subtype identity not available. (f) Dot plot shows that the expression of CT antigen TEX101 inversely correlates with DNMT1 in breast cancer. (g) Dot plot shows that the expression of CT antigen TEX101 inversely correlates with DNMT1 in head and neck cancer

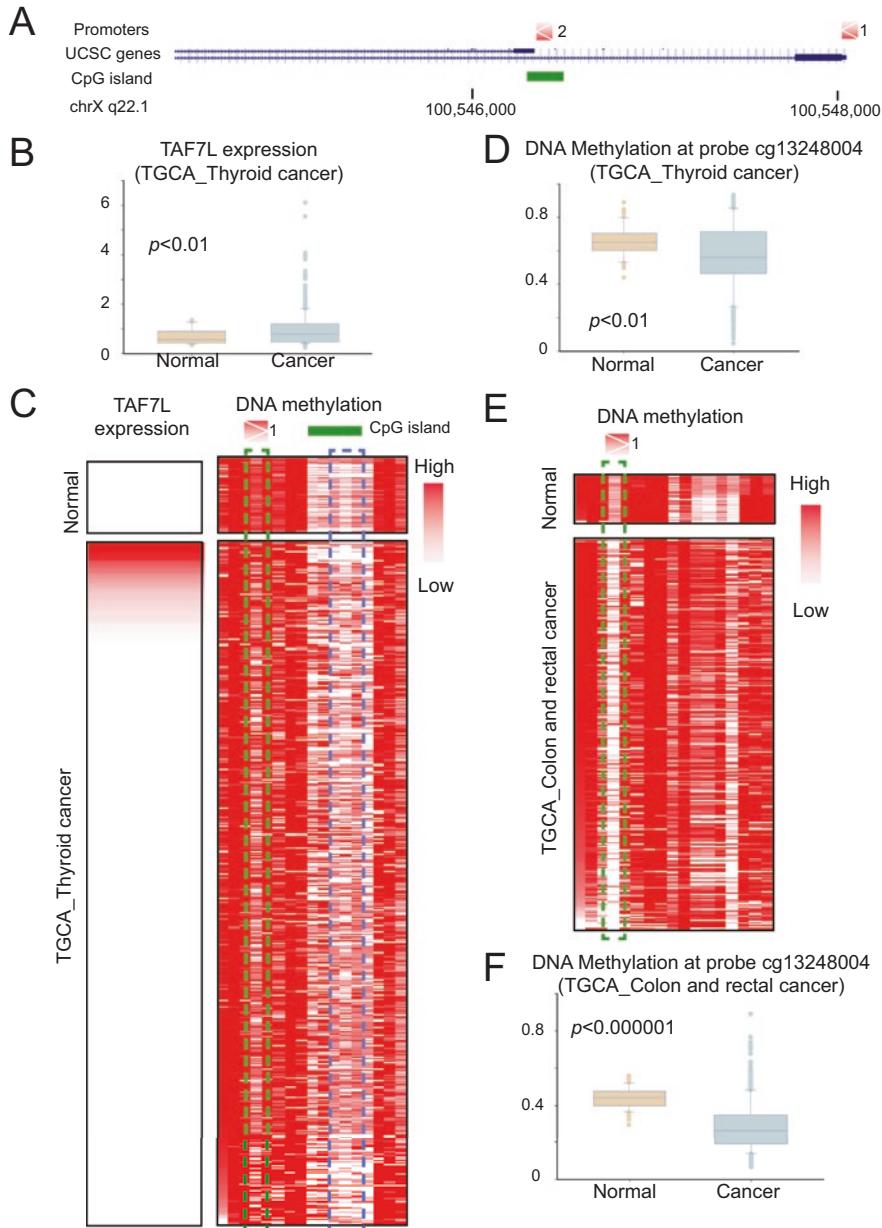


Fig. 6 The expression of CT antigen TAF7L is under the control of DNA methylation in its promoter and 5' CpG island. (a) View of CT antigen TAF7L in UCSC genome browser. TAF7L has two alternative promoters. The 5' CpG island associated with promoter 2. (b) Box plot shows that the expression of TAF7L is significantly elevated in thyroid cancer compared with normal tissues. (c) Heat map shows the expression of TAF7L in both normal and thyroid cancer together with the DNA methylation status of the 5' region of TAF7L. (d) Box plot shows that promoter 1 (green box in c) of TAF7L is significantly hypomethylated in thyroid cancer compared with normal tissue. (e) Heat map shows the DNA methylation status of the 5' region of TAF7L. (f) Box plot shows that promoter 1 (green box in e) of TAF7L is significantly hypomethylated in colon and rectal cancer compared with normal tissues

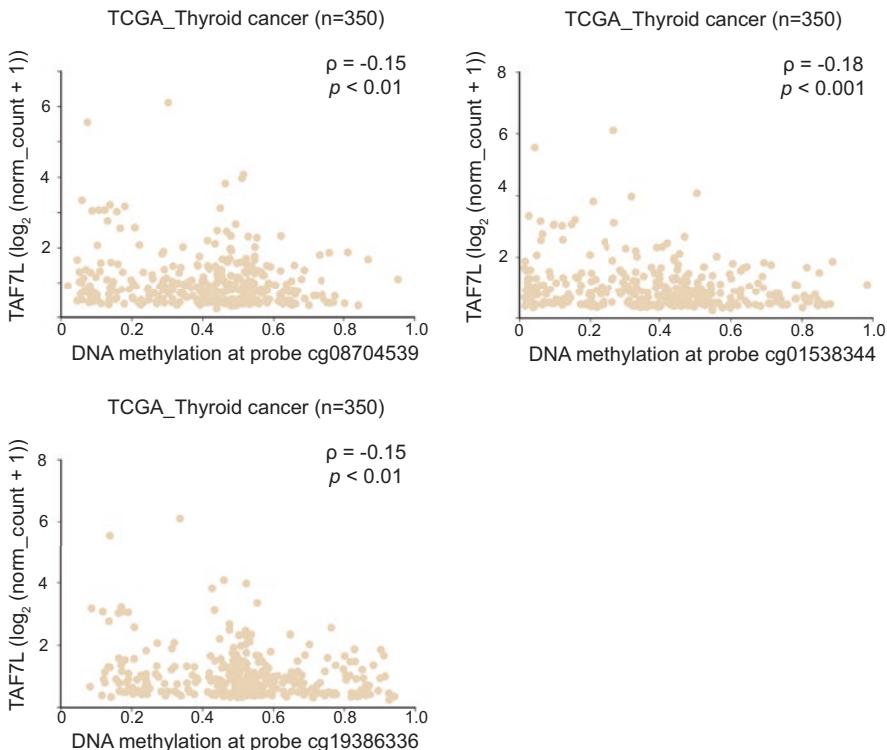


Fig. 7 The expression of CT antigen TAF7L is inversely correlated with DNA methylation of the 5' CpG island in thyroid cancer. Dot plots show that the expression of TAF7L negatively correlates with the DNA methylation status of its associated 5' CpG island in thyroid cancer. The exact location of each probe is described in the results session

antigens by setting the threshold of their expression in normal testes as RPKM (reads per kilobase of transcript per million mapped reads) ≥ 20 in the GTEx (The Genotype-Tissue Expression) Database to rule out CT antigens whose expression levels were already low in normal testes. This resulted in a list of 103 CT antigens. Gene ontology analysis suggested that several CT antigens were enriched in protein processing and metabolic pathways (Fig. 8b), both of which are important in tumorigenesis. We then further filtered the 103 CT antigens by setting the threshold of fold change in their expression at 4 with the idea that the remaining CT antigens should be mostly susceptible to regulation by TAF7L. Sixteen CT antigens passed the threshold of fold change (Fig. 8c). We then confirmed if they were under direct control of TAF7L by analyzing the ChIP-Seq data on TAF7L and RNA polymerase II (POL II) [63]. Peak annotations showed that approximately 94% of TAF7L peaks were within 1 kb of the promoters (Fig. 9a), consistent with a previous analysis [63]. Moreover, the 16 filtered CT antigens were occupied by TAF7L peaks, and these CT antigens were all actively transcribed as indicated by the co-localized POL II peaks

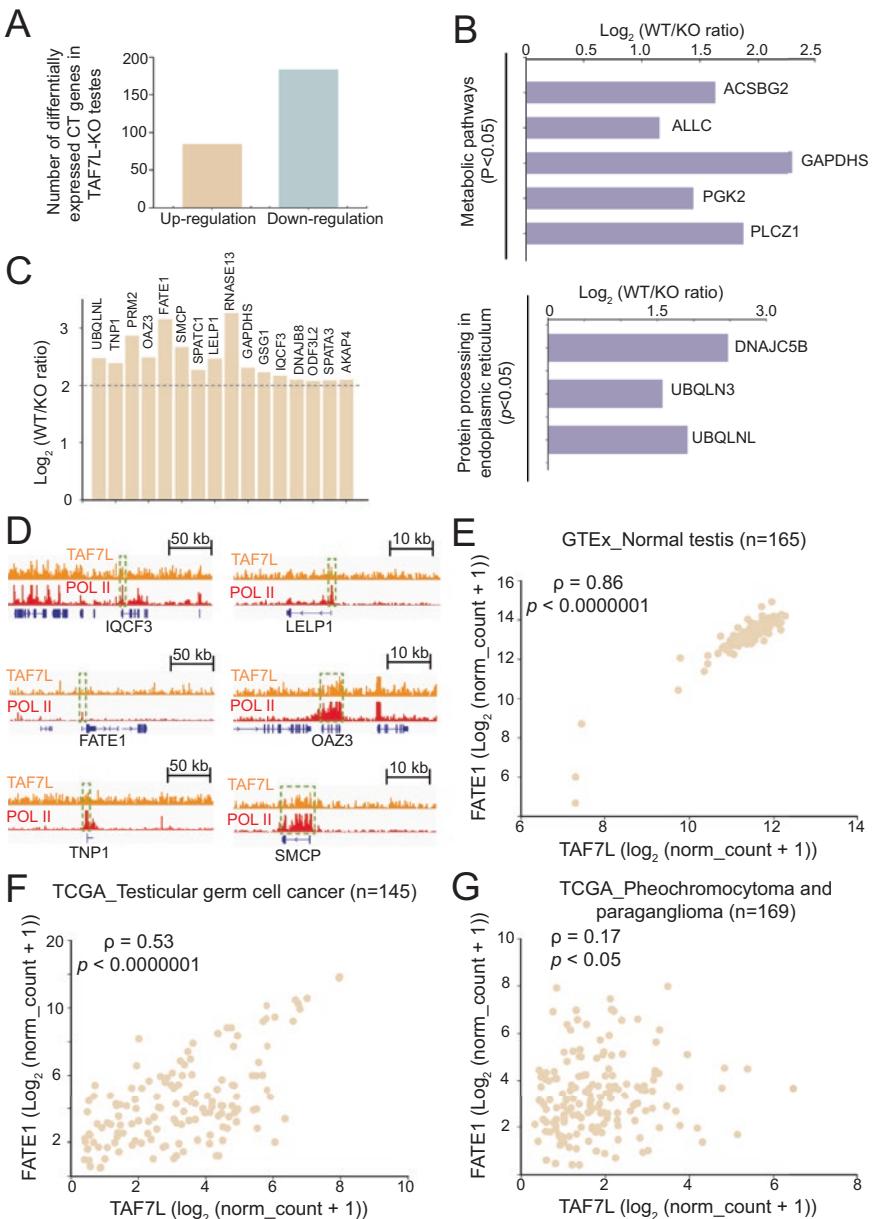


Fig. 8 CT antigen TAF7L controls the expression of a subset of CT antigens. **(a)** Bar graph shows that a total of 84 up-regulated and 183 down-regulated CT antigens are among the genes that were differentially expressed in TAF7L-deficient testes. **(b)** Down-regulated CT antigens identified in the TAF7L-deficient model were subject to functional annotations. Protein processing in the endoplasmic reticulum and metabolic pathways were enriched in the analysis. The fold change of each CT antigen is indicated. **(c)** Bar graph shows CT antigens with >4 -fold change in expression identified in the TAF7L KO model. **(d)** ChIP peak coverage of TAF7L and POL II suggests that TAF7L directly binds to CT antigens, which are actively transcribed as indicated by colocalized peaks of Fig. 8 (continued). TAF7L and POL II (green box). **(e)** Dot plot shows that the expression of CT antigen FATE1 significantly correlates with the expression of TAF7L in normal human testes. **(f)** Dot plot shows that the expression of CT antigen FATE1 significantly correlates with the expression of TAF7L in testicular germ cell cancer. **(g)** Dot plot shows that the expression of CT antigen FATE1 significantly correlates with the expression of TAF7L in pheochromocytoma and paraganglioma

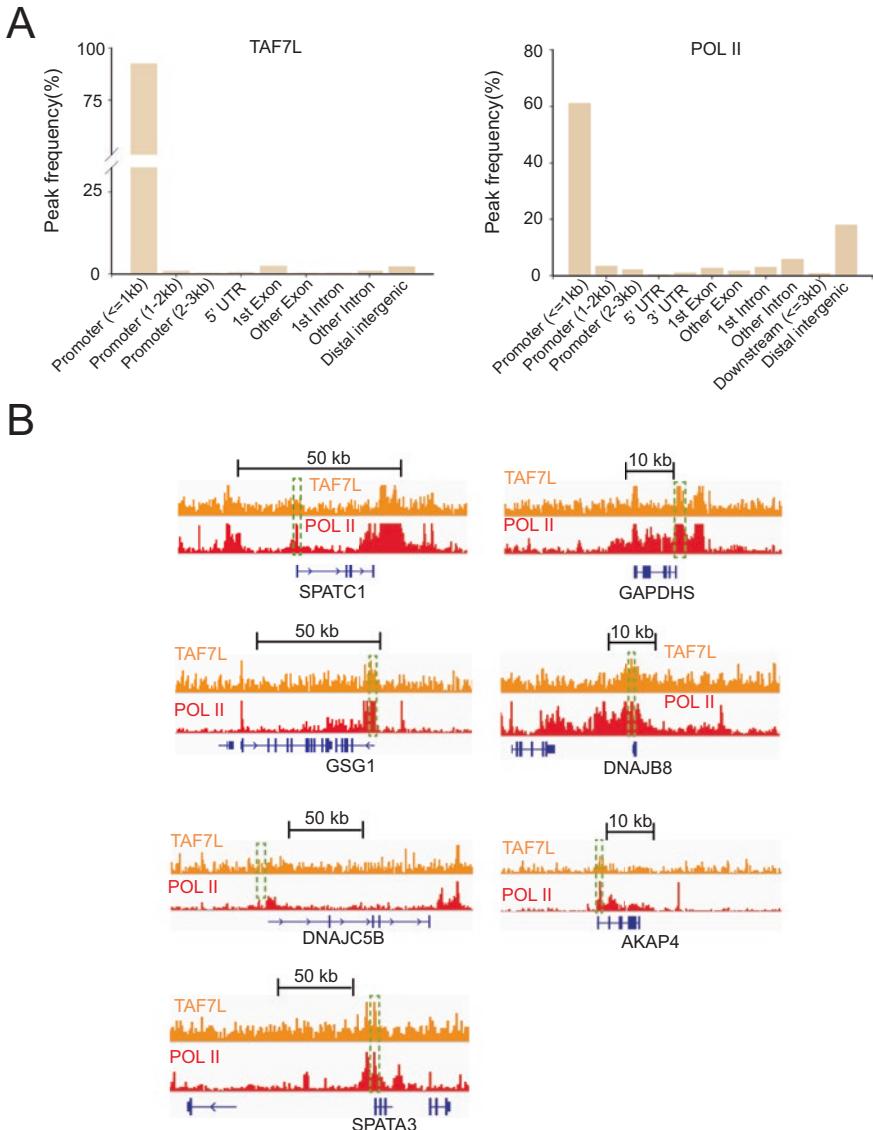


Fig. 9 CT antigen TAF7L regulates the expression of a subset of CT antigens. **(a)** Bar graphs summarize peak annotations of the ChIP-Seq data for both TAF7L and POL II. **(b)** ChIP peak coverage of TAF7L and POL II indicates that TAF7L directly binds to CT antigens, which are actively transcribed as indicated by the colocalized peaks of TAF7L and POL II (green box)

(Figs. 8d and 9b, green box). These data suggest that TAF7L regulates the expression of these CT antigens by directly binding to their promoters in testes.

We then set out to test if a similar regulatory mechanism between TAF7L and CT antigens is found in cancers. We focused on one of the CT antigens FATE1 (Fig. 8c). FATE1 has been reported to be overexpressed in hepatocellular carcinoma, as well as gastric and colon cancers [64]. Furthermore, FATE1 decreases the sensitivity of adrenocortical carcinoma cells to the chemotherapeutic drug mitotane, and its expression in adrenocortical carcinoma negatively correlates with patient survival [65]. We observed that FATE1 expression was highly correlated with TAF7L expression in normal human testes (Fig. 8e), consistent with our earlier findings (Fig. 8c). Interestingly, this positive correlation persisted in testicular germ cell cancer (Fig. 8f), as well as pheochromocytoma and paraganglioma (Fig. 8g), indicating that FATE1 expression is also under the influence of TAF7L in cancer.

Discussion and Concluding Remarks

The past decade has seen a tremendous surge in the number of high resolution next generation sequencing datasets generated from the fields of reproduction and cancer. New bioinformatics tools have also helped to systematically identify CT antigens, which have greatly expanded the pool of CT antigens from more than 200 to approximately 1000 [13]. At the time of this writing, a new study has reported yet another genome-wide identification of CT antigens, adding an additional 201 CT antigens to an already exhaustive pool of CT antigens [66]. With such an explosion of publicly available datasets, one may be surprised by how little we know about the molecular mechanisms underlying the function and regulation of these CT antigens throughout gametogenesis and tumorigenesis. The problem may no longer be a lack of available data to work on, but a lack of direction on where to look and how to analyze the data. The shared characteristics between gametogenesis and tumorigenesis have attracted considerable interest. It has been proposed that the expression of CT antigens during tumorigenesis is due to the reactivation of a gametogenic program in non-germline somatic cells [6, 12, 67]. If this is true, understanding the regulation of CT antigens during gametogenesis is likely to provide new insights on the regulation of these CT antigens during tumorigenesis.

By re-analyzing published datasets and data from multiple databases, we have re-examined the likely roles of CT antigens in cancers by providing the following information. First, a subset of CT antigens associated with SEs in three different cancer types. Of interest, ten of these CT antigens also associated with SEs in normal mouse testes. By analyzing ChIP-Seq data from human testes and several types of cancer, we expected to find additional conserved SE-associated CT antigens. Thus, it is tempting to speculate that these CT antigens acquired SEs via a conserved mechanism during tumorigenesis, which in turn drives their aberrant

expression in different cancer types. SEs have been reported to associate with key oncogenes [27, 68, 69]. We found that the CT antigen TPX2 associated with a SE in MM, GBM, and SCLC, as well as in normal testes. Moreover, TPX2 per se belongs to the human core fitness gene family. Previous studies have shown that certain inhibitors, such as the BET-bromodomain inhibitor JQ1 and CDK7 inhibitor, preferentially target SE-associated oncogenes in cancers [24, 70]. Thus, targeting TPX2 or its associated SE in cancer cells may prove to be an effective therapeutic approach.

Second, we showed that hypomethylation in SE regions may affect the ectopic expression of certain CT antigens in breast cancer. Hypomethylation/demethylation is known to occur throughout gametogenesis and tumorigenesis [47, 71]. Moreover, DNA hypomethylation participates in the activation of a subset of CT antigens in different cancer types [72, 73]. By extracting information from a study in which DNA methyltransferase DNMT1 was conditionally knocked out in PGCs, we found that DNMT1 can potentially suppress the expression of a new subset of CT antigens in cancers, such as TEX101, whose expression strongly correlates with cancer prognosis [66]. The demethylating agent 5-aza-2'-deoxycytidine has been shown to induce the expression of the CT antigen MAGE in both normal and malignant lymphoid cells [74]. In fact, 5-aza-2'-deoxycytidine inhibits DNA methylation by prohibiting DNMT1 access to the progressing replication fork during DNA replication [75]. The ablation of DNMT1 alone, but not DNMT3B alone, in HCT116 cells (a colorectal cancer cell line) activated CT antigens MAGE-A1, NY-ESO-1 and XAGE-1 [76]. Taken collectively, this information confirms our observations on the important role of DNMT1 in regulating CT antigen expression in cancer. Additionally, TAF7L is another CT antigen that was identified in the DNMT1 KO model, wherein the expression of TAF7L regulated by the status of DNA methylation in its promoter and the promoter-associated CpG island in cancers. This finding is not surprising given that demethylation of the CpG sites within the 5' regions of the CT antigen MAGE-A1 also correlates with its ectopic expression in cancer cells [77]. Thus, DNA hypomethylation may represent the primary mechanism of activation for a growing subset of CT antigens in cancers.

Finally, we examined a testis TAF7L KO model. TAF7L is a germ cell-specific transcription factor. We showed that TAF7L could directly bind to the promoter regions of many CT antigens to regulate their expression in normal testes, and also potentially in cancers. To our surprise, the role of TAF7L resembled that of the previously identified DNA-binding protein BORIS (also known as CTCFL). BORIS per se is a CT antigen whose expression, similar to TAF7L, is also regulated by DNA methylation in its promoter region in different cancer types [78, 79]. Of interest, BORIS can also directly bind to the promoters of other CT antigens, such as NY-ESO-1 in lung cancer cells, to regulate their expression [80]. Thus, there is a subset of CT antigens that function as transcriptional regulators of other CT antigens. Future analysis may identify additional CT antigens with similar roles to provide a more comprehensive outlook on the regulation of CT antigens in cancer.

Summary

In summary, we have shown that it is possible to extract useful information on the regulation of CT antigens during tumorigenesis by studying gametogenesis. This approach may also provide a unique gateway to probing the functions of these genes in future studies. By taking advantage of the ever-growing publicly available datasets, systematic analysis can, without a doubt, provide new insights into these CT antigens.

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Coxsackievirus and Adenovirus Receptor (CXADR): Recent Findings and Its Role and Regulation in Spermatogenesis



Yang Zhang and Wing-Yee Lui

Introduction

Recent studies of testicular cell type-specific CXADR knockout mice models suggested that CXADR is indispensable to spermatogenesis. Specific knockout of CXADR in Sertoli cells (SC-CXADR^{-/-} knockout) causes reproductive impairment [1]. CXADR is found at the inter-Sertoli cell junctions as well as the apical ectoplasmic specialization where elongate spermatids attached to Sertoli cell epithelium. Although CXADR is a junction component, but its role cannot be substituted by other junction proteins evidenced by SC-CXADR^{-/-} knockout displaying reproductive impairment, indicating that CXADR not only serves as a structural junction protein, but also exerts unique roles in spermatogenesis. In this view, we will summarize and highlight the novel findings of CXADR in the testis and discuss the recent findings of CXADR in other tissue models and in tumour progression. We hope this information can serve as a blueprint and provide some insights and direction to delineate the unknown functions of CXADR that are essential for spermatogenesis.

CXADR Isoforms and Their Molecular Structure

Cxadr gene gives rise to numerous splice variants with soluble and membrane-bound isoforms. So far, three soluble isoforms (hCXADR4/7, hCXADR3/7 and hCXADR2/7) and two membrane-bound isoforms (hCXADR1 and hCXADR2)

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have been identified in human. Three splice variants of *Cxadr* gene have been identified in mouse and the spliced transcripts result in three membrane-bound isoforms including mCXADR1 (Exons I–VIII), mCXADR2 (exons 1–VIIb) and mCXADR3 (exon I–VIIc) [2, 3]. hCXADR1 is equivalent to mCXADR1 and hCXADR2 is equivalent to mCXADR2.

Membrane CXADR isoforms comprise of an extracellular domain, a transmembrane domain and an intracellular domain. Having identical extracellular and transmembrane domains, their structural difference is mainly contributed by their intracellular domain generated by alternative splicing between Exons VII and VIII. CXADRs belong to the CTX subfamily of the Ig superfamily that bears two Ig-like domains named D1 Ig-variable domain and D2 Ig-constant domain at their extracellular domains [4–6]. The transmembrane domain is a single spanning helix of 21 amino acids. The intracellular domain contains motifs such as class I PDZ-binding motif, clathrin-adaptor protein (AP) recognition motif and a sorting motif at its C-terminus. The intracellular domain is important for post-translational modification of CXADR. In human, soluble CXADR isoforms with no transmembrane domain are the end results of excision of *Cxadr* exon 5. Soluble CXADR protein secreted from the cell can also interact with coxsackievirus and adenovirus [7].

Phenotypes of CXADR Knockout Mice

Conventional knockout (KO) of CXADR resulted in embryonic lethality associated with cardiac defects. Cardiomyocytes from CXADR KO mice at 10.5 days post coitum (dpc) exhibited apoptotic features, degenerated myocardial wall and thoracic haemorrhaging, leading to death between 11.5 and 13.5 dpc [8, 9]. These two very first CXADR KO studies confirmed that CXADR is crucial for embryonic heart development.

Two cardiac-specific CXADR KO mice models were then generated to elucidate the functional significance of CXADR in cardiac development and functions. Cardiomyocyte-specific CXADR deletion occurred at 10 dpc and after 11 dpc respectively mediated by Cre recombinase under the control of two different promoters lead to different outcomes. For cardiomyocyte-specific CXADR deletion at 10 dpc, mice died by 12.5 dpc with significant cardiac abnormalities such as hyperplasia of the left ventricular myocardium and abnormal sinoatrial valves. Cardiomyocyte-specific CXADR deletion appeared after 11 dpc, mice managed to survive to adulthood without notable cardiac abnormalities [2]. These studies suggested that a temporal expression of CXADR is essential for cardiomyocyte proliferation and differentiation.

Apart from heart, CXADR is ubiquitously expressed in many tissues and is believed to play crucial roles in various physiological functions. To evaluate the unique functions of CXADR in other tissues and cells, several cell-specific and drug-inducible CXADR knockout mice models have been generated by different research groups (Table 1). However, data from conditional knockout studies have

Table 1 Phenotypes of various CXADR knockout mouse models

Model	Time and site of Cre activation	Deleted exon	Phenotype(s)	Reference
Cxadr ^{-/-}	E0, whole body	Exon 1	Die between E11.5 and E13.5; reduced density of myofibrils; enlarged mitochondria; enriched glycogen storage strongly; pericardial edema formation	[9]
Cxadr ^{flox/flox} ; TNT-Cre	E9.5; cardiomyocyte	Exon 2	Lethal; hyperplasia of the proximal heart tube; engorgement of cardinal veins; absence of the sinoatrial valves	[2]
Cxadr ^{flox/flox} ; MHC-Cre	E11.5; cardiomyocyte	Exon 2	Viable	[2]
Cxadr ^{flox/flox} ; Cre-ERT TM	P3 weeks	Exon 2	Dilated intestinal tract; atrophy of the exocrine pancreas; abnormal thymopoiesis	[10]
Cxadr ^{flox/flox} ; Cre-ERT TM	E12.5	Exon 2	Lethal; subcutaneous edema, hemorrhage and embryonic; dilated subcutaneous lymphatic vessels; abnormal structure with gaps and holes present at lymphatic endothelial cell-cell junctions; erythrocyte leakage	[11]
Cxadr ^{flox/flox} ; Cre-ERT TM	E13.5	Exon 2	Viable	[11]
Cxadr ^{+/+}	E0, whole body	n.a.	Ventricular conduction slowing; reduced sodium channel availability; increased arrhythmia susceptibility on pharmacological electrical uncoupling	[12]
Cxadr ^{flox/flox} ; Cre-ERT TM	P8	Exon 2	Intact blood-testis barrier; uncompromised fertility.	[13]
Cxadr ^{flox/flox} ; Cre-ERT TM	P4–6 weeks	Exon 2	Intact blood-testis barrier; uncompromised fertility; CXADR-negative sperms can fertilize WT eggs	[13]
Cxadr ^{flox/flox} ; hNphs2-Cre	E14.0 onwards; Podocyte	Exon 1	Normal podocyte development, normal stress response	[14]
Cxadr ^{flox/flox} ; Myh6-Cre	E9.5; heart	Exon 1	Viable; No obvious labyrinth defects.	[15]
Cxadr ^{flox/flox} ; Tnnt2-Cre	E7.5; heart	Exon 1	Embryonic lethality by E12.5; Thinner placentas; Decreased labyrinth depth.	[15]
Cxadr ^{flox/flox} ; Sox2-Cre	E14.5; visceral yolk sac, allantois, labyrinth	Exon 1	Lethal between E11.5 and E12.5; Altered interheamal membrane (IHM) architecture; Reduced IHM branching; Flatter placentas.	[15]
Cxadr ^{flox/flox} ; Amh-Cre	E14.5; Sertoli cells	Exon 3	Reduced fertility with age; increased germ cell apoptosis; premature loss of elongated spermatids; compromised BTB function and apical ES structure; dysregulation of occludin and zonula occludens-1; altered β-catenin/Cdc42 signaling	[1]
Cxadr ^{flox/flox} ; Stra8-iCre	P3; male germ cells	Exon 3	Normal; no observable changes in reproductive functions	[1]

E embryonic, P postnatal

suggested that CXADR is redundant in some tissues. For example, CXADR is dispensable in kidney epithelial cells as the fact that podocyte-specific CXADR deletion model shows normal podocyte development and function and stress response [14].

Functions and Regulation of CXADR

CXADR as a Virus Receptor

Membrane-bound CXADR serves as a major viral receptor for adenovirus (AdV) species A and C-G and group B coxsackieviruses (CVB) [16–18]. AdV and CVB interact directly with CXADR via the extracellular D1 domain [17, 19]. Although extracellular D2 domain of CXADR exerts no direct interaction with AdV and CVB, D2 domain of CXADR is believed to play a role on the binding cooperativity and steric interaction between CXADR and the virus as the absence of D2 domain results in failing viral infection to the host [20–22].

In human, hCXADR1 and hCXADR2 bind to fiber knob of AdV and canyon-like receptor binding site of CVB [23], however, differential localization of hCXADR1 and hCXADR2 in polarized cells implicate their distinguishing roles as viral receptor. hCXADR1 is localized mainly at the apical membrane of the polarized airway epithelial cells and retinal pigment epithelium (RPE) that makes them more accessible to airborne AdV and/or CVB [24]. Studies have confirmed that pro-inflammatory cytokines such as IL-8 promotes the localization of hCXADR1 at the apical region and overexpression of the apical hCXADR1 enhances AdV entry in polarized human airway epithelial cells. Also, knockout of AP-1B in AdV-resistant MDCK cells enhanced the apical hCXADR1 localization and ADV infectivity [24]. hCXADR2 is primarily found at the basolateral surface of the polarized airway epithelium and the localization of hCXADR2 is regulated in part by MAGI-I, AP-1A and AP-1B. It is believed that the access of AdV and CVB to hCXADR2 is hindered by tight junctions located near the apical area, resulting in blockage of the virus entry. However, hCXADR2 helps facilitating the basal virus spread.

Among three soluble isoforms, hCXADR4/7 and hCXADR3/7 processing a complete D1 domain are able to interact with AdV and CVB. Studies have suggested that soluble hCXADRs may serve as a decoy molecule and interact with AdV and CVB that leads to a reduction on viral infectivity. For instance, a significant reduction of virus entry was observed in HeLa cells preloaded with hCXADR4/7 prior to CVB infection as preloaded hCXADR4/7 binds to canyon-like receptor binding site of CVB. Using mouse *in vivo* models, a significant inhibition of CVB3 infection in mouse myocardium and pancreas has been observed if exogenous hCXADR4/7 was injected into mice before or during the inoculation of CVB3. However, the biological importance of these two isoforms on decoying viral receptors in human body remains unclear.

CXADR as a Cell Junction Component

CXADR serves as a cell junction component that contributes to numerous developmental and cellular processes ranging from peri-implantation phase of embryos to tumour development and metastasis [25–27]. Typically, CXADR is colocalized and interacts with zonula-occludens (ZO-1) at the basolateral membrane in most epithelial cells. CXADR interacts with various tight and adherens junction proteins including, but not limited to, β -catenin, ZO-1 and MUPP-1 [4, 28, 29]. Homotypic CXADR interlocks localized at adherens and tight junctions is linked to actin filament and microtubules via ZO-1. Dynamic cytoskeletal reorganization mediated by CXADR results in the regulation of barrier permeability, cell adhesion and cell migration [4, 27].

Homotypic interaction of CXADR was firstly confirmed in CXADR-expressing CHO cells and such homotypic interaction mediates cell aggregation in non-polarized cells [4, 30]. Colocalization of CXADR and ZO-1 was identified in various polarized epithelial cells including human airway epithelial cell lines, human colonic cell line and MDCK cells [4, 28, 31]. Pull-down experiments have confirmed that CXADR exerts physical association with ZO-1 and β -catenin via either direct interaction or indirect interaction through other junction proteins [4, 28]. CXADR also elicits the ability to limit paracellular solute flow, indicating that CXADR could function as a tight junction component [4, 28]. Subsequent studies from other groups have reported that CXADR interacts with actin cytoskeleton in sub-cellular fraction of brain sample and podocytes [32, 33].

The functional significance of CXADR in barrier function and adhesion has been demonstrated in various developmental processes and tissues. For instance, CXADR plays a pivotal role in regulating the paracellular permeability barrier in trophectoderm of peri-implantation embryos and facilitating the interaction between implanting embryos and endometrium [26]. Embryos incubated with CXADR blocking antibody showed a remarkable increase in dextran-FITC influx in blastocoel and exhibited a significant decrease in blastocoel volume, suggesting that TJs constituted by CXADR are involved in maintenance of paracellular permeability barrier in trophectoderm and are crucial for blastocyst development [26]. CXADR plays a crucial role in early embryonic development as depletion of CXADR in mouse results in embryonic lethality at E11.5 and E13.5 in pertinent to malformation of cardiac tissue. It is found that CXADR exerts similar function as N-cadherin and involves in the formation of cell contacts between myocytes and the organization of myofibrils that is required for embryonic heart development [9]. Subsequent studies using cardiac-specific knockout have revealed that CXADR is indispensable for the localization of connexin 45 at the AV-node cell-cell junction and of β -catenin and ZO-1 at the ventricular intercalated disc that is required for normal AV-node conduction [34]. Studies using tamoxifen-inducible CXADR-deficient mouse model have uncovered that CXADR is crucial to the development of the lymphatic vasculature. It was found that CXADR is expressed in lymphatic but not vascular endothelial cells during mouse development. CXADR deficiency impairs the formation

of junctions between lymphatic endothelial cells and cell-cell adhesion and incomplete separation between the blood and the lymphatic vasculature during development, resulting in subcutaneous edema, haemorrhage and lethality [11].

Above-mentioned examples have clearly demonstrated that CXADR is a crucial tight and adherens junction component during tissue development. Apart from being junction building blocks, CXADR modulates the localization and bioavailability of other junction proteins at the sites, thus altering cell adhesiveness. For instance, overexpression of CXADR in MCF7 cells reduces the level of E-cadherin at cell-cell junctions and cell adhesion [35]. The mechanism of CXADR-dependent E-cadherin regulation in pertinent to cell adhesion has been explored further in human bronchial epithelial cells (HBEC). Studies have revealed that PKC δ -mediated phosphorylation of CXADR at the C-terminus inhibit/prevent Src-dependent E-cadherin endocytosis at cell junctions, thus stabilizing the junction [36].

In addition to homotypic interaction of CXADR, heterotypic interaction of CXADR and other Ig superfamily members have been reported to regulate cell migration and immune response [37, 38]. Junctional adhesion molecule-like protein (JAML) is the first identified junction protein that can interact heterotypically with CXADR. Proximal Ig domain of JAML in neutrophil can interact physically with D1 domain of CXADR in epithelial cells and such interaction is involved in neutrophil transepithelial migration [37]. The ligation and co-stimulation of JAML in $\gamma\delta$ T cells and CXADR in keratinocytes are essential for $\gamma\delta$ T cell activation in wound response [38]. Blockage of such heterotypic interaction inhibits wound healing process [38].

Regardless homotypic or heterotypic interaction, CXADR is undoubtedly an important junction protein to mediate cell-cell interaction and adhesion, eliciting various physiological functions.

CXADR as a Signalling Platform

Apart from serving as a junction component, CXADR exerts its range of actions through functioning as a signalling platform. Having such characteristic, the physiological significance of CXADR could be greatly enhanced as triggering distinct downstream signalling cascades regulate different physiological and cellular processes such as tissue homeostasis and gene transcription that could be independent to its role on cell adhesion.

In human epithelial cell, CXADR overexpression could regulate integrin function via triggering the activation of p44/p42 MAPK signalling cascade. Enhanced activation of $\beta 1$ and $\beta 3$ integrin further promotes the Ad5 viral binding to CXADR [39]. Upregulation of CXADR promotes cardiomyocyte hypertrophy via enhancement of β -catenin/Akt/GSK3 β nuclear signalling and upregulation of c-myc gene transcription. Enhanced β -catenin stabilization is achieved in part via CXADR-induced Akt-dependent suppression of GSK3 β [40].

Previous studies have reported that loss of CXADR is linked to cancer progression and poor outcome in various cancers [41–44], however, the underlying mechanism and the role of CXADR remain unexplored. Until recently, studies in luminal A breast cancer uncovered that CXADR is a key component of the signalosome in controlling the epithelial-mesenchymal plasticity [27]. Indirect interaction of CXADR with PTEN and PHLPP2 via MAGI-1 allows the formation of an Akt-inhibitory signalosome and inhibits Akt signalling that is essential for epithelial-mesenchymal transition (EMT). Knockout of CXADR in EpRas cells destabilizes PTEN, PHLPP2 and MAGI-1 signaling and hyperactivates Akt signalling, thus promoting EMT [27]. These findings also unfold the correlation of CXADR loss with poor survival in luminal A breast cancer [27].

Like other cell adhesion molecules (CAMs), ectodomain shedding of CXADR could be mediated by ADAM10 in U87 cells overexpressing CXADR followed by regulated intramembrane proteolysis. The regulated intramembrane proteolysis involves the recruitment of presenilin/ γ -secretase complex for the cleavage of CXADR intracellular domain (ICD). The released CXADR ICD fragments then undergoes nuclear translocation to mediate nuclear signalling [45]. Although the downstream effects triggered by the nuclear translocation of CXADR ICD fragments have not been explored in details, the hypothesis of CXADR ICD as functional nuclear signalling molecule remains possible for the following reasons. First, it is well-documented that the cleaved intracellular domains of other CAMs serves as nuclear signalling molecules [46]. Also, the existence of CXADR fragments have been identified in nuclear extracts of other cell types including HeLa cells [47]. Taken together, it is expected that CXADR itself may serve as signalling molecule upon precise proteolysis.

Role of CXADR in Spermatogenesis

RT-PCR, immunostaining and immunoblotting analyses performed in various laboratories have confirmed that testicular cells including Sertoli cells, germ cells and Leydig cells express CXADR [48–50]. RT-PCR and immunoblotting analyses have also shown that Sertoli cells express a higher level of CXADR compared to germ cells [50]. Immunostaining analyses have suggested that CXADR is highly expressed at Stage VIII of the seminiferous epithelial cycle with pronounced expression at the blood-testis barrier (BTB) and apical ectoplasmic specialization (ES) [49, 50]. Unique expression and localization of CXADR perfectly matched with the stage-specific events happened at Stage VIII where extensive restructuring of the BTB and apical ES occur. Thus, different research groups have then focused to unfold the potential role of CXADR on germ cell migration across the BTB and restructuring of apical ES with regards to spermatid maturation and detachment.

Studies from Mirza et al. revealed that two membrane-bound CXADRs have been found on different subcellular sites of the acrosomal region of spermatozoa [49]. CXADR is colocalized and interacts physically with JAM-C in testis and

isolated spermatozoa. It is suggested that interaction of CXADR with JAM-C form a complex in acrosome that may be crucial for sperm maturation [49]. However, these studies have not ruled out the possibility that the interaction of CXADR and JAM-C can also be contributed by the heterotypic interaction of CXADR in Sertoli cells and JAM-C in sperm.

Subsequent studies from Wang et al. confirmed the expression pattern of CXADR in testicular cells as report by Peters et al. earlier. Wang et al. have revealed Sertoli cells expressed much higher level of CXADR than germ cells [50]. In addition, CXADR is highly concentrated at the BTB and apical ES. CXADR is localized with ZO-1 and N-cadherin at low-density Sertoli cell culture, suggesting that CXADR is formed at cell junctions between Sertoli cells [50]. Expression of CXADR in Sertoli cells increases significantly during the formation of tight junctions in Sertoli cells [50]. In addition, siRNA knockdown of CXADR perturbs Sertoli cell TJ barrier as evidenced by a decrease in transepithelial electrical resistance (TER), while overexpression of CXADR in Sertoli cells promotes TJ barrier function [51]. CXADR knockdown-mediated TJ disruption is contributed not only by its own absence at the TJ barrier, but also its effects on regulating occludin bioavailability [51]. Knockdown of CXADR alters the phosphorylation status of occludin, and induces endocytosis of occludin [51]. In short, CXADR serves as a structural TJ component and a regulator to modulate TJ barrier function *in vitro*.

Immunostaining analyses have demonstrated that migratory germ cells situated near the BTB express high level of CXADR. JAM-A and JAM-B, but not JAM-C, is colocalized with CXADR in migratory germ cells at the BTB [52]. Mirza et al. have proposed that the interaction of CXADR with JAM may facilitate the migration of germ cells across the BTB as other JAM proteins such as JAML in leukocyte is known to interact with CXADR in epithelium to facilitate leukocyte transmigration [37].

Although *in vitro* results obtained from different research groups regarding the roles of CXADR in germ cells and Sertoli cells are not all aligned, all of the above studies suggested that CXADR could be a crucial molecule in spermatogenesis.

Regulation of CXADR in Testicular Cells

Studies have identified that hormone and cytokines are key molecules to regulate the function of cell junctions and barrier. Mirza et al. have reported that CXADR mRNA level in cultured Sertoli cells is increased by twofold [52]. Studies performed by Wang et al. have identified that TNF α , a key cytokine in spermatogenesis, downregulates CXADR protein level by 60% upon 4-day TNF α treatment [50]. Although FSH and TNF α could regulate the expression of CXADR in testis, no detailed regulatory mechanisms have been identified. Studies in our laboratory have revealed that IFN γ +TNF disrupts testicular cell adhesion by exerting a synergistic effect on CXADR downregulation [53]. We found that IFN γ +TNF treatment inhibits the binding of the basal transcription factors and promotes the binding of

negative regulators including NF κ B subunits and Sp1 to the CAR promoter region [53]. In addition, IFN γ +TNF also upregulates CAR protein degradation via ubiquitin-proteasome and NF κ B pathways [53]. Taken together, IFN γ +TNF down-regulates CXADR level via transcriptional and post-translational regulation.

Conditional CXADR Knockouts Unfold the Physiological Significance of CXADR

Generating conditional CXADR mouse models such as tamoxifen-inducible or cell type-specific knockout models allow us to unravel the *in vivo* function of CXADR in spermatogenesis. Sultana et al. generated the first *in vivo* tamoxifen-inducible CXADR knockout mouse model to study the function of CXADR in testis. Prepubertal mice at P8 were received a single dose of tamoxifen injection and killed at P9, P24, P49 and P90 [13]. Adult mice at the age of 4–6 weeks were received tamoxifen injection daily for 5 consecutive days and mice were analyzed 3, 6 or 12 weeks after the first tamoxifen injection. Surprisingly, these two groups of tamoxifen-induced CXADR conditional knockout mice display an intact BTB with proper cellular junctions and normal fertility GC-CXADR $^{-/-}$. Results from these conditional knockout studies suggested that CXADR is dispensable for testis development and spermatogenesis in mice.

Although tamoxifen-induced CXADR knockout mice showed no short-term effect on spermatogenesis, late-onset effects on spermatogenesis have not been ruled out. Previous studies of conditional occludin knockout have clearly demonstrated that knockout of occludin showed no early-onset effects, but testicular atrophy occurs and becomes very severe when the knockout animals reach 40–60 weeks (=280–420 days) old [54]. Also, the tamoxifen-induced CXADR deletion was initiated in P9 and P28, the effect of CXADR in embryonic testis development and its sequential consequence in adult testis development have not been taken into account. Therefore, no apparent disruptive effects on spermatogenesis in tamoxifen-induced CXADR knockout mice is not too surprising.

To overcome/address the above-mentioned concerns, our laboratory made an effort to generate cell type-specific CXADR knockout mice [1]. They are Sertoli cell-specific CXADR (SC-CXADR $^{-/-}$) and germ cell-specific CXADR (GC-CXADR $^{-/-}$) knockouts. CXADR $^{\text{flox/flox}}$ mice are crossed with Amh-Cre mice or Stra8-Cre mice to obtain SC-CXADR $^{-/-}$ and GC-CXADR $^{-/-}$ mice respectively. Cre-recombinase activity has been reported in SC as early as E14.5 and in GC at P3 respectively. Cell type-specific knockout of CXADR has been confirmed and validated by genotyping and flow cytometry analyses. Fertility tests, BTB integrity assays and morphological analyses have been performed in SC-CXADR $^{-/-}$ and GC-CXADR $^{-/-}$ mice at various time points (P45-P300) to investigate the cell type-specific knockout effect of CXADR in testicular functions.

Our studies have confirmed that SC-CXADR, but not GC-CXADR, is crucial for spermatogenesis [1] (Fig. 1). SC-CXADR^{-/-} mice display reduced fertility, disrupted BTB, reduced testis-to-body ratio and notable premature loss of germ cells from the seminiferous epithelium from d120 and onwards, suggesting that deletion of CXADR in SC impairs spermatogenesis. BTB is disrupted in SC-CXADR^{-/-} mice as evidenced by the influx of FITC-biotin to the adluminal compartment and dysregulation of occludin and ZO-1 at the BTB [1].

Apart from BTB disruption, flow cytometry analyses detected a significant diminishment of the subhaploid H peak from the SC-CXADR^{-/-} testicular cell sample when compared to the wild-type one [1]. Data from flow cytometry analyses match perfectly with the premature loss of germ cells from the seminiferous epithelium observed in histological analyses. Also, SC-CXADR^{-/-} mice exhibit disorganized F-actin at the apical ES, whereas extensive and organized F-actin network is associated with spermatid heads at stages VI and VIII in control testis [1]. These observations suggested that SC-CXADR plays a crucial role in the apical ES function that allows the proper attachment and interaction between spermatids and Sertoli cell epithelium (Fig. 1).

Similar analyses and assays have been performed in GC-CXADR^{-/-} mice up to the age of d300. However, no apparent change in fertility and tubule morphology [1]. Taken together, we confirmed that SC-CXADR, but not GC-CXADR, is indispensable for spermatogenesis.

Omics Analyses of SC-CXADR^{-/-} Testes

We have unravelled the regulatory mechanisms underlying SC-CXADR^{-/-} knock-out on reproductive impairment through integral omics analyses using the whole SC-CXADR^{-/-} testis sample [1]. Bioinformatics analyses have enabled us to identified numerous potential key molecules and signalling networks that are required for SC-CXADR-mediated reproductive functions [1]. For instance, gene ontology (GO) enrichment analyses suggested that many biological processes as indicated by the GO terms such as cell death, microtubule-based process and movement are significantly altered. KEGG enrichment analyses revealed that several canonical signalling pathways such as PI3K-Akt signalling pathway, Wnt signaling and focal adhesion are significantly perturbed based on transcriptomic and proteomic analyses.

Our bioinformatic analyses suggested that β -catenin and Cdc42 are potential downstream mediators of SC-CXADR-regulated reproductive processes. To test this possibly, we examined their protein levels in SC-CXADR^{-/-} testis sample. We found that a significant increase in protein levels of non-phosphorylated β -catenin (active β -catenin), a reduction of active Cdc42 and F-actin disorganization in SC-CXADR^{-/-} testis, indicating that activation of β -catenin signalling and Cdc42 inhibition [1]. Overexpression of constitutive active of Cdc42 in MSC-1 cells having CXADR knockdown could partially redistribute non-phosphorylated β -catenin

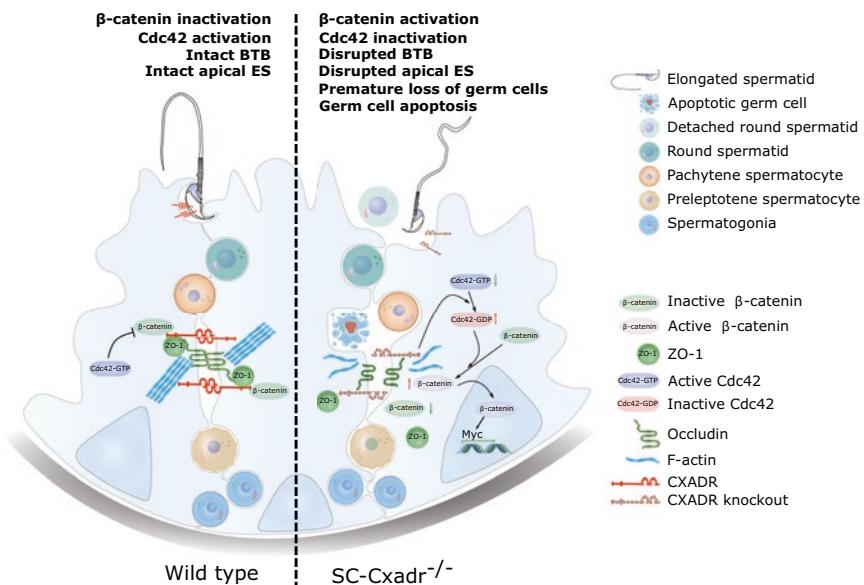


Fig. 1 A diagrammatic presentation of the role of CXADR in Sertoli cells based on the findings from Sertoli cell-specific CXADR knockout mouse (SC-CXADR^{-/-} KO) studies. CXADR is a structural component of the blood-testis barrier (BTB) and ectoplasmic specialization (ES). BTB is constituted by tight junction and the basal ectoplasmic specialization (ES). Apical ES (a special type of anchoring junction) is formed between Sertoli cell and elongating spermatids. In wild-type mouse testis, CXADR enables the proper formation of BTB and apical ES via CXADR-mediated β -catenin inactivation and Cdc42 activation. When SC-CXADR is knocked-out, BTB and apical ES are disrupted with mislocalization of occludin and ZO-1 at the BTB and F-actin disorganization at both BTB and apical ES, resulting in germ cell apoptosis and premature loss of haploid spermatids. SC-CXADR knockout inactivates Cdc42, thus altering the status of inactive β -catenin (phosphorylated β -catenin) to active β -catenin (non-phosphorylated form). Active β -catenin translocates from cytoplasm to nucleus to modulate gene transcription such as activation of myc gene transcription. Upward arrow means an increase in its protein level; downward arrow means a decrease in its protein level.

from the cytoplasm to the site of cell-cell contact, restore the organization of F-actin and partially block Myc gene transcription [1]. In these studies, we CXADR modulates cell adhesion and gene transcription through β -catenin inactivation and Cdc42 activation (Fig. 1).

Future Perspectives and Concluding Remarks

It is no doubt that CXADR is a multifunctional molecule. It is indispensable in some developmental and cellular processes including embryonic cardiac development, spermatogenesis and EMT transition, but its role can be substituted by other proteins in some cells and tissues such as podocytes, suggesting that CXADR displays

unique functions and signalling cascades in heart, testis and tumors. Studies have been performed to unravel the physiological roles of CXADR and its regulation in spermatogenesis. Recent findings from testicular cell type-specific knockout approach confirmed the importance of CXADR in Sertoli cells, but not germ cells. The newly identified CXADR-mediated β -catenin/Cdc42 signalling cascades showcases the importance of CXADR in triggering novel regulatory mechanism in the regulation of cell junctions and gene transcription. The omics data analyses have suggested that CXADR knockout triggers and alters numerous signalling pathways and biological processes. Further studies are warranted to address the many questions that remain unaddressed. For example, apart from β -catenin and Cdc42, what other downstream mediators are involved in CXADR-mediated signalling cascades to activate different cellular responses? Whether CXADR-mediated signalling cascades crosstalk with other signalling pathway to refine the cellular responses? Whether soluble CXADR is present in the testis and the mechanism and signaling involved in cleavage of CXADR? Can soluble CXADR functions as an autocrine molecule in the testis to coordinate the events occur at the apical and basal compartments? What regulatory mechanisms are involved in recycling and degradation of CXADR in the Sertoli cells? How CXADR in Sertoli cells regulates germ cell development? The above-mentioned area may provide important information to unravel the *in vivo* functions of CXADR in Sertoli cells and in spermatogenesis.

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Leydig Cell and Spermatogenesis



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Abbreviation

AKR1C14	3 α -hydroxysteroid dehydrogenase
cAMP	Adenosine 3'5'-cAMP
CYP11A1	Cytochrome P450 cholesterol side chain cleavage enzyme
CYP17A1	Cytochrome 17 α -hydroxylase/17,20-lyase
hCG	Human chorionic gonadotrophin
HSD17B3	17 β -Hydroxysteroid dehydrogenase isoform 3
HSD3B	3 β -Hydroxysteroid dehydrogenase
LHCGR	Luteinizing hormone/chorionic gonadotrophin receptor
NAD $^+$	Nicotinamide adenine dinucleotide
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
SCARKO	Conditional knockout androgen receptor in Sertoli cell
SRD5A1	Steroid 5 α -reductase 1
SRY	Sex-determining region Y protein

Introduction

Testosterone is the main androgen secreted by Leydig cells in mammals. This steroid is necessary for spermatogenesis. Many data were available in rat, mouse and human Leydig cells. In this chapter, Leydig cell development, regulation, and its function for spermatogenesis are discussed.

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The Development of Leydig Cells

The German scientist Franz Leydig first described a cell type with steroid-producing potential in the interstitial compartment of mammalian testes in 1850 [1]. This interstitial cell was named as the Leydig cell later. In mammalian males, Leydig cells are present in the interstitial area of the testis, surrounded by the seminiferous tubules (Fig. 1) [2]. These cells are void and multigonal in shape. The Leydig cell of mammals has smooth endoplasmic reticulum, which is abundant like other steroidogenic cells such as adrenal cells. These cells also contain numerous mitochondria. The Leydig cell of mammals also contains lipid droplets. Unlike rats and mice, another typical cytological feature of human Leydig cells is Reinke crystal, which is an indicator of reduced steroidogenic capacity during cell aging [3].

The Fetal Leydig Cell

There are two generations of Leydig cells in both rats and mice: fetal Leydig cell and adult Leydig cell [2, 4, 5]. Fetal Leydig cells are differentiated from stem Leydig cells. Although the exact origin of fetal Leydig cells are still under debate, they were believed to be originated from mesenchymal cells and cells in the mesonephros [6]. The genetic X and Y chromosomes determine sex of an embryo at fertilization [7]. The Y chromosome is required for fetal testis differentiation [8]. The sexual

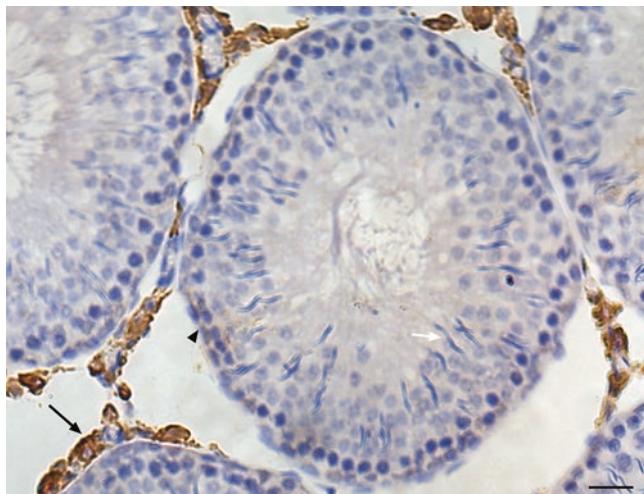


Fig. 1 Immunohistochemical staining of adult rat testis section. Immunohistochemical staining of cytochrome P450 cholesterol side chain cleavage enzyme, a biomarker of Leydig cells. Black arrow designates the Leydig cell. Black arrowhead designates the Sertoli cell, which support spermatogenesis. White arrowhead designates a sperm. Bar designates 20 μ m

differentiation of the fetus starts when the gonads differentiate [9], and in the case of males, male sexual differentiation of the fetus begins when the fetal testis differentiates. However, initially, gonads are identical in XY and XX embryos and the gonads are referred as the indifferent gonads [10].

Around gestational Day 12 in mice or 14 in rats and gestational Week 6 in humans [9, 11], the bipotential gonad appears and it develops into the fetal testis under the action of sex-determining region Y protein (SRY) produced from its gene on Y chromosome [12]. SRY is a transcription factor and binds to specific regions of DNA to regulate testis-specific gene expression [8, 13, 14]. The first appearance in the Sertoli cells, and then the stem cells of fetal Leydig cells begin to migrate and start to differentiate in the interstitium of the testis. The differentiation of fetal Leydig cells from stem cells is believed to be regulated by Sertoli cell-secreted factors, such as desert hedgehog [15] and platelet derived growth factors [16], and aristaless-related homeobox [17]. During the later gestation, the number of fetal Leydig cells gradually increases and they form clusters and express steroidogenic enzymes and reach a maximum secretion of androgens during the late gestation [18]. Fetal Leydig cells involute gradually after birth [19]. There is still controversy about the fate of fetal Leydig cells in the postnatal testis [18]. A few fetal Leydig cells are believed to persist in adult mouse testis [20]. However, the contribution to testosterone secretion in the adult testis by fetal Leydig cells is minimal [19].

In humans, seminiferous tubules are formed within the gonadal blastema and create interstitial parts by gestational Week 6 [21]. Fetal Leydig cells are differentiated from undifferentiated mesenchymal cells (potential stem Leydig cells) within these interstitial compartments on gestational Week 8 [10]. The number of fetal Leydig cells increases gradually, reaching a maximum by gestational Week 14–15 [21]. Due to the formation of fetal Leydig cells, the androgen concentrations in the fetal testis change in parallel with the increase of fetal Leydig cell number. Unlike rats and mice, the numbers of fetal Leydig cells, serum testosterone levels, some steroidogenic enzyme expression begin to decline [22, 23]. The fetal Leydig cell number is approximately 60% lower than the prenatal peak at the birth [22, 23]. The primary function of fetal Leydig cells is the secretion of testosterone, which stimulates the development of both the internal and external genitalia of the male fetus [2].

The Neonatal Leydig Cell

Unlike rats and mice, humans have additional generation of Leydig cells during the neonatal period, referred as the neonatal Leydig cell. The number of neonatal Leydig cells again increases and reaches a peak at 2–3 months after birth, leading to a peak in serum testosterone concentrations. This type of Leydig cells are typical, containing abundant smooth endoplasmic reticulum, mitochondria and lipid-droplets [24, 25]. Although the exact origin of neonatal Leydig cells is still unclear, it is believed that neonatal Leydig cells differentiate from stem Leydig cells under the brief surge of pituitary activities. Then, neonatal Leydig cell number rapidly

regresses by the end of the first year of age [26]. Since then to the first decade, The Leydig cells in human testis are in quiescence with absence of well-developed Leydig cells and the interstitial area of the postnatal human testis contains stem Leydig cells or progenitor Leydig cells, which are spindle-shaped. These cells are believed to be source of adult Leydig cells because they are able to increase steroidogenic activity under the stimulation of human chorionic gonadotrophin, which also binds to the surface of luteinizing hormone/chorionic gonadotrophin receptor (LHCGR) [27–29].

The Adult Leydig Cell

Adult Leydig cells are differentiated from stem Leydig cells during the second week of age in mice and rats after they commit into spindle-shaped progenitor Leydig cells. Progenitor Leydig cells have a few smooth endoplasmic reticulum and mitochondria but have some lipid-droplets and they are abundant around postnatal Day 21 in rodents [2, 30, 31]. They express some androgen synthetic enzymes such as cytochrome P450 cholesterol side chain cleavage enzyme (CYP11A1), 3β -hydroxysteroid dehydrogenase/ $\Delta 5\text{-}4$ isomerase (HSD3B), and cytochrome P450 17α -hydroxylase/ $17,20$ -lyase (CYP17A1) but lack last-step testosterone synthetic enzyme 17β -hydroxysteroid dehydrogenase isoform 3 (HSD17B3) [32, 33]. Thus, progenitor Leydig cells are capable of only making androstenedione, the precursor of testosterone [32]. Progenitor Leydig cells also contain a fair amount of androgen metabolic enzymes, steroid 5α -reductase 1 (SRD5A1) and 3α -hydroxysteroid dehydrogenase (AKR1C14) [32, 33]. Therefore, androstenedione formed is metabolized into androstanedione by SRD5A1 and further into androsterone by AKR1C14 [32]. Progenitor Leydig cells are almost unresponsive to luteinizing hormone stimulation because they almost have truncated LHCGR [30, 32]. Around postnatal Day 28–35, progenitor Leydig cells differentiate into ovoid immature Leydig cells [2]. Immature Leydig cells have increased amount of smooth endoplasmic reticulum and mitochondria and numerous lipid-droplets [31]. However, the smooth endoplasmic reticulum in immature Leydig cells are still under developmental stage [31]. Immature Leydig cells have all four androgen synthetic enzymes (CYP11A1, HSD3B, CYP17A1, and HSD17B3), thus they can make testosterone [32, 34]. However, immature Leydig cells still contain high levels of SRD5A1 and AKR1C14, thus SRD5A1 converting testosterone into dihydrotestosterone, which is further converted into 5α -androstane- $3\alpha,17\beta$ -diol, as the major secreted androgen [32, 34]. Around postnatal Day 49 and after, immature Leydig cells mature into adult Leydig cells, which are large and void and they have well developed smooth endoplasmic reticulum and many mitochondria and have almost no lipid droplets [30, 31]. Adult Leydig cells have all four androgen synthetic enzymes (CYP11A1, HSD3B, CYP17A1, and HSD17B3) and are able to make testosterone. However, the SRD5A1 is silenced in adult Leydig cells, thus testosterone cannot be metabolized further [32]. Interestingly, rat adult Leydig cells contain a small amount of

cytochrome P450 2A1, thus they can metabolize testosterone into 7α -hydroxytestosterone [35] and however, testosterone is still the major end androgen in adult Leydig cells [32]. In rat testis, stem and progenitor stem Leydig cells have high proliferative capacity and they have higher expression of cyclin A2 [36, 37]. Although immature Leydig cells have decreased proliferative capacity and decreased expression of cyclin A2 [36, 37], they still can divide once and make a maximum of about 23×10^6 cells per testis after postnatal Day 56 [38].

Adult Leydig cells in humans are developed from stem Leydig cells from about 10 years of age and development is complete by 13 years of age [39]. Adult Leydig cells increases and reaches a maximum of about 5×10^8 cells per testis in the early 20s [24] and they mainly secrete testosterone with the plasma levels of average 6 ng/mL during adulthood. The primary function of adult Leydig cells is the synthesis of androgen, which promotes the development of the second sexual characteristics of males, stimulates spermatogenesis and maintains protein synthesis at adulthood.

Steroidogenesis in Leydig Cells

The major function of adult Leydig cells is to secrete testosterone. The steps of testosterone synthesis include the enzymatic activities of four enzymes: CYP11A1, HSD3B, CYP17A1, and HSD17B3 [32]. In some precursor cell types such as progenitor and immature Leydig cells, SRD5A1 and AKR1C14 are expressed [32].

The Sources of Cholesterol in Leydig Cells

Cholesterol is the starting material for making testosterone in rat, mouse and human Leydig cells. In rats, mice and humans, cholesterol is absorbed primarily via lipoprotein in the circulation via high-density lipoprotein, which binds to the membrane receptor, scavenger receptor class B member 1, for uptake [40–42]. Cholesterol can also be taken in via lipoprotein in the circulation, after binding to the low-density lipoprotein receptor for uptake [43]. Cholesterol can be also de novo synthesized from acetyl CoA in the smooth endoplasmic reticulum via a series of enzymatic reactions: (1) acetyl CoA units are linked to form 3-hydroxy-3-methylglutaryl coenzyme A; (2) 3-hydroxy-3-methylglutaryl coenzyme A is catalyzed into mevalonate; (3) mevalonate is converted to isopentenyl pyrophosphate; (4) isopentenyl pyrophosphate is lined to 30-carbon squalene; and (5) squalene cyclizes to lanosterol and further metabolized to form cholesterol [44]. Cholesterol is also capable of being obtained from the liberation of esters in lipid droplets by cholesterol esterase [45].

Cholesterol Transportation Within Leydig Cells

The first enzyme to use cholesterol is CYP11A1, which is located in the inner membrane of the mitochondrion. Cholesterol cannot pass through the aqueous mitochondrial lumen to reach the CYP11A1 in the inner membrane of mitochondrion. It is believed that cholesterol is transported by some carrier proteins. One of the most important carrier proteins is steroidogenic acute regulatory protein [46, 47], which transports cholesterol together with peripheral benzodiazepine receptor [48]. However, the role of peripheral benzodiazepine receptor in steroidogenesis is still controversial. CRISPR/Cas9-mediated deletion of peripheral benzodiazepine receptor in mouse MA-10 Leydig cells does not alter steroidogenesis [49] and but alters mitochondrial fatty acid oxidation without altering mitochondrial membrane potential [50]. Another study shows that the peripheral benzodiazepine receptor disruption causes reduction of both steroidogenesis and mitochondrial membrane potential [51]. However, global deletion of peripheral benzodiazepine receptor in mice does affect Leydig cell steroidogenesis [52–54].

Androgen Synthetic Pathways

In Leydig cells from rats, mice, and humans, all steroids need CYP11A1 for the first catalysis from substrate cholesterol to generate pregnenolone. After that, there is a clear species difference in the steroidogenic pathways between rodents and humans. In rodents, the Δ^4 pathway (pregnenolone \rightarrow progesterone \rightarrow 17α -hydroxyprogesterone \rightarrow androstenedione \rightarrow testosterone) is the preferable pathway (Fig. 2). In the Δ^4 pathway, pregnenolone is preferably bounded by HSD3B, catalyzing the formation of progesterone. In human Leydig cells, the Δ^5 pathway (pregnenolone \rightarrow 17α -hydroxypregnenolone \rightarrow dehydroepiandrosterone \rightarrow androstenedione \rightarrow testosterone) is the preferable pathway (Fig. 2).

CYP11A1 Catalysis

Cholesterol is the substrate of CYP11A1, which converts it into pregnenolone. CYP11A1 is present in the inner membrane of mitochondrion [55]. A single gene (*Cyp11a1* in rodents and *CYP11A1* in humans) encodes CYP11A1 [56–58]. The reaction of CYP11A1 requires a mitochondrial electron transfer system, which consists of adrenodoxin and adrenodoxin reductase [55]. CYP11A1 catalyzes three sequential oxidative reactions of cholesterol, and each oxidative reaction needs one molecule of oxygen and one molecule of nicotinamide adenine dinucleotide phosphate (NADPH) [55, 59]. The first oxidative reaction happens at C22, then the second oxidative reaction happens at C20 to produce [20, 22] R-hydroxycholesterol,

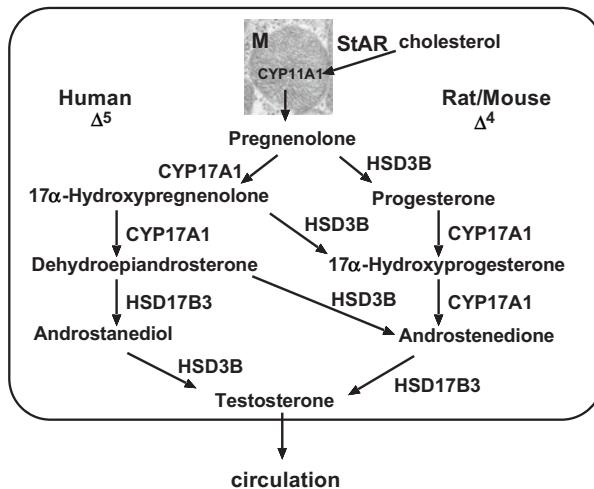


Fig. 2 The Δ^4 and Δ^5 steroidogenic pathways in rodent and human testis. Rat or mouse Leydig cell takes Δ^4 steroidogenic pathway while human Leydig cell takes Δ^5 steroidogenic one. *M* mitochondrion, *StAR* steroidogenic acute regulatory protein, *CYP11A1* cytochrome P450 cholesterol side chain cleavage enzyme, *CYP17A1* cytochrome 17 α -hydroxylase/17,20-lyase, *HSD3B* 3 β -hydroxysteroid dehydrogenase, *HSD17B3* 17 β -hydroxysteroid dehydrogenase isoform 3

which is unstable and is cleaved between C20 and C22 to produce pregnenolone and isocaproaldehyde [60, 61].

HSD3B Catalysis

Pregnenolone is believed to diffuse from mitochondria into smooth endoplasmic reticulum, where HSD3B is located. In rats, two genes (*Hsd3b1* and *Hsd3b2*) encode respective HSD3B isoforms [62]. In rat Leydig cells, type I HSD3B is predominant isoform [62]. In mice, the corresponding counterpart is *Hsd3b6* gene, which encodes HSD3B6 [63]. Two human *HSD3B* genes with 81.9% identity were cloned: *HSD3B1* mainly exists in placenta and *HSD3B2* predominantly occurs in human Leydig cells [64]. HSD3B has two steps of catalysis: dehydrogenation and isomerization of a double bond in the steroid molecule and it requires nicotinamide adenine dinucleotide (NAD^+) as the coenzyme [62]. Rodent and human HSD3B take different pathway for catalysis. Rodent HSD3B uses pregnenolone as the substrate to dehydrogenize it at 3 β -hydroxyl group of this steroid. Pregnenolone has a double bond between carbons 5 and 6 and the isomerase activity of HSD3B converts the double bond between carbons 4 and 5 in progesterone (Fig. 2). In human Leydig cells, HSD3B catalyzes the CYP17A1 products, 17 α -hydroxypregnenolone and dehydroepiandrosterone, into 17 α -hydroxyprogesterone and androstenedione (Fig. 2), respectively [65].

***CYP17A1* Catalysis**

A single gene (*Cyp17a1* in rodents and *CYP17A1* in humans) encodes CYP17A1 [66–68]. This enzyme has two activities: 17 α -hydroxylase and C17,20-lyase activities. CYP17A1 is located in the smooth endoplasmic reticulum. CYP17A1 catalysis depends on the Δ^4 (rodent) or Δ^5 (human) pathway. In the Δ^4 pathway, CYP17A1 catalyzes progesterone to 17 α -hydroxyprogesterone by 17 α -hydroxylase activity and the later further into androstenedione by C17,20-lyase (Fig. 2) [69]. Each reaction requires coenzyme, NADPH [55], which transfers electrons via cytochrome P450 oxidoreductase [55]. In the Δ^5 pathway, CYP17A1 catalyzes pregnenolone into 17 α -hydroxyprogesterone and the later further into dehydroepiandrosterone (Fig. 2). CYP17A1 takes either Δ^4 (rodent) or Δ^5 (human) pathway, depending on the species and tissue location. Human CYP17A1 has a higher affinity for 17 α -hydroxypregnenolone and has almost no C17,20-lyase activity with 17 α -hydroxyprogesterone [68]. However, rodent CYP17A1 can catalyze both 17 α -hydroxypregnenolone and 17 α -hydroxyprogesterone [68].

***HSD17B3* Catalysis**

Although there many 17 β -hydroxysteroid dehydrogenase isoforms [70], the rat [32] and mouse *Hsd17b3* [71] and human *HSD17B3* [72] encode HSD17B3, which is the isoform in Leydig cells for the last-step of testosterone synthesis. HSD17B3 is located in the smooth endoplasmic reticulum. HSD17B3 catalyzes androstenedione into testosterone. HSD17B3 catalysis requires NADPH as its coenzyme. The production of testosterone is considered an end-product in adult Leydig cells.

***SRD5A1* Catalysis**

In the rodent precursor cells, mainly progenitor and immature Leydig cells, SRD5A1 is highly expressed [32, 34, 73]. Rat [74] and mouse [75] *Srd5a1* as well as human SRD5A1 [76] encode SRD5A1. SRD5A1 is located in the smooth endoplasmic reticulum of Leydig cells [77]. SRD5A1 catalyzes the conversion of testosterone into the more potent androgen, dihydrotestosterone under the assistance of coenzyme, NADPH. SRD5A1 in progenitor and immature Leydig cells also catalyzes androstenedione into androstanedione [32].

AKR1C14 Catalysis

In the rodent Leydig cells, AKR1C14 is expressed and down-regulated with the development of rat Leydig cells during puberty [31, 32, 78]. Mouse Leydig cells also express AKR1C14 [34]. Rat [79] and mouse [80] *Akr1c14* encode AKR1C14. AKR1C4 is located in the cytoplasmic part of Leydig cells [81]. AKR1C14 catalyzes the conversion of dihydrotestosterone into weak androgen 5 α -androstane-3 α , 17 β -diol [32]. It also catalyzes the conversion of androstanedione into weak androgen, androsterone [32].

Testosterone Secretion

When testosterone is synthesized after HSD17B3 catalysis in the smooth endoplasmic reticulum, it passively diffuses out of Leydig cells via concentration gradient. In the interstitial fluid after diffusion, testosterone is bound to androgen binding protein, a protein secreted by Sertoli cells [82]. Bound androgen binding protein-testosterone is transported into the rodent seminiferous tubules and epididymis [82]. When entering the circulation, testosterone in the blood is bound to some plasma proteins. In humans, two types of plasma proteins bind to testosterone: sex hormone binding globulin and albumin. Sex hormone binding globulin is secreted by human liver, and is a high-affinity testosterone binding protein with a KD of 1 nM and albumin is a low-affinity testosterone binding protein with a KD of 1000 nM. The biological activity of testosterone is free testosterone levels in the serum, which depends on sex hormone binding globulin and albumin levels.

Regulation of Leydig Cell Development and Function

Adult Leydig cells were differentiated from stem Leydig cells. Stem Leydig cells have been identified in rats [36], mice [83], and humans [84]. In the rat model, many growth factors such as platelet-derived growth factor-AA [36, 85], platelet-derived growth factor-BB [85], leukemia inhibitory factor [36], epidermal growth factor [36], fibroblast growth factor 1 [86], fibroblast growth factor 2 [85, 87], fibroblast growth factor 16 [88], nerve growth factor [89], insulin-like growth factor 1 [85], desert hedgehog [85], activin A [85, 90], and kit ligand [36, 85, 91] stimulate the proliferation of stem Leydig cells, while other factors, including platelet-derived growth factor -AA [92], nerve growth factor [89], desert hedgehog [85], insulin-like growth factor 1 [93], androgen [85], fibroblast growth factor 12 [94], and parathyroid hormone-related protein [95] stimulate the differentiation of these cells (Fig. 3). In mouse stem Leydig cells, platelet-derived growth factor-AA and platelet-derived growth factor-BB, and desert hedgehog seem also to regulate its

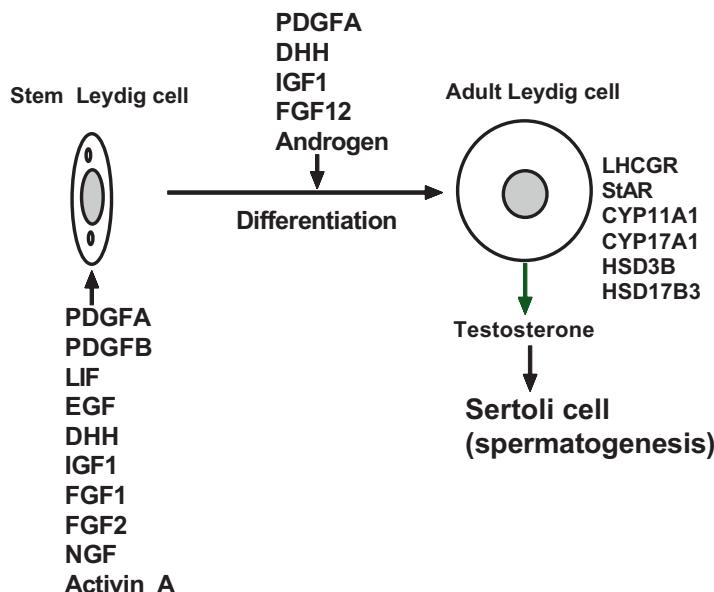


Fig. 3 Illustration of hormones and growth factors to regulate Leydig cell development. PDGFA, PDGFB, LIF, EGF, DHH, IGF1, FGF1, FGF2, NGF, and activin A stimulate stem Leydig cell proliferation. PDGFA, DHH, IGF1, FGF12, androgen stimulate stem Leydig cell differentiation in the Leydig cell lineage. *PDGFA* platelet-derived growth factor-A, *PDGFB* platelet-derived growth factor B, *LIF* leukemia inhibitory factor, *EGF* epidermal growth factor, *DHH* desert hedgehog, *IGF1* insulin growth factor-like 1, *FGF1* fibroblast growth factor 1, *FGF2* fibroblast growth factor 2, *NGF* nerve growth factor

development, as the knockout of platelet-derived growth factor A [96], platelet-derived growth factor receptor α [97] and platelet-derived growth factor β [97] and desert hedgehog [98] causes involution and absence of adult Leydig cells (Fig. 3).

When stem Leydig cells enter the Leydig cell lineage, luteinizing hormone and other growth factors seem to positively regulate its differentiation in rat and mouse models. LHCGR is expressed in progenitor, immature and adult Leydig cells [30]. Luteinizing hormone binds to the LHCGR in progenitor and immature Leydig cells, inducing their proliferation [37]. The action of luteinizing hormone to induce proliferation of these precursor cells of Leydig cells, possibly via interacting epidermal growth factor receptor/ERK1/2 pathways [99, 100].

In adult Leydig cells, luteinizing hormone is the major regulator of steroidogenesis. There are acute and chronic effects for luteinizing hormone. The acute effects take place within minutes [47]. This process acts through bound luteinizing hormone-LHCGR, triggering intracytoplasmic adenylate cyclase to increase adenosine 3'5'-cAMP (cAMP) to mobilize steroidogenic acute regulatory protein for cholesterol transportation [47]. Besides cAMP signaling, other signaling pathways including release of calcium, efflux of chloride ions, and production of arachidonic acid [101]. Luteinizing hormone also has chronic trophic actions on immature and

adult Leydig cells, up-regulating the expression of many steroidogenesis-related genes, including *Lhcgr*, *Scarb1*, *Cyp11a1*, and *Cyp17a1* [32, 102]. The chronic action of luteinizing hormone possibly exerts via cAMP/PKA/cAMP responsive element binding protein [2].

Onset and Maintenance of Spermatogenesis by Testosterone

Spermatogenesis takes place in the seminiferous tubules to eventually release of spermatozoa in the testis. The detailed process of spermatogenesis is reviewed in the other chapters. The effects of Leydig cell on spermatogenesis mostly act via secreting hormones, mainly androgen. The importance of androgen for the regulation of spermatogenesis is proven by pharmacological treatment of androgens and the conditional knockout androgen receptor.

Pharmacological Treatment of Androgens

Spermatogenesis depends on action of androgens. Luteinizing-immunization to deplete luteinizing action in Leydig cells induces the reduction of testis weight due to blocked spermatogenesis, indicating the importance of androgens for spermatogenesis [103]. Hypophysectomized rats develop testicular involution due to disrupted spermatogenesis and the androgen administration before hypophysectomy is capable of preventing these effects [104]. Using a drug ethane dimethane sulfonate to delete Leydig cells in adult rats and administration of high doses of androgen, Sharpe et al. showed that Leydig cell factors other than testosterone are not essential for maintenance of spermatogenesis in rats [105]. Testosterone is able to maintain the spermatogenesis in intact rats [106, 107], in estradiol-inhibited rats [108], and in gonadotropin-releasing hormone vaccine rats [109].

Clinical study in hypogonadotropic hypogonadal patients demonstrates that testosterone can partially maintain spermatogenesis and even fertility in some cases [110]. Testosterone and hCG have been demonstrated to initiate spermatogenesis in hypogonadotropic hypogonadal patients although the sperm production was much lower in many patients [111–113].

Androgen Action on Sertoli Cells

Germ cells themselves do not express androgen receptor [114, 115]. Indeed, germ cell conditional androgen receptor knockout mice have normal spermatogenesis [116]. Therefore, androgen action is most likely via indirect somatic cell-mediated mechanism. These somatic cells include Sertoli cells, myoid cells, and Leydig cells.

Androgen receptor is expressed in Sertoli cells [117, 118], myoid cells [114, 119, 120], and Leydig cells [30, 121]. The effects of androgen on spermatogenesis via androgen receptor have been demonstrated in Sertoli cell conditional androgen receptor knockout mice. Using Sertoli cell specific anti-Müllerian hormone promoter (only expressed in Sertoli cells) to drive Cre recombinase to create two Sertoli cell androgen receptor conditional knockout models: androgen receptor exon 2 deletion in Sertoli cell (SCARKO) [122] and S-AR^{-Y} mice [123]. Both knockout models have a normal phenotype of external male reproductive tract phenotype but blocked spermatogenesis at the level of meiosis [122, 123]. The defects of spermatogenesis in SCARKO and S-AR^{-Y} mice are as severe as those in androgen depletion in wild-type mice, indicating that androgen acts mostly via genomic androgen-dependent pathway. This is further confirmed by the severe spermatogenesis arrest in Sertoli cell conditional knockout of androgen receptor deleting exon 3, which encodes the DNA-binding domain [124], like SCARKO and S-AR^{-Y}. This indicates that non-genomic action of androgen receptor plays a minor role in the regulation of spermatogenesis by Sertoli cells. A mouse model with decreased androgen receptor (AR^{flox(ex1-neo)Y}) shows germ cells can complete meiosis but fails to complete spermiogenesis [125]. This finding supports the contention that androgen is also required for spermatogenesis beyond meiosis.

Conclusion

Leydig cells are critical cell types in the testis and they differentiate from stem Leydig cells. They control sperm cell meiosis and spermiogenesis beyond meiosis via secreting androgen, which acts on androgen receptor in Sertoli cells in the regulation of spermatogenesis.

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Motor Proteins and Spermatogenesis



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Introduction

The blood-testis barrier (BTB) in the adult mammalian testis is a unique blood-tissue barrier which restricts paracellular (between cells; i.e., gate-keeper function of the BTB) and transcellular (across cells; i.e., fence function of the BTB) transport (or diffusion) of water, electrolytes, nutrients, cytokines and biomolecules including paracrine and autocrine factors between adjacent Sertoli cells at the base of the seminiferous tubules, also known as the Sertoli cell barrier [1–6] (Fig. 1). Interestingly,

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microvessels found in the interstitial space between seminiferous tubules contribute relatively little barrier function to the BTB in the testis of rodents, primates and humans (Fig. 1) [5, 9]. The BTB also divides the seminiferous epithelium into the basal and the adluminal (apical) compartments as noted in Fig. 1. As such, meiosis I/II and all the cellular events pertinent to post-meiotic development take place behind the BTB in a specialized microenvironment (Fig. 1), whereas mitotic proliferation of spermatogonia and differentiation/transformation of type A and type B spermatogonia to earlier spermatocytes take place in the basal compartment [10–12]. The BTB is a highly dynamic blood-tissue barrier since preleptotene spermatocytes, once derived from type B spermatogonia in the basal compartment rodents, are to be transported across the BTB in late Stage VII through early Stage IX of the epithelial cycle while differentiating into leptotene spermatocytes, which can be transformed into zygotene and pachytene spermatocytes to prepare for meiosis. Studies have shown that the BTB in the rodent testis is constituted by the actin-based tight junction (TJ) between adjacent Sertoli cells, reinforced by a testis-specific actin-rich adherens junction (AJ) type called basal ectoplasmic specialization (ES), and supported by the actin-based gap junction, but also intermediate filament-based desmosome [13–19]. Once haploid spermatids are formed through meiosis, they are also being transported across the seminiferous epithelium in the adluminal compartment before fully developed step 19, 16, and 12 spermatids in the testis of rats, mice, and humans, respectively, are transformed to spermatozoa via spermogenesis [12, 14, 20] as these cells are lacking the ultrastructures found in motile

Fig. 1 (continued) BTB (for basal ES) but also spermatid transport across the epithelium (for apical ES). These germ cells, namely spermatocytes and developing spermatids are the cargoes which are to be transported “directionally”, either to be base or to the adluminal edge of the seminiferous epithelium, due to the polarized nature of the actin- and MT-based cytoskeletons through the MT- or actin-dependent motor proteins. For instance, dynein 1 moves cargoes to the minus (−) end of MTs, and kinesin 15 to the plus (+) end of MTs; whereas myosin VIIa moves cargoes to the plus (+) end of actin filaments and myosin VI to the minus (−) end of actin filaments. In brief, the actin- and MT-based tracks found in Sertoli cells work in concert to support the directional transport of germ cells across the seminiferous epithelium using the corresponding actin- and MT-based motor proteins. Even though germ cells located outside the Sertoli cell actin- and MT-cytoskeletons, the ES provides the means by which these germ cells anchor tightly onto the Sertoli cell cytoskeleton-based tracks to facilitate their transport across the epithelium. Through these actions of corresponding motor proteins, proper germ cell and cargo transports can take place across the seminiferous epithelium during the epithelial cycle to support spermatogenesis

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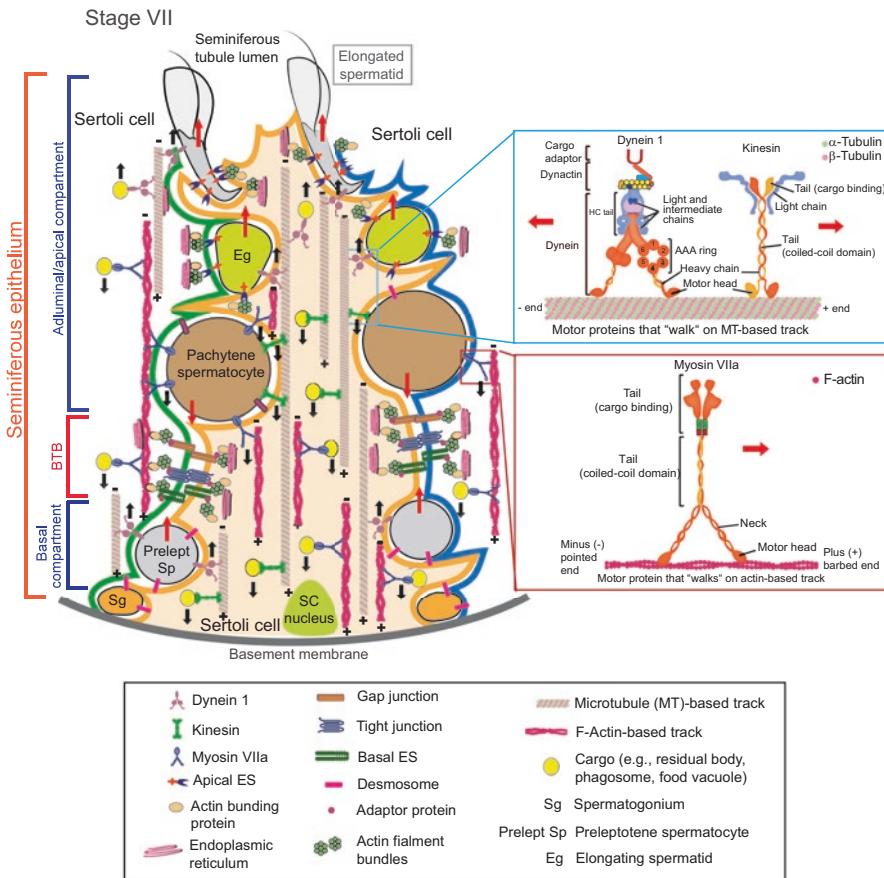


Fig. 1 Schematic drawing of the cross-section of a typical Stage VII seminiferous tubule from adult rat testes. The seminiferous epithelium across the tubule is constituted by adjacent Sertoli cells which, in turn, support germ cells at different stages of their development during spermatogenesis as noted herein with a Sertoli:germ ratio of about 1:30–50 [7, 8] (left panel). Between adjacent Sertoli cells near the basement membrane are the specialized junctions, namely the actin-based tight junction (TJ), basal ES (ectoplasmic specialization, a testis-specific adherens junction type) and gap junction, which together with the intermediate filament-based desmosome constitute the blood-testis barrier (BTB). The BTB also divides the seminiferous epithelium in the rat testis and other mammalian testes into the basal and the adluminal (apical) compartments, such that meiosis I/II and all subsequent events of post-meiotic spermatid development take place behind the BTB. The most notable structural features are the microtubule (MT)-based and actin-based tracks that stretch across the seminiferous epithelium. These tracks support the corresponding MT- and actin-based motor proteins (see the insets on the right panel) to provide cellular transport of cargoes as discussed in this review. For F-actin, besides serving as the track-like structures to support cellular transport, actin filaments that lay parallel to the Sertoli cell plasma membrane are assembled as bundles which appeared as aggregates of rod-like structures in cross-sections of the testis, both at the Sertoli-spermatid site called apical ES and also at the Sertoli cell-cell interface at the BTB called basal ES. The ES is not only an important ultrastructure to support spermatid and Sertoli cell adhesion, they are crucial to support preleptotene spermatocyte transport across the

cells, namely the lamellipodia and filopodia [21]. Spermatozoa are then line-up at the edge of the seminiferous tubule lumen to undergo spermiation in Stage VIII of the epithelial cycle in rodents versus VI in humans, respectively, which is composed of a tightly regulated series of biochemical and cellular events involving multiple signal and regulatory proteins [13, 22–24]. The testicular sperm emptied into the epididymis are then undergo another series of maturation processes, rendering them capable of fertilizing the egg.

Studies have shown that BTB dynamics that support preleptotene spermatocyte transport across the immunological barrier, and the subsequent haploid spermatid transport across the seminiferous epithelium, are tightly regulated cellular events. These involve several biologically active peptides released at the basement membrane but also at the Sertoli-spermatid adhesion site known as the apical ES via proteolytic cleavage of the structural proteins at these two sites, namely the F5-, the NC1- and the LG3/4/5-peptide [9, 25–27]. These bioactive peptides, in turn, are working in concert with a number of signaling proteins such as mTORC1/rpS6/Akt1/2 and FAK-Y407, and cytoskeletal regulatory proteins such as Arp3, Eps8, +TIPs and –TIPs to modulate BTB and ES dynamics [9, 27, 28]. The ultrastructures and the biomolecules that support germ cell transport are the actin- and MT-based cytoskeletons, as well as the corresponding actin- and MT-based motor proteins. In brief, motor proteins are the “vehicles” that carry the “cargoes”, namely preleptotene spermatocytes and spermatids, utilizing the corresponding actin or microtubule (MT)-based cytoskeletons as tracks to transport developing germ cells and other organelles (e.g., residual bodies, phagosomes, cell vacuoles, endocytic vesicles) to their corresponding “destination” across the seminiferous epithelium (Fig. 1). Furthermore, this requires intricate involvement of both actin- and MT-based cytoskeletons to support cargo transport across the seminiferous epithelium. However, much of this information remains unknown. In this review, we provide a timely discussion on latest findings in this area of research regarding the role of motor proteins in supporting cargo transport across the seminiferous epithelium using the rat testis as a study model. We also highlight some of the specific research areas that deserve attentions in future studies, which should be helpful to understand the underlying mechanism(s) of idiopathic male infertility.

Sertoli Cell Cytoskeletons in the Testis

In the seminiferous epithelium of adult rodent testes, similar to other mammalian organs, the two prominent cytoskeletons are the intrinsically polarized actin- and microtubule (MT)-based cytoskeletons which are composed of globular subunits of actin and α -tubulin/ β -tubulin oligomers, respectively (Fig. 1) [29–33]. These polarized structures also serve as tracks to support specific motor proteins for directional transport of cargoes across the seminiferous epithelium. On the other hand, the intermediate filament-based cytoskeleton constituted by vimentin [16, 34] and the septin-based cytoskeleton [35] are both apolar structures, thus, they do not support motor proteins for directional cargo transport along their filaments.

Actin-Based Cytoskeleton

A functional actin-based track is composed of linear actin filaments (i.e., filamentous actin, F-actin) derived from polymerized globulin (G)-actin subunits, with the fast-growing barbed (+) end near the base of the seminiferous epithelium, closest to the basement membrane, and the slow-growing pointed (−) end near the seminiferous tubule lumen (Fig. 1) [36, 37]. In brief, polymerization of a linear actin filament occurs by incoming ATP-bound G-actin subunits at the fast-growing barbed (+) end involving actin nucleation proteins (e.g., formin 1, spire 1). The ATP-bound G-actin subunits are rapidly dephosphorylated to ADP-bound G-actin and are all found at the slow-growing pointed (−) end near the tubule lumen (Fig. 1) [37, 38]. The actin-based tracks are most notable in late Stage VIII of the epithelial cycle that stretch across the seminiferous epithelium and align perpendicular to the basement membrane [38] (Fig. 1). However, F-actin are also prominently noted at the apical ES and basal ES/BTB wherein the actin filaments are aligned parallel to the Sertoli cell plasma membrane and appear as bundled structures in cross-sections of the tubules. As such, these actin filaments appear as “rod-like” structures in cross-sections of the tubules at the apical ES and basal ES/BTB sites, thereby reinforcing cell adhesion (Fig. 1). ES in the testis also plays a crucial role to support germ cell transport as preleptotene spermatocytes (at the basal ES) and developing spermatids (at the apical ES) tightly anchored onto the actin filament bundles at the ES, and with the MTs located nearby [18, 33], which are located in close proximity to the plasma membrane of the Sertoli cell. Thus, these cells are separated only by their apposing Sertoli cell-cell or Sertoli-germ cell plasma membranes [3, 39]. Thus, even though these germ cells, namely preleptotene spermatocytes or haploid elongate spermatids, are located “outside” the Sertoli actin filament and MT networks, they are anchor onto these cytoskeletons through the unusual adhesion of ES between these adjacent cells, which are considered as cargoes to the Sertoli cell at the site. Due to this intrinsic polarized nature of the actin filaments, the actin-based plus (+) end-directed motor protein myosin VIIa, and the actin-based minus (−) end-directed myosin VI are capable of moving cargoes either to the base or to the tubule lumen across the epithelium, respectively (Fig. 1).

MT-Based Cytoskeleton

Microtubules (MTs) are also polarized ultrastructures in which a microtubule is composed of 13 laterally associated protofilaments of α - and β -tubulin heterodimers, with a hollow lumen wherein the plus (+) fast growing end is near the basement membrane and the minus (−) slow growing end near the tubule lumen (Fig. 1) [40–43]. Due to the intrinsic polarized nature of MTs, the MT-based minus (−) end-directed motor protein dynein 1 and the plus (+) end-directed motor protein kinesins (e.g., kinesin 15) can move cargoes to the corresponding minus or plus end of MTs, respectively [44–47].

Motor Proteins

Motor proteins are a class of molecular motors that bind to either microtubule (MT)- or actin-based tracks. They are capable of converting chemical energy through the hydrolysis of ATP to generate the mechanical force necessary to transport cargoes along the track across cell cytoplasm. Herein, we discuss several motor proteins that have been studied in the testis pertinent to support spermatogenesis. Besides serving as an update, this summary also provides the basis for future studies regarding the role of motor proteins in supporting germ cell and cargo transport across the seminiferous epithelium.

MT-Based Motor Proteins: Dynein and Kinesin

Dynein

Dynein is a family of motor proteins that use MT-based track in retrograde sliding movement towards the minus (-) ends of microtubules [47, 48]. In brief, a dynein motor protein transports cargoes towards the center of the cell or seminiferous tubule lumen in the testis. There are two major classes of dyneins, cytoplasmic and axonemal dyneins, which are classified according to their function and structure differences. Dynein 1 is a cytoplasmic dynein of about 1.5 megadaltons (MDa) (Fig. 2; Fig. 3A), involved in intracellular transport, mitosis, cell polarization and directional cargo transport. For instance, dynein 1 carries the cargo (e.g., spermatid) by “walking” along the MT-track in the Sertoli cell. Even though spermatids locate outside the Sertoli cell, but they are tightly anchored onto the MT-track in the Sertoli cell at the apical ES (or preleptotene spermatocyte anchored onto the MT-track in the Sertoli cell at the basal ES), which is a known adhesion ultrastructure that supports spermatid or preleptotene spermatocyte transport [3, 17]. There are 15 types of axonemal dyneins to support ciliary (e.g., dynein 2) and flagellar movement [48–51] such as sperm flagella that confers sperm progressive motility. Axonemal dyneins support the beating of flagella and cilia through rapid and efficient sliding movements of MTs [52]. In this context, it is of interest to note that mechanical movement of hair cells in cochlea is supported by the motor protein prestin [53, 54] which is different from the dynein family motor proteins. A functional dynein motor protein is considerably larger and more complex than kinesin or myosin motors, and it is composed of two heavy chains and a variable number of associated intermediate chains, light intermediate chains and light chains (Fig. 3A). For instance, dynein 1 is a dimeric protein composed of two identical heavy chains with a large molecular mass (Mr) of 500 kDa each. Each HC, in turn, binds to a light intermediate chain (LIC), an intermediate chain (IC), and three light chains (LCs) of LC7, LC8, and Tctex 1 (Fig. 3A). Thus, dynein 1 is a dimer of dimers. Each heavy chain is composed of three functional domains: a coiled-coil stalk with MT binding domain (MTBD) containing a globular motor head at the C-terminus, an AAA+ ring containing six AAA+ modules that organized into a doughnut-like structure, and a

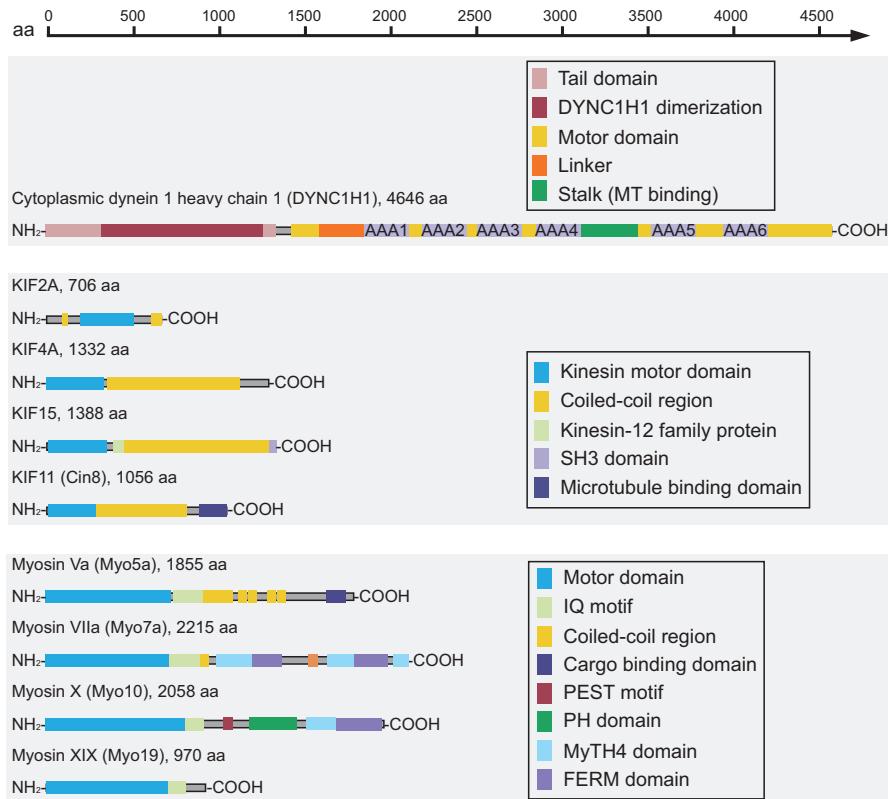
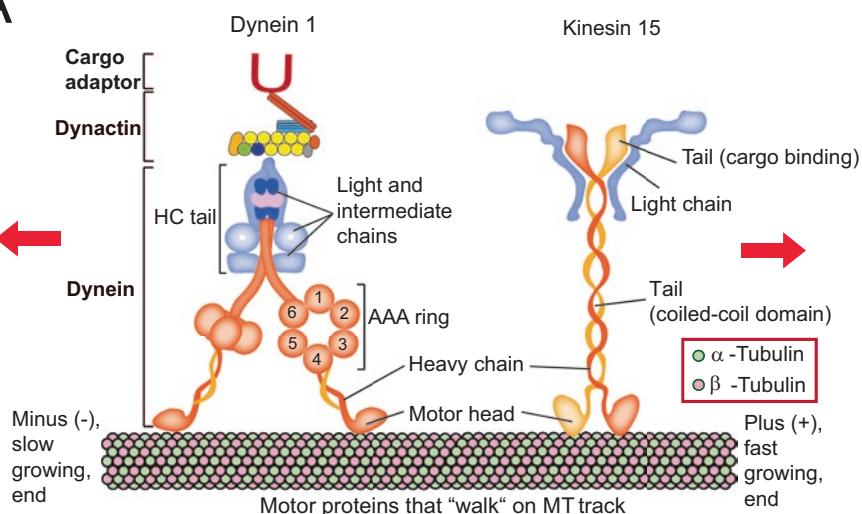


Fig. 2 Schematic illustration on the functional domains of the microtubule-based motor proteins dyneins and kinesins, and actin-based motor proteins myosins. The different functional domains of motor proteins dyneins, kinesins and myosins are noted in corresponding panels. This figure was prepared based on earlier reports [55–58]. Abbreviations: DYNC1H1, dynein cytoplasmic 1 heavy chain 1; KIF, kinesin; MT, microtubule; SH3, SRC homology 3 domain; IQ motif, isoleucine and glutamine motif is a basic unit containing about 23 amino acids; PEST motif, a motif rich in proline (P), glutamic acid (E), serine (S) and threonine (T); PH domain, pleckstrin homology domain; MyTH4 domain, Myosin Tail Homology 4 domain; FERM domain, F for 4.1 protein, E for ezrin, R for radixin and M for moesin

cargo-binding tail at by N-terminus (Figure 3A). The AAA+ ring can hydrolyze ATP hence converting chemical energy into mechanical force to support cargo transport [59]. In the testis, dynein 1 interacts with a protein complex called dynactin and cargo adaptor to form a functional motor protein called the dynein-dynactin-adaptor complex that supports spermatid transport on MT-based cytoskeleton. Dynein I also transports various cellular cargoes along MT towards the minus (−) end of MT tracks [60]. Cargoes transported by cytoplasmic dynein include endosomes [61], lysosomes [62], phagosomes [63], melanosomes [64], peroxisomes [65], lipid droplets [66], mitochondria [67] and vesicles from the endoplasmic reticulum (ER) destined for the Golgi [68]. These cargo transports hence regulate the intracellular function of cells and tissues through different cell signaling pathways.

A



B

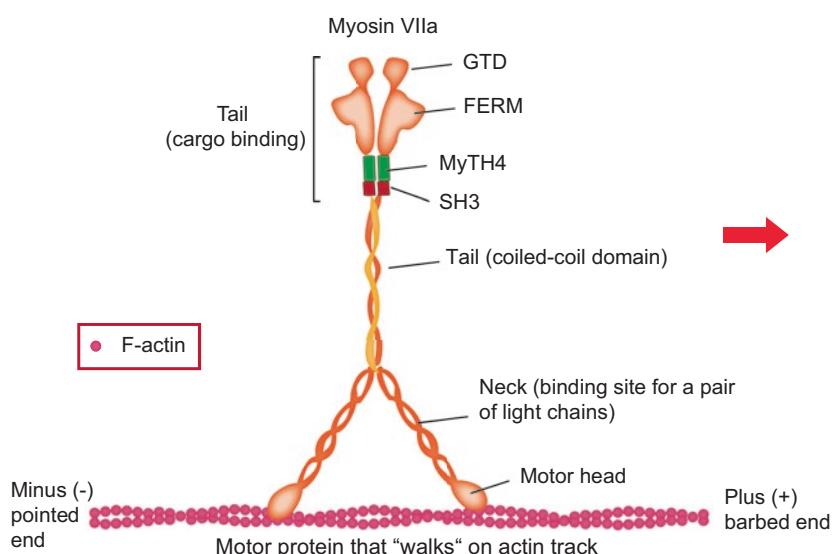


Fig. 3 Schematic illustrations on the structural components of the functional motor proteins dynein 1, kinesin 15 and myosin VIIa. (A) A functional dynein motor protein (e.g., dynein 1) complex is composed of the dynein, dynactin and the cargo adaptor (left panel). The dynein motor protein consists of two monomers. Each monomer is composed of a heavy chain (HC) motor and several other subunits: an intermediate chain, a light intermediate chain, and three light chains called LC7 (light chain 7) LC8 and Tctex. Each HC has the N-terminus at the tail and the C-terminal motor unit contains six AAA (ATPase Associated with Cellular Activities) domains, AAA1 to AAA6, and organized into a ring-like structure, which in turn connects to the microtubule binding domain (MTBD) in the motor head at the C-terminus which also binds onto the microtubule. AAA1

In the rat testis, dynein 1 is necessary to confer Sertoli cell TJ-permeability barrier function since its knockdown by RNAi perturbs the TJ-barrier function due to gross defects of F-actin and microtubules (MTs) across the Sertoli cell cytosol wherein both cytoskeletons become extensively truncated [69]. These defects, in turn, perturb the distribution of BTB-associated proteins at the site, including the cell adhesion complexes CAR/ZO-1 and N-cadherin/β-catenin [69]. Furthermore, dynein 1 knockdown also perturbs the polymerization activities of F-actin and MTs [69], possibly due to defects in transporting machineries (e.g., actin or MT polymerization proteins) necessary to support cytoskeletal nucleation. More important, the loss of dynein 1 function by RNAi also perturbs the BTB function *in vivo* since the barrier no longer restricts the diffusion of small molecular biotin across the immunological barrier [69]. Multiple defective sperms are also noted in the epididymis including extensive defects in spermatid heads, tail, and sperm morphology due to defects of intracellular trafficking to support the assembly of essential cellular components during spermiogenesis [69]. The importance of dynein-based motor proteins is also noted in Table 1 since its KO in mice led to embryonic lethality.

Kinesin

Kinesin is a group of related motor proteins that use MT track in anterograde movement, to transport cargoes towards the plus (+) ends of MTs [96–98] (Fig. 2). In brief, a kinesin motor protein transports cargoes away from the center of the cell, usually to cell peripheries to support cell homeostasis, or to the base of the seminiferous epithelium in the testis (Fig. 1). Kinesin superfamily members in humans and

Fig. 3 (continued) is the major site of ATP hydrolysis with other AAA sites play the regulatory roles. AAA1 converts the chemical energy (from ATP hydrolysis via ATPase) to mechanical force which is transmitted to the HC tail at the N-terminal region. Dynein 1 interacts with its cofactor called dyneactin (which also composed of multiple subunits as earlier reviewed [47]) to form the functional dynein-dyneactin complex. This complex in turn interacts with the cargo adaptor to form a functional motor protein to support cargo transport. On the *right* panel is the kinesin motor protein (e.g., kinesin 15) which is also a dimeric protein, composed of two monomers. Each monomer has a heavy chain (HC) with its N-terminal region contains the motor head which is the site for ATP hydrolysis to generate the chemical energy to be transmitted to the mechanical energy via the tail to propel cargo transport at the C-terminal region. The motor head at the N-terminal region also contains the microtubule binding domain. This is followed by the α-helical coiled-coil domain that constitutes the stalk and ends with the C-terminal tail of cargo binding. **(B)** A functional actin-based plus (+) end directed motor protein myosin (e.g., myosin VIIa) is also a dimeric protein comprised of two monomers. Each monomer has a heavy chain (HC) that begins with the motor head at its N-terminal region which contains the ATP hydrolysis motor domain and the actin-binding domain. This is followed by the neck region that transmits the chemical energy derived from ATP hydrolysis at the motor head to the tail cargo binding site through the coiled-coil domain in the tail. The neck region has a pair of light chains which facilitates the transmission of chemical energy to the cargo propelling mechanical force at the tail cargo binding site. The C-terminal tail region contains the FERM (F, 4.1 protein; E, ezrin; R, radixin; M, moesin), MyTH4 Myosin tail homology 4) and SH3 (SRC homolog 3) domains, and the globular tail domain (GTD) at the C-terminus to support cargo binding

rodents are organized into 14 families [99, 100]. A functional kinesin motor protein is a tetrameric protein, comprised of two heavy chains and two light chains (Fig. 3A). Each heavy chain has a globular motor head where microtubule binding and ATP hydrolysis take place at its N-terminal region, which in turn generate the energy via ATPase that converts chemical energy into mechanical force to elicit cargo transport. The head region is connected by a short neck linker to a long intertwined coiled-coil stalk, to be followed by the tail at its C-terminal region (Fig. 3A). A light chain associates with a tail which serve as the adapter for binding to a cargo while moving along the MT track towards the MT plus (+) end to facilitate cargo (e.g., spermatid, residual body, phagosome) transport [49, 97, 101] (Table 1). Kinesins typically move cargoes in the direction of MT plus (+) end on MT tracks, such that cargo is transported from the center of the cell to its periphery (i.e., anterograde movement). However, some kinesins (members of the kinesin-5 family), such as kinesin-14, move cargoes to the MT minus (-) end along the MT tracks wherein the motor region is located at the C-terminal region of the heavy chain [102]. On the other hand, kinesin-5 Cin8 (members of the kinesin-5 family) is a bidirectional kinesin which can move a cargo towards the microtubule minus (-) end when works alone but to the plus (+) end in an ensemble with a team of motors [103]. Emerging evidence has shown that kinesins are crucial to support tumorigenesis. For instance, KIF18A promotes invasion and metastasis by activating Akt and MMP-7/MMP-9-related signaling pathways [104] whereas kinesins also support proliferation, cell differentiation, aggressiveness and epithelial-mesenchymal transition of tumor cells [105–109]. A recent report has demonstrated the importance of kinesin-9 in conferring progressive motility in mouse spermatozoa since a deletion of 16 bp nucleotides of the *Kif9* gene in mice (*Kif9*^{-/-}) using CRISPR/Cas9 led to defects in flagellar movement of sperm tails [110]. Studies have also shown that kinesin-7 CENP-E is crucial to support chromosome alignment and genome stability of spermatogenic cells (e.g., spermatogonia and spermatocytes) during mitosis and meiosis [111], whereas kinesin-5 Eg5 supports spindle assembly and chromosome alignment of mouse spermatocytes [112]. Nonetheless, much work is needed to better understand the role of kinesins in supporting spermatogenesis in the testis. However, as noted in Table 1, deletion of one of the several kinesins in mice led to embryonic lethality, illustrating the physiological significance of kinesin-based motor proteins in supporting cellular function.

F-actin-Based Motor Proteins: Myosins

Myosins

Myosins are the only known actin-based motor proteins in mammalian cells and tissues including the testis [47, 113]. There are 18 classes of myosin superfamily members known to date based on phylogenetic analysis of their motor domain, and at least 40 myosin genes have been identified [57, 114]. By converting chemical energy via hydrolysis of ATP at the myosin motor head to mechanical energy, which

Table 1 Phenotypes in mice following specific knockout (KO) of different motor protein genes

Gene name	KO type	Phenotype(s)	References
<i>Mdhc7</i> ^{-/-} [Mouse dynein heavy chain-7 KO]	Global	Asthenozoospermia	Neesen et al. [70]
<i>Dync2h1</i> ^{-/-} [Dynein cytoplasmic 2 heavy chain 1 KO]	Global	Preweaning lethality	Adams et al. [71]
<i>Dnal1</i> ^{-/-} [Dynein, axonemal, light chain 1 KO]	Global	Preweaning lethality	Adams et al. [71]
<i>Dnah2</i> ^{-/-} [Dynein, axonemal, heavy chain 2 KO]	Global	Male infertility; abnormal locomotor activation and impaired glucose tolerance	Adams et al. [71]
<i>Mdnah5</i> ^{-/-} [DNAH5 KO]	Global	Primary ciliary dyskinesia and hydrocephalus	Ibanez-Tallón et al. [72]
<i>Dnah6</i> ^{-/-} [Dynein, axonemal, heavy chain 6 KO]	Global	Enlarged heart and abnormal kidney morphology	Adams et al. [71]
<i>Dnah17</i> ^{-/-} [Dynein, axonemal, heavy chain 17 KO]	Global	Male infertility and sparse hair in female mice	Adams et al. [71]
<i>Drc7</i> ^{-/-} [Dynein regulatory complex subunit 7 KO]	Global	Male infertility and abnormal behavior; cardiovascular system phenotype	Adams et al. [71]
<i>Dynlrb1</i> ^{-/-} [DYNLRB1 KO]	Global	Embryonic lethality at E8.5	Harada et al. [73]
<i>Kif1b</i> ^{-/-} [KIF1B KO]	Global	Preweaning lethality	Adams et al. [71]
<i>Kif2c</i> ^{-/-} [KIF2C KO]	Global	Preweaning lethality	Adams et al. [71]
<i>Kif3a</i> ^{-/-} [KIF3A KO]	Global	Embryonic lethality at E10.5	Takeda et al. [74]
<i>Kif3b</i> ^{-/-} [KIF3B KO]	Global	Embryonic lethality at E12.5	Nonaka et al. [75]
<i>Kif3c</i> ^{-/-} [KIF3C KO]	Global	Embryonic lethality at E15.5	Adams et al. [71]
<i>Kif5a</i> ^{-/-} [KIF5A KO]	Global	Preweaning lethality	Adams et al. [71]
<i>Kif5b</i> ^{-/-} [KIF5B KO]	Global	Embryonic lethality at E9.5–11.5	Tanaka et al. [76]
<i>Eg5</i> ^{-/-} (<i>Kif11</i> ^{-/-}) [KIF11 KO]	Global	Embryonic lethality at E2.5–3.5	Castillo and Justice [77], Chauviere et al. [78]
<i>Kif16b</i> ^{-/-} [KIF16B KO]	Global	Embryonic lethality at E4.5	Ueno et al. [79]

(continued)

Table 1 (continued)

Gene name	KO type	Phenotype(s)	References
<i>Kif18a</i> ^{-/-} [KIF18A KO]	Global	Testis atrophy and germinal cell aplasia	Liu et al. [80]
<i>Kif18b</i> ^{-/-} [KIF18B KO]	Global	Preweaning lethality	Adams et al. [71]
<i>Kif20a</i> ^{-/-} [KIF20A KO]	Global	Preweaning lethality	Adams et al. [71]
<i>Kif21a</i> ^{-/-} [KIF21A KO]	Global	Embryonic lethality at E12.5	Adams et al. [71]
<i>Kif21b</i> ^{-/-} [KIF21B KO]	Global	Abnormal neuroanatomy such as microcephaly; brain dysfunction such as negative effect on neuron and synaptic transmission; social, learning and memory deficits.	Kannan et al. [81], Muhia et al. [82], Gromova et al. [83], Morikawa et al. [84]
<i>Kif26a</i> ^{-/-} [KIF26A KO]	Global	Megacolon and dysfunction of nociceptive responses; preweaning lethality	Wang et al. [85], Zhou et al. [86]
<i>Kif26b</i> ^{-/-} [KIF26B KO]	Global	Preweaning lethality	Adams et al. [71]
<i>Kif28</i> ^{-/-} [KIF28 KO]	Global	Preweaning lethality	Adams et al. [71]
<i>Myo1e</i> ^{-/-} [Myosin IE KO]	Global	Impaired renal function	Krendel et al. [87]
<i>Myo1h</i> ^{-/-} [Myosin IH KO]	Global	Preweaning lethality	Adams et al. [71]
<i>Myo2a</i> ^{-/-} [NMHC IIA KO]	Global	Embryonic lethality at E7.5	Conti et al. [88]
<i>Myo2b</i> ^{-/-} [NMHC IIIB KO]	Global	Embryonic lethality at E14.5	Tullio et al. [89], Ma et al. [90]
<i>Myo3a</i> ^{-/-} [Myosin XVIIIA KO]	Global	Abnormal behavioral response to light and increased urine magnesium level	Adams et al. [71]
<i>Myo3b</i> ^{-/-} [Myosin XVIIIB KO]	Global	Convulsive seizures and decreased leukocyte cell number	Adams et al. [71]
<i>Myo7a</i> ^{-/-} [Myosin VIIA KO]	Global	Abnormal neuroanatomy; decreased body weight; metabolic disorder; abnormal bone morphology and structure and persistence of hyaloid vascular system	Adams et al. [71]
<i>Myo9a</i> ^{-/-} [Myosin IXA KO]		Hydrocephalus and abnormal development of ventricular system	Abouhamed et al. [91]

(continued)

Table 1 (continued)

Gene name	KO type	Phenotype(s)	References
<i>Myo10</i> ^{-/-} [Myosin X KO]	Global	Embryonic semi-lethality. Abnormal coat/hair pigmentation, curly tail, abnormal eye development, and webbed digits	Heimsath et al. [92]
<i>Myo15</i> ^{-/-} [Myosin XV KO]	Global	Absent pinna reflex; increased total body fat amount and decreased red blood cell distribution width	Adams et al. [71]
<i>Myo18a</i> ^{-/-} [Myosin XVIIIA KO]	Global	Embryonic lethality at E13.5	Horsthemke et al. [93]
<i>Myo18b</i> ^{-/-} [Myosin XVIIIB KO]	Global	Embryonic lethality at E10.5	Ajima et al. [94]
<i>Myo3d</i> ^{-/-} <i>Myo3b</i> ^{-/-} [Myosin IIIA KO and Myosin IIIB KO]	Global	Deafness	Lelli et al. [95]

in turn is used to propel cargo to be transported along the actin-based tracks, which are most notable in late Stage VIII tubules across the seminiferous epithelium in the testis [38]. Besides the regular myosins noted in mammalian cells, there is an emerging long-tailed unconventional class of myosins, namely myosin 1E and myosin 1F [115]. In general, each myosin has a Mr of 520 kDa, consisting of six subunits: two 220 kDa heavy chains, and two pairs of light chains (20 kDa for each light chain) (Fig. 3) [116]. Thus, there are two monomers in a functional myosin motor protein, with each monomer consists of a heavy chain and a pair of light chains to a total of three subunits. Each heavy chain, in turn, can be divided into distinctive head, neck and tail domains (Fig. 3B). The globular head domain interacts with actin filaments (i.e., actin-based track) through its actin binding site at the N-terminal region which also contains the ATPase site, capable of hydrolyzing ATP to convert the chemical energy to mechanical energy to propel cargo transport. The neck region of each heavy chain serves as a linker, which also transduces force generated by the catalytic motor domain at the head region. The neck region also provides the binding site for a pair of light chains, which are distinct protein subunits that interact with the neck region (Fig. 3B). The C-terminal tail contains a relatively long α -helical coiled-coil domain and at its C-terminal region, it contains the sequential SH3 (SRC homology 3), MyTH4 (myosin tail homology 4), FERM (F, 4.1 protein; E, ezrin; R, radixin; M, moesin) domains and the globular tail domain (GTD) at its C-terminus. GTD domain is supported by the FERM, MyTH4 and SH3 domains, and GTD also recognizes different cargoes through direct interactions or mediated through adaptor proteins, such as vezatin in the testis [117] (Fig. 3B). Most myosins (e.g., myosin VIIa) walk along actin filaments to the actin plus (+) end, but Myosin VI moves cargoes to the minus (-) end of actin tracks [113]. Myosin VIIa is a member of the

myosin superfamily found in testis and other tissues [118] In testes, actin filament bundles constitute the ectoplasmic specialization, which also serve as the attachment site for cell adhesion protein complexes (e.g., N-cadherin- β -catenin, occludin-ZO-1, nectin-afadin). It also supports the transport of spermatids or organelles (i.e., cargoes) by serving as the track [12, 18]. Studies of myosin VIIa in the testis have shown that the knockdown of myosin VIIa in the testis *in vivo* by RNAi perturbs the organization of F-actin, but also MT tracks, across the seminiferous epithelium wherein these cytoskeletal tracks are extensively truncated [119]. These disruptive changes are likely the results of a considerably reduction in actin and MT polymerization activity in Sertoli cells [119] due to defects in intracellular protein trafficking. These defects also lead to formation of multiple defective sperms with gross changes in their morphology including round-shaped epididymal sperm heads, consistent presence of cytoplasmic droplets in the head region, and structural defects of sperm necks [119]. These findings are also consistent with earlier reports which have shown that KO of myosin motor proteins lead to embryonic fatality in mice (Table 1), and its mutation or genetic variations in humans also lead to defects in brain and heart development due to defects in intracellular protein trafficking.

Concluding Remarks and Future Perspectives

Herein, we summarize findings regarding the role of MT- and actin-based motor proteins in supporting mammalian spermatogenesis. As seen in studies using genetic models through gene deletion in mice (Table 1), and genetic mutations or gene variants in humans (Table 2), embryonic lethality (in mice) and serious pathological conditions (in humans) are noted in Tables 1 and 2, illustrating the significance of these motor proteins in cells and tissues, besides the testis. However, there are main questions remain. For instance, what are the biomolecules that trigger the use of specific plus (+) end or minus (-) directed motor proteins to initiate cargo transport of germ cells or other organelles to support spermatogenesis through different epithelial cycles? What is the mechanism(s) in place that selects the use of actin- or MT-based tracks or both? How does actin- and MT-based cytoskeletons coordinate with each other to streamline the transport of cargoes using their tracks to support spermatogenesis? It is now known that the several locally produced biomolecules, namely the F5-, NC1- and LG3/4/5-peptide, that regulate spermatogenesis exert their regulatory effects through their corresponding downstream signaling molecules on cytoskeletal organization. What is the mechanism(s) by which these biomolecules select the appropriate cytoskeleton, namely the F-actin or MT cytoskeleton, to execute their function? The answers to many of these questions will be helpful to understand and better manage unexplained male infertility. In brief, an intensive race is on to search for answers to some of these questions in the years to come, such as the role of many genes known to regulate spermatogenesis to support motor protein function [191]. It is likely that the use of scRNA-seq and scATAC-seq coupled with transcriptome profiling and bioinformatics analyses will provide

Table 2 Pathological conditions in humans with mutation(s) and/or genetic variations of motor genes

Mutation(s) or genetic variations	Human diseases/pathological conditions	References
DYNC1H1 (Dynein Cytoplasmic 1 Heavy Chain 1)	Malformations of brain, Charcot-Marie-Tooth disease (CMT) and spinal muscular atrophy	Poirier et al. [120], Weedon et al. [121], Harms et al. [122], Vissers et al. [123], Willemsen et al. [124], Chen et al. [125]
DYNC2H1 (Dynein Cytoplasmic 2 Heavy Chain 1)	Asphyxiating thoracic dystrophy (ATD) and short rib polydactyly syndrome (SRP) Type III	Dagoneau et al. [126], El Hokayem et al. [127], Schmidts et al. [128], [129]
DNAI1 (Dynein intermediate chain 1)	Asthenozoospermia (AZS) and primary ciliary dyskinesia (PCD)	Zuccarello et al. [130], Zariwala et al. [131]
DNAH5 (Dynein Axonemal Heavy Chain 5)	Asthenozoospermia (AZS)	Zuccarello et al. [130]
DNAH11 (Dynein Axonemal Heavy Chain 11)	Asthenozoospermia (AZS)	Zuccarello et al. [130]
KIF1A (Kinesin Family Member 1A)	Hereditary spastic paraparesis (HSP), cognitive Impairment, spastic Paraparesis, axonal neuropathy, and cerebellar atrophy	Blackstone [132], Lee et al. [133]
KIF1B (Kinesin Family Member 1B)	Charcot–Marie–Tooth type 2 (CMT2)	Hirokawa and Tanaka [134]
KIF1C (Kinesin Family Member 1C)	Hereditary spastic paraparesis (HSP)	Caballero Oteyza et al. [135]
KIF3B (Kinesin Family Member 3B)	Autosomal-Dominant Ciliopathy	Cogne et al. [136]
KIF3C (Kinesin Family Member 3C)	Infantile spasms syndrome (ISs)	Dimassi et al. [137]
KIF4A (Kinesin Family Member 4A)	Hydrocephalus internus	Meier et al. [138]
KIF5A (Kinesin Family Member 5A)	Hereditary spastic paraparesis (HSP), Charcot–Marie–Tooth type 2 (CMT2), amyotrophic lateral sclerosis (ALS), myoclonus and neonatal onset progressive leukoencephalopathy, slowly progressive atypical motor syndrome	Reid et al. [139], Goizet et al. [140], Crimella et al. [141], Liu et al. [142], Brenner et al. [143], Duis et al. [144], Rydzanicz et al. [145], Filosto et al. [146]
KIF6 (Kinesin Family Member 6)	Neurodevelopmental defects and intellectual disability	Konjikusic et al. [147]
KIF12 (Kinesin Family Member 12)	Congenital anomalies of the kidney and urinary tract (CAKUT)	Westland et al. [148]

(continued)

Table 2 (continued)

Mutation(s) or genetic variations	Human diseases/pathological conditions	References
KIF14 (Kinesin Family Member 14)	Abnormal development in brain, kidney, ureter and female genital organs	Meier et al. [138]
KIF15 (Kinesin Family Member 15)	Braddock–Carey Syndrome (BCS)	Sleiman et al. [149]
KIF16B (Kinesin Family Member 16B)	Novel autosomal-recessive intellectual disability syndrome	Alsaifi et al. [150]
KIF21A (Kinesin Family Member 21A)	Congenital fibrosis of the extraocular muscle type 1 (CFEOM1)	Yamada et al. [151]
KIF21B (Kinesin Family Member 21B)	Brain malformations, including corpus callosum agenesis (ACC) and microcephaly	Asselin et al. [152]
KIF26B (Kinesin Family Member 26B)	Autosomal dominant spinocerebellar ataxia and pontocerebellar hypoplasia	Nibbeling et al. [153], Wojcik et al. [154]
MYH2 (Myosin-2)	Myopathy, proximal, and ophthalmoplegia (MYPOL)	Martinsson et al. [155]
MYH3 (Myosin-3)	Arthrogryposis and contractures, ptterygia, and variable skeletal fusions syndrome	Chong et al. [156], Carapito et al. [157], Scala et al. [158], Cameron-Christie et al. [159], Toydemir et al. [160], Tajsharghi et al. [161]
MYL3 (Myosin light chain 3)	Cardiomyopathy, familial hypertrophic 8 (CMH8)	Poetter et al. [162], Olson et al. [163], Richard et al. [164], Jay et al. [165]
MYH6 (Myosin-6)	Atrial septal defect 3 (ASD3), cardiomyopathy, sick sinus syndrome 3 (SSS3)	Ching et al. [166], Carmiel et al. [167], Holm et al. [168]
MYH7 (Myosin-7)	Cardiomyopathy, familial hypertrophic 1 (CMH1)	Fananapazir et al. [169], Rayment et al. [170], Bundgaard et al. [171], Blair et al. [172], Richard et al. [164], Erdmann et al. [173], Van Driest et al. [174], Houg et al. [175]
MYH8 (Myosin-8)	Carney complex variant (CACOV)	Veugelers et al. [176]
MYH9 (Myosin-9)	Macrothrombocytopenia and granulocyte inclusions with or without nephritis or sensorineural hearing loss (MATINS), deafness, Alport syndrome and cataract	Seri et al. [177], Heath et al. [178], Kunishima et al. [179], Seri et al. [180], Arrondel et al. [181], Deutsch et al. [182], Seri et al. [183], Mhatre et al. [184], Lalwani et al. [185]

(continued)

Table 2 (continued)

Mutation(s) or genetic variations	Human diseases/pathological conditions	References
MYH10 (Myosin-10)	Intellectual disability (ID), brain malformations and/or congenital diaphragmatic hernia (CDH).	Tuzovic et al. [186], Hamdan et al. [187]
MYH11 (Myosin-11)	thoracic aortic aneurysm/aortic dissection (TAAD) and patent ductus arteriosus (PDA)	Zhu et al. [188]
MYH14 (Myosin-14)	Peripheral neuropathy, myopathy, hoarseness, and deafness	Donaudy et al. [189], Choi et al. [190]

many of the missing information in this race to tackle male infertility (or fertility) in the years to come. Declaration of Conflicts of Interest The authors have nothing to declare,

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Male Infertility in Humans: An Update on Non-obstructive Azoospermia (NOA) and Obstructive Azoospermia (OA)



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Introduction

Infertility is an emerging global health issue facing married couples [1, 2]. More than 10% of couples are unable to conceive their own child(ren), and approximately half of these cases are contributed by men [3, 4]. Azoospermia, defined as the complete absence of sperm from the ejaculate, is a major factor in male infertility. Azoospermia can be categorized into obstructive azoospermia (OA), comprising about 40% of the cases, and non-obstructive azoospermia (NOA), constitutes the other 60%. Obstruction in the ductal system is the cause of OA [5]. For NOA, the cause is the failure of the testicles to produce mature sperm so that no sperm are found in the ejaculate. NOA can be classified clinically into four types: NOA-I, no spermatozoa; NOA-II, no spermatids; NOA-III, no spermatocytes; and NOA-SCO (Sertoli cell-only), no spermatogenic cells of any types; in the ejaculate [6]. The definition of these four types of NOA is based on diagnostic analysis of the testes, hormonal analysis (e.g., FSH, testosterone) in plasma or serum, and physical examination [7]. OA patients are characterized by an obstructed flow of spermatozoa along the male genital tract. However, OA patients have normal spermatogenesis in the testis. The etiology of NOA is more complex, which can be divided into primary and secondary testicular failure. The primary testicular failure refers to pathology localized to the testis, including chromosomal/genetic abnormalities, Klinefelter's

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syndrome, testicular tumor, undescended testes, and others. The secondary testis failure is caused by the abnormal secretion of gonadotropins from the pituitary gland, which contribute to insufficient stimulation of Leydig cells and Sertoli cells in the testis to maintain normal spermatogenesis. As a result, Sertoli cells are unable to secrete adequate factors including hormones (e.g., estradiol-17 β), and Leydig cells fail to provide enough local testosterone to support normal spermatogenesis [8, 9].

Genetic Mutation(s)

The diagnostic tools of male infertility are limited, and clinicians are racing to identify more predictive biomarkers. Several crucial fundamental laboratory analyses, including semen analysis, anti-sperm antibodies, Y-microdeletion analysis, Karyotype analysis, microarray technologies, and endocrine-based laboratory investigations are currently being used to support clinicians in diagnosing, categorizing, and treating male factor infertility [10]. To date, seminal fluid, containing the highest concentration of biomolecules, is often used as a standard biomarker for the evaluation of male infertility, including DNA fragmentation index, anti-sperm antibody and sperm fluorescence in situ hybridization (Table 1) [16]. Azoospermia often involves gene mutations. However, it is difficult to determine the genetic component of male infertility because more than 2000 genes have been shown to be involved in human spermatogenesis [17]. In recent years, novel high-throughput approaches have developed to study pertinent genes that are mutated in azoospermic men, including genomic hybridization-arrays (arrays-CGH), genome wide association studies (GWAS) and next generation sequencing (NGS). Whole genome sequencing (WGS), an advanced technology, has brought an unprecedented opportunity to explore the genetic basis of infertility, which also makes it possible to perform studies of large cohorts of patients [18]. Whole exomes sequencing is also widely used to identify potential pathogenic and novel genes pertinent to infertile

Table 1 Current basic research biomarkers to identify OA and NOA

Methods	Assay content	Reference(s)
Semen analysis	Coagulation, color, viscosity, pH, volume, sperm agglutination, sperm counts, sperm concentration, sperm motility, sperm morphology, and sperm viability	[10, 11]
Antisperm antibodies	Sperm agglutination and cervical mucus penetration	[12, 13]
Acrosome reaction	Sperm penetration assay (Spa)	[14, 15]
Karyotype analysis	Genetic composition of Turner Syndrome	[10]
Y-microdeletion analysis	Light microscopy to evaluate the appearance of chromosomes	
Microarray technologies	Copy number variations, gene expression levels, and Single Nucleotide Polymorphisms (SNPs)	

males. Copy number variation (CNV) and single nucleotide polymorphisms are also the risk factors associated with male infertility [19]. Nonetheless, relatively few studies have provided functional and biological evidence to validate the variants as pathogenic genes.

Accumulating evidence has shown that *TEX11* and *TEX15* are mutated in infertile men of NOA and also meiotic arrest, analogous to the mutation mouse model [20, 21]. In one study of 40 Japanese patients with idiopathic NOA by conducting sequence analysis of 25 known disease-associated genes using next-generation sequencing and genome-wide copy-number analysis. The results revealed that oligogenic mutations, including *SOHLH1* and *TEX11* and monogenic mutation, which accounted for more than 10% of cases of idiopathic NOA (Table 2).

Table 2 A summary of selected mutated genes involved in male infertility

Gene	Name	Phenotype of infertility	Reference(s)
<i>TEX11</i>	Testis expressed 11	Meiotic arrest	[20–23]
<i>TEX15</i>	Testis expressed 15	NOA	[20, 21]
<i>SOHLH1</i>	Spermatogenesis and oogenesis specific basic helix-loop-helix 1	NOA	[22]
<i>TAF4B</i>	TATA-box binding protein associated factor 4b	NOA	[24]
<i>ZMYND15</i>	Zinc finger MYND-type containing 1	NOA	[24]
<i>SYCE1</i>	Synaptonemal complex central element protein 1	Meiotic arrest	[25, 26]
<i>DMC1</i>	DNA meiotic recombinase 1	NOA	[27]
<i>CFTR</i>	CF transmembrane conductance regulator	Maturation arrest	[28]
<i>TDRD9</i>	Tudor domain containing 9	Apoptosis of hSSC	[29]
<i>FOXP3</i>	Forkhead box P3	Apoptosis of hSSC	[30]
<i>PAK1</i>	P21 (RAC1) activated kinase 1	Meiotic arrest	[31]
<i>STAG3</i>	Stromal antigen 3	NOA	[32, 33]
<i>PABPC1</i>	Poly(A) binding protein cytoplasmic 1	NOA	[34]
<i>PABPC3</i>	Poly(A) binding protein cytoplasmic 3	NOA	[34]
<i>EPAB</i>	Poly(A) binding protein cytoplasmic 1 like	NOA	[34]
<i>ADGRG2</i>	Adhesion G protein-coupled receptor G2	Meiotic arrest	[35]
<i>MEIOB</i>	Meiosis specific with OB-fold	Meiotic arrest	[36]
<i>TEX14</i>	Testis expressed 14	Meiotic arrest	[36]
<i>DNAH6</i>	Dynein axonemal heavy chain 6	NOA	[36]
<i>TDRD7</i>	Tudor domain containing 7	NOA	[37]
<i>WT1</i>	WT1 transcription factor	NOA	[38]
<i>USF1</i>	Upstream transcription factor 1	NOA	[39]
<i>SYCP3</i>	Synaptonemal complex protein 3	NOA	[40]
<i>SPINK2</i>	Serine peptidase inhibitor Kazal type 2	NOA	[41]
<i>USP26</i>	Ubiquitin specific peptidase 26	NOA	[42]
<i>BCORL1</i>	BCL6 corepressor like 1	NOA	[43]
<i>DZIP1</i>	DAZ interacting zinc finger protein 1	NOA	[44]
<i>SYCP2</i>	Synaptonemal complex protein 2	NOA	[45]
<i>CFAP65</i>	Cilia and flagella associated protein 65	NOA	[46]
<i>RNF212</i>	Ring finger protein 212	NOA	[33]

Furthermore, submicroscopic copy-number variations (CNVs) on the autosomes and X chromosome may contribute to NOA, which require additional validation [22]. In 2014, truncating mutations in *TAF4B* and *ZMYND15* were reported in the azoospermic brothers of two families by exome sequencing. The two genes were shown to have an important role in spermatogenesis in mice, and they were also the first genes identified in the azoospermic men [24]. Also, using exome analysis and Sanger sequencing, a splice site mutation in *SYCE1* was found in two NOA patients in a consanguineous Iranian Jewish family [25]. *SYCE1*, a component of the central element of the synaptonemal complex, was shown to be a crucial interacting/regulatory protein between proteins *SYCE2* and *RAD51*, and its deletion led to meiosis arrest in *SYCE1* null mouse [26]. He et al. discovered *DMC1*, a meiosis-related gene, was crucial to support meiosis since its missense mutation led to meiotic arrest at the zygotene stage during human spermatogenesis. This *DMC1* mutation was identified from the male patient's family by whole-exome sequencing [27]. Cystic fibrosis transmembrane conductance regulator (CFTR), a crucial gene in supporting spermatogenesis, was recently found to be involved in NOA [28]. In another study of five azoospermic infertile NOA patients, recessive deleterious mutation in *TDRD9* was identified which contributed to sperm maturation arrest. Similar clinical phenotype was also observed in the *Tdrd9* global knockout mice [29]. Additionally, mutations and polymorphisms in *HIWI2* were detected in idiopathic NOA, which were crucial for piRNA biogenesis and function in supporting spermatogenesis [47]. piRNA pathway is a fundamental component of spermatogenesis which ensures male fertility and genome integrity [48]. Furthermore, *PABPC1*, *PABPC3*, and *EPAB*, the poly(A)-binding protein genes, are differentially expressed in NOA patients when compared to normal men, implying their involvement in NOA [34]. It is known that development of the spermatogonial stem cells (hSSC) is essential for human spermatogenesis, and *FOXP3* pathogenic variants affected the proliferation and apoptosis of hSSC, causing male infertility [30]. Similar to *FOXP3*, a reduced expression of *PAK* was noted in NOA patients, which thus inhibited apoptosis and promoted proliferation of hSSC through PDK1/KDR/ZNF367 and ERK1/2 and AKT pathways [31]. Also, genetic variants in *STAG3*, a meiosis-specific protein, has been reported to cause meiotic arrest in both male and female mice; however, its genetic variants in humans led to premature ovarian failure in women, but not in infertility in men [49] (Table 2).

DNA Methylation

Emerging evidence has shown that DNA methylation, sperm-borne and epigenetic abnormalities in chromatin dynamics, may contribute to male infertility [50]. Epigenetic modifications take place frequently during spermatogenesis, including large-scale demethylation of the genome to allow for sex-specific resetting by DNA methylation and histone modifications. DNA methylation, a heritable epigenetic modification, and a widely investigated epigenetic marker plays an essential role in

regulating genes during human spermatogenesis, which mainly takes place in the fifth position of cytosine bases and followed by guanine (CpG). Male germ cells acquire DNA methylation beginning at mitotic and meiotic germ cells, and it is completed at the stage of the pachytene phase of meiosis [51, 52]. Abnormal DNA methylation in sperm may contribute to male infertility and pass onto offspring, who may become more susceptible of developing illnesses later on in life [53]. Several studies have reported that there is a significant difference on DNA methylation levels between normal and infertile men, which also leads to lower sperm count, reduced semen volume and lower sperm progressive motility [54]. Studies have also shown that DNA methylation can be induced by environmental factors, including exposure to endocrine disrupting chemicals [e.g., perfluoroctanesulfonate (PFOS), phthalates] and heavy metals (e.g., cadmium, mercury, lead), nutritional status, air pollution, smoking, and unhealthy lifestyle, since these are contributing factors to gene-specific and global DNA methylation [55, 56]. For example, cadmium, an environment toxicant that exists as CdCl₂ in the environment has been shown to reduce fetal growth by hypomethylation of the *PCDHAC1* promoter region, which leads to a positive expression of *PCDHAC1* [57]. Air pollutants, containing massive different environmental exposures, was also found to alter DNA methylation levels of the genes encoding the mitogen-activated protein kinase (MAPK) regulatory network and other blood-based proteins [58, 59]. Male infertility is also influenced by smoking via epigenetic pathways [60]. Smoking was also shown to have a strong influence on DNA methylation, which alters the CpG methylation patterns in the regions of MAPK8IP and TKR genes, leading to reduced sperm count, motility, and defects in sperm morphology [61]. In some azoospermia sperm samples, there was a considerable increase in the levels of KCNQ1OT1 (KCNQ1 Opposite Strand/Antisense Transcript 1, is a long non-coding RNA gene found in the KCNQ1 locus), compared to the normal group [62]. Also, global methylation level of sperm DNA did not affect the pregnancy rate in IVF, but it affected embryo development when global DNA methylation level was below a threshold value [63]. Furthermore, smokers displayed hypomethylation of reproductive related genes, including Nme2, Trim27, ICR, H19, SNRPN, Sort and Pebp1, which negatively impeded spermatogenesis and sperm motility [64–67].

Also, distinctive DNA methylation modifications are noted in promoters and repeat elements during spermatogenesis [68]. As a crucial transcription factor for mitochondrial biogenesis, nuclear respiratory factor (NRF)-1 cooperates with DNA methylation to directly regulate the expression of various germ cell-specific genes, including *Asz1* [69]. The hypermethylation at the promoter of SOX30 contributes to its silencing of expression in NOA, and the decreasing level of SOX30 is related to the severity of NOA disease. Furthermore, the absence of *Sox30* in mice led to male infertility with a complete lack of spermatozoa, which impaired testis development and spermatogenesis. However, *Sox30* does not influence ovary development and female fertility [6]. Hypermethylation of the MAEL promoter increased the expression of the transposable element LINE-1, leading to a decrease in the appearance of MAEL, and the methylation of the MALE promoter in infertility men correlates

with the severity of spermatogenic failure [70]. On the other hand, aberrant methylation of the GTF2A1L promoter did not affect fertilization rates, but its expression was reduced in NOA patients.

A recent case-control study in NOA and OA patients by investigating the differences and conservations in DNA methylation based on genome-wide DNA methylation and bulk RNA-Seq between these groups for transcriptome profiling. These results have shown that the genome modification of testicular cells from NOA patients is disordered, and the reproductive related gene expression is considerably different [71]. Four functional regions (CGI, gene body, promoter, and TEs) were identified and it was noted that the NOA patient's entropy values in these regions were considerably lower *versus* the OA group. Meanwhile, the methylation level of the OA patients was lower, and the gene expression level was higher than the NOA patients. Likewise, the methylation level of Dazl gene, an RNA binding protein deleted in azoospermia [72], in OA patients was lower than that of the NOA patients. A series of reproductive genes, including testis and ovary-specific PAZ domain gene1 (*Topaz1*), the nuclear receptor *NR5A1*, and the vertebrate-conserved RNA-binding protein gene *DND1*, all displayed lower NDA methylation level and higher gene expression level in OA patients compared to NOA, which are related to the development of spermatogonia that may contribute to male infertility. Transposons are often silenced by DNA methylation, and some functional transposons exhibited higher enrichment scores in NOA patients, including ALU, ERV1, HAT, and MIR. These findings are summarized in Table 3.

Chromosomal Aberrations and Y chromosome (Yq) Microdeletions

Genetic disorders are one of the primary causes of azoospermia, including chromosomal abnormalities, monogenic disorder, multifactorial genetic diseases, and epigenetic disorders, which also constitute the genetic basis of reproductive failure [73]. The prevalence of chromosomal aberrations in azoospermic patients was between 15% and 25% [28, 74–76]. Klinefelter syndrome and its variants (47, XXY and mosaics 46, XY/47, XXY) is the most frequent chromosomal anomaly in NOA, whereas oligozoospermia is more prevalent in men with autosomal structural defects [77]. Klinefelter syndrome (KS), identified over 70 years ago, also remains one of the prevalent causes of infertility, which is typified by small testes, hypogonadotropic hypogonadism, and cognitive impairment. As a syndromic disease, KS is associated with cardiovascular abnormalities, autoimmune diseases, metabolic disorders and cognitive or psychiatric health problems, which may also increase the risk of death [78, 79]. The average prevalence of KS is 152 per 100,000 newborn males, based on several larger cytogenetic chromosome surveys in countries around the world as noted in 2017 [80]. However, KS is often insufficiently diagnosed, and treatment is limited mostly to testosterone therapy, which overcomes some but not

Table 3 Summary of the study involved in DNA methylation and mRNA expression

Gene	Full name	Gene in chromosome
Hypermethylation and low mRNA expression		
<i>ANKRD60</i>	Ankyrin repeat domain 60	20
<i>TMPRS11E</i>	Transmembrane serine protease 11E	4
<i>PADI3</i>	Peptidyl arginine deiminase 3	1
<i>GPR149</i>	G protein-coupled receptor 149	3
<i>C8B</i>	Complement C8 beta chain	1
<i>SLC45A2</i>	Solute carrier family 45 member 2	5
<i>GJA8</i>	Gap junction protein alpha 8	1
<i>OR5AC2</i>	Olfactory receptor family 5 subfamily AC member 2	3
<i>CELA1</i>	Chymotrypsin like elastase 1	12
<i>CCDC144A</i>	Coiled-coil domain containing 144A	17
<i>C10orf142</i>	Long intergenic non-protein coding RNA 2881	10
<i>GPR25</i>	G protein-coupled receptor 25	1
<i>TEX13B</i>	Testis expressed 13B	X
<i>HIST3H3</i>	Histone cluster 3	1
<i>TAF1L</i>	TATA-box binding protein associated factor 1 like	9
<i>RGPD1</i>	RANBP2 like and GRIP domain containing 1	2
<i>NME8</i>	NME/NM23 family member 8	7
<i>FRG2C</i>	FSHD region gene 2 family member C	3
<i>ELOA2</i>	Elongin A2	18
<i>NDUFA13</i>	NADH:ubiquinone oxidoreductase subunit A13	19
<i>HIST1H2AA</i>	Histone cluster 1	6
<i>TGIF2LY</i>	TGFB induced factor homeobox 2 like Y-linked	Y
<i>ZNF723</i>	Zinc finger protein 723	19
<i>CSNK1A1L</i>	Casein kinase 1 alpha 1 like	13
<i>AL162231</i>	Galactose-1-phosphate uridylyltransferase	9
<i>ABRA</i>	Actin binding Rho activating protein	8
<i>CMTM1</i>	CKLF like MARVEL transmembrane domain containing 1	16
<i>RGPD3</i>	RANBP2 like and GRIP domain containing 3	2
<i>C10orf82</i>	Chromosome 10 open reading frame 82	10
<i>UBXN10</i>	UBX domain protein 10	1
Hypomethylation and high mRNA expression		
<i>ID3</i>	Inhibitor of DNA binding 3	1
<i>S100A1</i>	S100 calcium binding protein A	1
<i>GJC2</i>	Gap junction protein gamma 2	1
<i>TNFRSF14</i>	TNF receptor superfamily member 14	1
<i>MGP</i>	Matrix Gla protein	12
<i>C1QC</i>	Complement C1q C chain	1
<i>ANGPTL1</i>	Angiopoietin like 1	1
<i>ISLR</i>	Immunoglobulin superfamily containing leucine rich repeat	15
<i>DCN</i>	Decorin	12
<i>B3GALT2</i>	Beta-1,3-galactosyltransferase 2	1

all comorbidities [77]. Y chromosome harbors a large number of genes that are necessary for testis development and function. The azoospermia factor (AZF) deletions impaired spermatogenesis, which is also a major molecular cause of male infertility [81]. Meanwhile, Y chromosome (Yq) microdeletions constitute a significant cause of male infertility. European infertile men are less susceptible to Yq microdeletions compared to East Asian and Americans infertile men. Y chromosome is composed of a short arm (Yp), a long arm (Yq), and two pseudo autosomal regions (PARs), which are separated by a centromere [82]. Studies have demonstrated that the deletion of human PARs in men reduced recombination in PARs, leading to sterility [83, 84]. This thus increases the frequency of sex chromosome aneuploidy in sperm, contributing to X-chromosome monosomy (Turner syndrome) or XXY (Klinefelter syndrome) in the offspring [85, 86]. X-chromosome monosomy (Turner syndrome), accounted for approximately 2% of all conceptions, is due to a partial or total loss of the second sexual chromosome, leading to an abnormal development phenotype, including typical dysmorphic stigmata, sexual infantilism, short stature, and partial organs and metabolic abnormalities [87]. However, the TS phenotype may be associated with a genomic imbalance from the absence of genes linked to the second sex chromosome and altered regulation of gene expression that triggered by epigenetic factors. Thus, both copy number variations and epigenetic changes are crucial contributing factors in the TS phenotype [87]. Epigenetic alterations in pericentromeric heterochromatin may also contribute to reconstructing of chromatin conformation, leading to chromosomes that have defects in their ability to align, attach to mitotic spindle fibers, and segregate during mitosis [88].

Concluding Remarks and Future Perspectives

DNA methylation as an epigenetic marker which plays an important role in male spermatogenesis. In this chapter, we discuss the importance of DNA methylation and gene expression that contribute to NOA and OA. We also summarized the several important reproductive genes in NOA and OA that show different DNA methylation and expression level. Also, we discuss findings based on the use of advanced technology to detect genetic mutations in NOA vs. OA that lead to male infertility. More studies are needed by increasing the sample sizes to integrate multiple epigenomic and RNA-seq analysis, which will help in the identification of epigenetic markers and genes pertinent to the regulation of fertility and infertility. Future investigations using single cell (sc) RNA-seq, scATAC-seq and epigenomics will be important to define the etiology and pathogenesis in NOA and OA [89, 90].

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Androgen Actions in the Testis and the Regulation of Spermatogenesis



William H. Walker

The regulation of spermatogenesis by androgens was reviewed recently [1, 2]. The goal of this chapter is to summarize the most important functions of androgens in the testis and to discuss major new published progress.

Testosterone Production and Levels in the Testis

Testosterone is a steroid hormone capable of diffusing through the plasma membrane of cells to interact with the intracellular androgen receptor (AR, NR3C4), a member of the steroid hormone receptor family located in the cytoplasm or nucleus that is encoded by a single gene on the X chromosome. In men, testosterone is produced predominately by Leydig cells located in the interstitial regions of the testis between the seminiferous tubules that contain the developing germ cells. The binding of luteinizing hormone to receptors on the surface of Leydig cells causes the activation of genes encoding steroidogenic enzymes that are responsible for the production and secretion of testosterone. Due to the presence of the Leydig cells, testosterone concentrations in the testes of men (340–2000 nM) are 25 to 125-fold greater than that in serum (8.75–35 mM). Testosterone levels are similarly elevated in the rodent testis. Thus, testosterone is the major androgen in the testis exceeding the levels of dihydrotestosterone (DHT) by 15- to 40-fold [3–7].

Production of spermatozoa and male fertility are dependent upon testosterone [8, 9]. The bioavailable levels of testosterone in the testis far exceed that required for

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function, as the binding affinity of AR for testosterone is approximately 1 nM and regulation of gene expression via AR binding requires 1–10 nM [10]. The physiological importance of high testosterone levels in the testis is not well understood, because qualitative spermatogenesis (the continuous process of developing spermatozoa from a population of spermatogonial stem cell) can be initiated and maintained in rodents with levels that are 10–25% of normal adult levels (reviewed in [11]). However, high testosterone levels are required to maintain quantitatively normal spermatogenesis because sperm counts decrease at a logarithmic rate as testosterone levels fall below 70 nM in the rodent [12].

Testosterone is aromatized to estrogen by Leydig, Sertoli and germ cells but the concentration of estradiol in the seminiferous tubule fluid of rat and rhesus monkeys (less than 1 nM) is much less than that of testosterone (reviewed in [13]). Thus far, there is no evidence that estrogen acts to directly affect processes required for spermatogenesis. Elimination of estrogen receptor beta (ER β) results in no testicular phenotype and knock out of ER β and ER α showed no testicular phenotype in early development. The global ER α knock out mouse model showed that the receptor is required for fertility, but the process of spermatogenesis was not directly affected. In older ER α knock out mice, inhibition of water reabsorption in the efferent ducts and epididymis resulted in fluid back-up into the seminiferous tubules that disrupted spermatogenesis [13]. Together, the results from the ER knock out mice indicate that estrogen signaling is not essential for the process of spermatogenesis.

A Concise Review of Spermatogenesis and the Processes that Require Testosterone

Spermatogenesis occurs within the seminiferous tubules in the testes. The seminiferous tubules are surrounded by peritubular myoid (PTM) cells that provide factors required to support spermatogenesis, while also contracting to propel spermatozoa through the tubules toward the rete testis and the epididymis. Within the seminiferous tubules, the somatic Sertoli cells extend from the basement membrane in the interior of the seminiferous tubule to the lumen opening at the center of the tubule, while branching around and supporting the developing germ cells.

Germ cell development begins with the differentiation of gonocytes (aka prospermatogonia) and their movement to the basement membrane of the seminiferous tubules followed by gonocytes differentiating further to become spermatogonial stem cells. The continuous process of spermatogenesis is initiated when spermatogonial stem cells divide to produce B spermatogonia (non-human primates and men) or undifferentiated spermatogonia (rodents) (Fig. 1). These spermatogonia undergo a series of mitotic divisions with incomplete cytokinesis. As a result, chains of developing germ cells sharing a single cytoplasm form along the basement membrane. With ongoing divisions, the spermatogonia lose their undifferentiated qualities and obtain differentiation markers. A final mitotic division that occurs in the

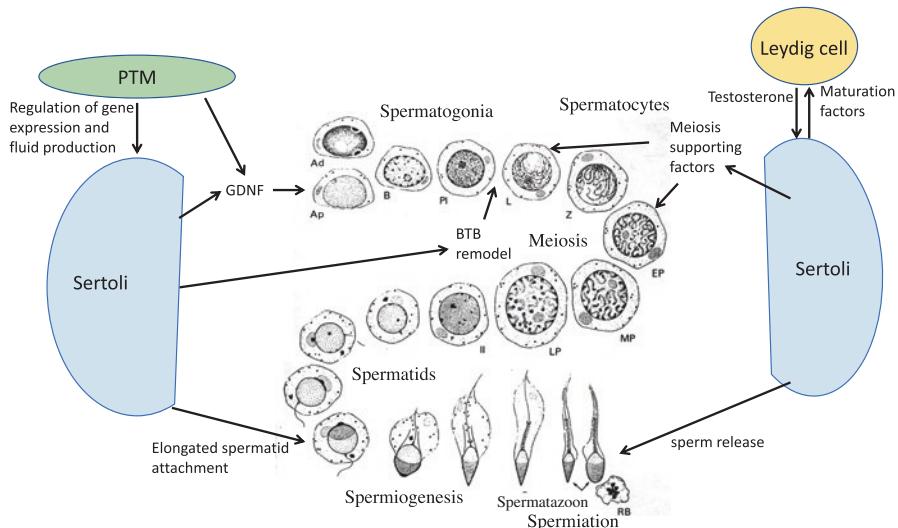


Fig. 1 Androgen mediated functions in the testis. The process of human spermatogenesis is shown with A pale (Ap) and A dark (Ad) spermatogonial stem cells, differentiating B spermatogonia (B), preleptotene leptotene, zygotene, (Pl. L, Z) as well as early, mid and late pachytene (EP, MP, LP) and Meiosis II spermatocytes, spermatids and spermatozoa and residual bodies (RB). Androgen-mediated products and functions of peritubular myoid cells (PTM, Sertoli cells and Leydig cells are shown. Adapted from [14]

presence of retinoic acid signaling allows for the production of preleptotene spermatocytes that are programmed to undergo meiosis. The preleptotene spermatocytes detach from the basement membrane to begin their movement to the lumen and “pass through” the blood-testis barrier (BTB). The BTB is a collection of adhesion junctions required to maintain spermatogenesis that are formed near the basement membrane between adjacent Sertoli cells. The BTB is dissolved above the preleptotene spermatocyte and reformed below as the germ cell begins its movement toward the lumen of the tubule [15]. After passing through the BTB, the meiotic cells continue through the lengthy leptotene, zygotene, pachytene and diplotene stages of prophase I followed by the rapid completion of meiosis I and II cell divisions that result in haploid round spermatids. The spermatids undergo extensive differentiation (spermiogenesis) including condensation of the nucleus and DNA, termination of transcription, alteration of adhesion protein contacts with Sertoli cells, elimination of much of the cytoplasm, acrosome formation, cell elongation and development of a flagellum. Spermatogenesis is completed with the release of mature elongated spermatids as spermatozoa (spermiation) into the lumen of the tubule.

The deprivation of testosterone in rodents results in blocking germ cell development in meiosis and the release of mature spermatids (reviewed in [9]) (Fig. 1). Decreased intratesticular testosterone levels or elimination of AR disrupts three major processes in the testis that are required for spermatogenesis and male fertility.

First, the BTB cannot be maintained resulting in the loss of meiotic and post-meiotic germ cells [16, 17]. Second, round spermatids are prematurely released as they are differentiating into elongated spermatids [18–20]. Third, mature spermatozoa cannot be released resulting in the phagocytosis of the germ cells by Sertoli cells [18].

Testosterone Signaling Pathways

Testosterone actions are mediated via the 110 kDa AR that has numerous well-characterized structurally and functionally distinct domains (reviewed in [21, 22] including the poorly conserved N-terminal domain (NTD) encoded by exon 1 that contains a proline rich region required for interactions with Src kinase [23, 24]. There is also a highly conserved DNA-binding domain (DBD) including two zinc fingers encoded by exons 2 and 3 that recognize specific DNA sequences called androgen response elements (AREs). A ligand binding domain (LBD) also contains a nuclear export sequence that is encoded by exons 4–8. Another portion of exon 4 encodes a short hinge region that separates the LBD from the DBD and contains a ligand-dependent localization signal (NLS). Domains encoding transcription activation functions are located in the N terminus (AF-1) and in the LBD (AF-2).

There are at least two mechanisms by which testosterone signaling is mediated, the classical and the non-classical pathways [25, 26]. The classical pathway is limited to the regulation of gene expression. In this pathway, testosterone diffuses through the plasma membrane and binds to AR that is sequestered by heat shock proteins in the cytoplasm. A conformational change in AR causes the receptor to be released from the heat shock proteins after which, AR translocates to the nucleus where it binds to androgen response elements (AREs) in gene regulatory regions, recruits co-regulator proteins and regulates gene transcription. Activation of the classical pathway requires at least 30–45 min to detect the first increases in gene transcription with protein-mediated effects occurring even later [27].

The non-classical (also called non-genomic) pathway characterized in Sertoli cells, causes the activation of kinase signaling pathways and Ca^{2+} influx as well as regulation of gene expression. Non-classical pathway actions are rapid and can be detected within 1 min. Thus far, two non-classical pathways have been described in Sertoli cells. In the first pathway, testosterone (10–100 nM) stimulation of cultured Sertoli cells isolated from rats transiently increases AR localization to the plasma membrane and triggers the direct association of the proline-rich region of AR (amino acids 352–359) and the SH3 domain of Src tyrosine kinase [28, 29]. Testosterone-mediated activation of Src causes the phosphorylation and stimulation of the EGF receptor (EGFR) [28]. Stimulation of EGFR is required to activate the MAP kinase cascade (Raf, MEK, ERK) that in turn activates p90^{Rsk} kinase to phosphorylate the CREB transcription factor [28]. AR was found to be essential to drive this non-classical testosterone pathway. Studies using the TM4 mouse Sertoli cell line suggest that androgen stimulation also activates AKT, GSK α , GSK β and p70 S6 kinases and that localization of AR to the membrane is dependent upon activation of

ERK and AKT kinases [30]. Stimulation of cultured Sertoli cells with levels of testosterone (10–250 nM) that are similar to or lower than those found in the testis (200–800 nM) [4, 7, 9] activated the non-classical pathway including the phosphorylation of ERK and CREB and increased the transcription of CREB-regulated genes including CREB itself, LDH-A and EGR [31]. Thus, the non-classical pathway also regulates gene expression downstream of kinase activation. Activation of Src, ERK and CREB was detected within 1 min with gene expression regulated at later time points. Increased protein phosphorylation could be sustained for at least 12 h [31].

Assays of non-classical testosterone signaling *in vivo* showed that treatment of adult rats with a GnRH antagonist to decrease intratesticular testosterone levels by 77% resulted in a corresponding 68% lower level of phosphorylated ERK kinase (p-ERK) in Sertoli cells within stage VII seminiferous tubule cross sections. Subsequent injections of testosterone that increased intratesticular testosterone to normal levels restored p-ERK levels in stage VII tubules to control levels within 1 h [32]. The rapid testosterone-mediated increase in p-ERK levels is not likely to result from classical testosterone signaling because accumulation of testosterone in the testis would require some latent period and testosterone mediated gene expression and protein production requires more than 45 min [27]. These studies indicate that testosterone activates the non-classical testosterone pathway in Sertoli cells *in vivo*.

There is evidence to suggest that both the non-classical and classical signaling pathways are required to maintain spermatogenesis. Adenovirus constructs expressing either a peptide that inhibits the interaction of AR and Src kinase (non-classical inhibitor) or a modified AR protein that acts as dominant negative inhibitor of AR-mediated transcription (classical inhibitor) were delivered into the seminiferous tubules and Sertoli cells of rats. Both inhibitors caused the mis-localization of the tight junction protein Claudin 11 away from the BTB region and disrupted the integrity of the BTB. In addition, the non-classical inhibitor resulted in the loss of spermatocytes in adult mice. Similar studies indicated that the non-classical inhibitor blocked the progression of meiosis and the production of spermatocytes. In contrast, the major effect of the classical inhibitor was the premature release of meiotic and post meiotic germ cells. The combined effects of both inhibitors resulted in a synergistic disruption of spermatogenesis. Together, these studies indicate that both signaling pathways contribute to the maintenance of spermatogenesis [32].

A second rapid acting non-classical pathway has been described in which testosterone causes depolarization of the Sertoli cell within 30 seconds due to the closing of K⁺_{ATP} channels via G protein-mediated activation of phospholipase C. As a result, there is rapid influx of Ca²⁺ and the activation of intracellular signaling factors (reviewed in [33]). This second non-classical pathway has only been described in immature Sertoli cells. It is not known whether AR is required to activate the pathway or whether the pathway contributes to Sertoli cell function in mature Sertoli cells. Other studies performed in the Sertoli cell line 93RS2 that lacks AR showed that testosterone acted via the Zn²⁺ transporter ZIP9 to activate ERK1/2, CREB and ATF1, stimulate claudin-1 and claudin-5 expression and enhance tight junction formation between Sertoli cells [34]. Thus far, it has not been determined whether the

ZIP9-mediated signaling pathway is relevant to Sertoli cells present *in vivo* that express AR. In other studies of testes isolated from three-day old rats that display no detectable AR in Sertoli cells, testosterone at concentrations that are higher than intratesticular levels (1 μ M), caused depolarization of Sertoli cells [35]. These findings raise the possibility that developing Sertoli cells *in vivo* may be able to respond to testosterone prior to the expression of detectable AR.

The Absence of Testosterone Signaling Blocks Testis Descent Causing Azoospermia

One of the first processes regulated by testosterone during development is the completion of testis descent into the scrotum. In rodents and humans with androgen insensitivity, the testis remains in the inguinal canal or groin [36]. Due to the exposure of the testis to increased intra-abdominal temperatures, azoospermia occurs because spermatogenesis is incomplete and testicular cancer incidence is increased [37].

Androgens act to regress the cranial ligament suspensory apparatus in the fetus that attaches the gonads to the abdominal wall. As a result, the testes are free to descend towards the scrotum [38]. This transabdominal phase of testis descent is mediated by INL3 (reviewed in [37]). However, androgens are required for completion of testis descent (the inguinoscrotal phase) that is guided by the growth and contractions of the gubernaculum that results in the pulling of the testis into the scrotum. For this process, androgens mediate the masculinization of the sensory nucleus of the genitofemoral nerve in the L1 or L2 dorsal root ganglia that supplies the scrotum before gubernaculum growth. In response to androgen, the sensory branch of the genitofemoral nerve releases the neurotransmitter calcitonin gene-related peptide (CGRP) that causes rhythmic contractions of the gubernaculum that pulls the testis into the scrotum (reviewed in [37, 39, 40]). Although AR expression has been detected in the rat lumbar spinal cord immediately before the onset of maximal androgen action for testicular descent [41], AR was not detected in the nuclei of the genitofemoral nerve until after the temporal window of androgen action [42]. Thus, the mechanisms by which androgen mediates CGRP secretion and the completion of testis descent remains to be determined.

Cell- and Temporal-Specific Expression of AR in the Testis

Adult testis cells expressing AR include PTM, Leydig, Sertoli, arteriole smooth muscle and vascular endothelial cells. In PTM cells, AR is expressed from the fetal period through adulthood in men and rodents [43–46]. Fetal and adult Leydig cells express AR at constant levels [47]. Sertoli cells in rodents first express AR three to

5 days after birth [48–51]. In men, AR is expressed at low levels in Sertoli cells beginning about 5 months after birth and expression remains weak until 4 years of age [43–46]. In the adult rodent, AR levels are low in Sertoli cells throughout most the cycle of the seminiferous epithelium except during stages VI–VIII when AR levels peak [48, 52, 53]. AR expression in men is also stage-specific with the highest levels of AR expressed during stage III of the six stages [54].

The stage-specific peaks in AR expression that are observed in Sertoli cells correspond with the initiation of testosterone-dependent processes that are essential for spermatogenesis, including formation of specialized adhesion junctions with elongating spermatids and the release of mature sperm that occur in stage VIII. The higher levels of AR also coincide with the appearance of preleptotene spermatocytes and the initiation of meiosis during stages VII–VIII. Thus, it is possible that testosterone-mediated paracrine signals from Sertoli cells to preleptotene spermatocytes may be responsible for the production of factors that are required later to complete meiosis. There is less information available regarding the potential importance of limiting AR levels and testosterone signaling in Sertoli cells during the remaining stages of cycle of the seminiferous epithelium.

Androgen-Mediated Autocrine and Paracrine Signaling Between Somatic Cells in the Testis

Sertoli cells: The proliferation of Sertoli cells is, in part, regulated by testosterone-mediated paracrine signals from other somatic cells. In the testicular feminized mouse (*Tfm*) model that has an inactivating AR mutation in all cells, the number of Sertoli cells was reduced and testosterone was found to contribute to Sertoli cell proliferation in fetal and early neonatal life [55]. ARKO mice, lacking AR in all cells, have fewer Sertoli cells at all ages from birth to adult. SCARKO mice having AR eliminated specifically in Sertoli cells displayed normal or slightly lower numbers of Sertoli cells only in older mice [56, 57]. Similar studies showed that Sertoli cell numbers were reduced at PD2 in ARKO but not SCARKO mice [58]. These findings suggest signals mediated by testosterone actions from other cells support Sertoli cell expansion.

Leydig cells: Fetal Leydig cells do not express AR and thus are not directly influenced by testosterone [59]. Furthermore, the lack of testosterone-mediated signals from other cells apparently does not affect fetal Leydig cells in rodents because at PD5, before adult Leydig cells arise, Leydig cell numbers remained stable in both ARKO and SCARKO models [56].

Maturation of adult Leydig cells requires AR-mediated signals. Adult Leydig stem cells initiate AR expression and become responsive to autocrine testosterone signaling at their stem/progenitor stage prior to birth [60, 61]. In adult *Tfm* mice, Leydig cells were reduced by 60%, were not fully differentiated and had decreased levels of steroidogenic enzymes [62]. The lack of Leydig cell differentiation is not

associated with cryptorchidism but occurs in response to the lack of androgen-mediated signals [62]. Similarly, fewer Leydig cells are present in ARKO mice due to a decrease in the population of adult Leydig cells that begins about PD7. This result is consistent with earlier reports that AR is required for the development of adult Leydig cell [63]. One possible explanation for the altered Leydig cell development is that in the absence of AR, needed growth signals for Leydig cells including perhaps PDGF-A are not secreted from Sertoli cells [64]. This idea is consistent with other evidence that androgen-mediated signals to adult Leydig cell progenitors contribute to their differentiation into immature Leydig cells [65].

In PD20 and adult SCARKO mice, the number of adult Leydig cells remained normal in one study [56] but decreased in another [64]. Thus, the effects of decreased androgen-mediated signals from Sertoli cells on Leydig cell expansion remains unresolved. However, the only group to study mice overexpressing AR in Sertoli cells showed that with elevated testosterone signaling differentiation of adult Leydig cells was accelerated and steroidogenesis was increased as evidenced by the accumulation of lipid droplets but, the number of fetal and adult Leydig cells was reduced due to decreased proliferation. Perhaps as a compensatory mechanism, there was Leydig cell hypertrophy. These findings indicated that AR actions in Sertoli cells regulate the proliferation and differentiation of Leydig cells [66, 67].

A mouse model in which AR is knocked out specifically in 75% of adult Leydig stem/progenitor cells (LCARKO mice) revealed that autocrine signaling via the Leydig cell AR was not required to attain normal numbers of Leydig cells [68]. However, in the absence of Leydig cell AR, there was degeneration of the seminiferous epithelium and apoptosis of Leydig cells in older mice. In addition, the LCARKO mice displayed a reduction in the number of spermatocyte and spermatids that begins about PD35 and progresses at least through PD80. Apoptosis of Leydig cells also was observed in men aged 20 or 36 years (but not younger) having complete androgen insensitivity syndrome. Together, these findings indicate that AR function in Leydig cells is needed for the lifelong support of spermatogenesis [68].

Pertubular myoid cells: Conditional knock out of AR in PTM cells using a Cre recombinase driven by the promoter of the transgelin smooth muscle protein 22 α gene resulted in a 57% reduction in sperm count but normal germ cell distribution ratios and normal fertility. The expression of Sertoli cell genes required for cell junctions, transport of nutrients to germ cells and tissue remodeling also were inhibited [69]. These results suggested that PTM cell-derived AR-mediated signals are important for maintaining normal levels of Sertoli cell functions that are required for spermatogenesis. A later study determined that the CRE construct used was not efficiently expressed in PTM cells in the testis, which may have accounted for the partial decrease in sperm production [70]. A second conditional AR knock out model using CRE recombinase driven by the myosin, heavy polypeptide 11, smooth muscle (Myh11) promoter (Myh11-Cre) resulted in an 86% reduction in germ cells, few sperm in the epididymis and infertility [71]. The relative number of spermatogonia as assessed by average volume/testis progressively decreased and reached a 62% reduction versus wild type by 100 days after birth. Reductions in the number

of spermatocytes were more severe than observed in SCARKO mice. The expression of some AR-regulated genes in Sertoli cells was decreased and the size of the seminiferous epithelium lumen was reduced. Sertoli cell numbers, maturation markers and the expression of AR were not affected. These observations suggest that there are signals from the PTM cell to the Sertoli cell that influence gene expression and the production of seminiferous tubule fluid that carries nutrients to germ cells and developing sperm [71].

Vascular endothelial cells and arteriole smooth muscle cells: Mice having AR knocked out specifically in vascular epithelial cells did not display compromised spermatogenesis [72]. Knock out of AR in arteriole smooth muscle cells also did not grossly alter spermatogenesis but did subtly impair Leydig cell function and testicular fluid exchange, perhaps by regulating the local microvascular blood flow within the testis [73].

Testosterone Is Required to Maintain the Blood-Testis Barrier

The blood-testis barrier (BTB) consists of numerous cellular junction and adhesion proteins between Sertoli cells [16, 17]. The BTB is present near the basement membrane of the seminiferous tubule and separates the basal compartment that has access to circulatory system from the luminal compartment that is dependent upon the Sertoli cell to provide nutrients and growth factors. All germ cell development beyond the leptotene stage of meiosis occurs in the specialized luminal environment beyond the BTB. Disruption of the BTB halts spermatogenesis in rodents [74, 75]. However, the extent to which BTB disruptions affect fertility in men is less well understood.

Tight junctions, a major determinant of BTB integrity, were strengthened in cultured Sertoli cells after stimulation with testosterone [76–78]. Testosterone induced the expression of the mRNA encoding a major tight junction protein N-cadherin and increased the localization of the tight junction protein claudin-11 to Sertoli-Sertoli contact sites [78, 79]. In addition, testosterone signaling increased steady-state levels of clatherin and clatherin association with tight junction proteins occludin and claudin 11. Claudin 3 is another adhesion protein that is induced by androgen and localizes to newly forming tight junctions at the BTB [16] but is not essential for fertility [80]. Testosterone also facilitates the transcytosis and recycling of tight junction proteins that are associated with the dissolution and reformation of the BTB as spermatocytes transit through the barrier. This function of testosterone signaling allows the rapid reformation of the BTB that occurs below the spermatocytes [81, 82].

In SCARKO mice, the BTB is present but formation is delayed, and the barrier is incomplete [17]. Claudin 11 and occludin, major transmembrane components of the BTB as well as the mRNAs encoding Tjp1, Tjp2 iso1, Tjp2 iso2 and Tjp2 iso3 that link the transmembrane tight junction proteins to the cytoskeleton are down-regulated in SCARKO mice [17, 83, 84]. Rats injected with flutamide to block AR

actions had decreased expression of the Cx43 gap junction mRNA and protein and the loss of Cx43 localization to the BTB. Furthermore, the tight junction associated ZO-1 protein was mis-localized away from the cell surface to the Sertoli cell cytoplasm after flutamide treatment [85]. Treatment of hypogonadal (*hpg*) mice with DHT restored the expression and localization of claudin 3, Cx-43 and claudin 11 to the BTB [86].

Inhibition of either classical or non-classical testosterone signaling disrupted the BTB *in vivo*. The injection of mouse testis seminiferous tubules with adenovirus constructs expressing inhibitors of either pathway resulted in decreased expression of claudin 11 at the BTB and increased transit of a biotin tracer through the BTB and into the lumen of the tubules [32]. The finding that the non-classical pathway is required for maintaining the BTB is consistent with previous studies showing that components of the non-classical pathway (ERK and Src family kinases) contribute to maintaining the BTB [87–89]. Preservation of the BTB by classical testosterone signaling is supported by previous studies showing that AR induces the expression of genes encoding BTB-associated proteins including *N-Cadherin*, *Claudin 11*, *Occludin*, *Gelsolin*, *Jam3* and *Claudin 3* [16, 17, 84]. However, presently it is not known whether the expression of the individual genes encoding BTB proteins is enhanced by classical or non-classical signaling *in vivo*.

Over-Expression of AR in Sertoli Cells Results in Fewer Sertoli and Germ Cells

A gain of function mouse model in which AR levels were increased by an AR transgene expressed only in Sertoli cells resulted in lower numbers of Sertoli cells due to precocious maturation that limited the proliferation window for Sertoli cell proliferation due to the premature expression of AR that was detected as early as PND 2. As a result, testes were smaller because the fewer Sertoli cells present could support only smaller populations of germ cells but, the development of the germ cells was accelerated [67]. The same mouse model having the transgenic AR expressed at even higher levels were infertile (or severely subfertile) with a 70% reduction of Sertoli cells and normal ratios of spermatogonia and spermatocytes to Sertoli cells but with elevated ratios of spermatids to Sertoli cells. The increased germ cell load of the remaining Sertoli cells was associated with increased numbers of apoptotic germ cells and premature release of spermatids, which likely contributed to the observed reduced sperm output [90].

Does Testosterone Regulate the Number of Gonocytes and Spermatogonia?

Whether testosterone regulates the migration and number of gonocyte germ cells in the fetal testis remains controversial. Evidence supporting the idea that AR actions do not influence gonocyte development was found in mice having the AR knocked out in all cells. In these ARKO mice, gonocyte migration to the basement membrane of seminiferous tubules was not altered. Also, gonocyte transformation into spermatogonia stem cells and the expansion of spermatogonia was not dependent upon AR based on the finding that wild type and ARKO mice had similar numbers of germ cells from E 17.5 through P10 [91]. The idea that testosterone signaling via AR does not regulate gonocyte development was supported by another study of 1-day-old mice in which gonocytes are the only germ cells present. At this age, the number of germ cells, Sertoli, and Leydig cells were not altered in ARKO mice [56]. A different study assayed the number of germ cells in 2-day-old mice found that the number of gonocytes present in ARKO mice exceeded those present in wild type [58]. However, the apparent difference in ARKO versus wild type gem cell numbers at 1 and 2 days after birth may be accounted for by the use of two different strategies to compare the number of cells.

Although an earlier study did not detect AR in fetal germ cells including gonocytes [92], there is evidence that active AR is expressed in fetal gonocytes. Immunoreactive AR was found in gonocyte nuclei at E 15.5 dpc and AR transcripts were present in gonocytes purified from E15.5 and E17.5 dpc mouse testis. Furthermore, a functional assay of AR responsiveness suggests that an AR is active in fetal gonocytes at these times [93, 94]. Further studies showed that *Tfm* mice had 40% more gonocytes at E17.5. The increase in gonocytes was associated with an increased percentage of these cells that were proliferating at E15.5 but not at later stages of development. In addition, the number of gonocytes in testis explants from wild type mice was decreased by treatment with DHT [94]. Together, these data argue that testosterone acts to limit the number of gonocytes during fetal development in mice. However, until similar studies are repeated using mice lacking AR only in germ cells, the possibility remains that testosterone effects are being mediated via other AR-expressing cells such as PTM cells.

In prepubertal boys having AR mutations that cause androgen insensitivity syndrome (AIS), there is germ cell maturation delay as gonocytes are uncharacteristically observed in the testes of AIS patients until puberty [95, 96]. In AIS patients, there also is a dramatic loss of germ cells beginning after 2 years of age with few or no premeiotic germ cells present in cross sections of seminiferous tubule by 10 years of age [95]. These data further support the idea that androgen signaling in boys regulates gonocyte and germ cell development prior to puberty and the initiation of spermatogenesis.

Evidence against Expression of Functional AR in Germ Cells after Birth

There have been periodic reports of AR being expressed in adult rodent germ cells [97–101]. However, there is general consensus that AR is either not present or not essential in germ cells after birth [43, 102]. Three functional studies indicate that if expressed, AR is not required in germ cells to complete spermatogenesis: (1) spermatogonial stem cells isolated from *Tfm* mice were able to complete spermatogenesis after transplantation into wild type mice [103], (2) spermatogenesis is not affected in knock out mice in which AR expression is eliminated beginning in early meiosis [104], (3) chimeric mice having both AR defective and wild-type germ cells produced pups from the AR defective germ cells [105].

Expansion of Spermatogonia Is Associated with Testosterone Signaling

Androgen-mediated signals contribute to the expansion of germ cells after birth because by PD5, when only spermatogonial stem cells and the first differentiating spermatogonia are present, germ cell numbers were reduced in ARKO mice by approximately 60% [56]. In contrast, the number of spermatogonia in *hpg* mice that have low endogenous levels of testosterone were not increased by testosterone treatment initiated at day 21 and continuing for 6 weeks [106]. These apparently contradictory results could be explained by the need for testosterone signals soon after birth, perhaps by indirectly supporting the population of spermatogonial stem cells that are expanding at that time.

A progressive decrease in spermatogonia and marked reductions in spermatoocytes and spermatids that cause the mice to become azoospermic was observed in PTM cell-specific AR knock out (PTM-ARKO) mice [71]. Glial-derived neurotrophic factor (GDNF) is a potential AR-regulated signal secreted from PTM cells that is required to maintain spermatogonia. GDNF acts through the GFRA1 and RET co-receptor complex to support the proliferation and maintenance of spermatogonial stem cells [107–110]. Although one group detected GDNF only in Sertoli cells of the adult mouse testis [111], GDNF was shown by others to be expressed in PTM cells [112–114], testicular endothelial cells [115] as well as Sertoli cells [111, 116, 117].

There is evidence that *Gdnf* is an AR target gene in PTM cells. A consensus ARE is located within the mouse *Gdnf* promoter. However, thus far no studies have been reported that assayed AR binding or regulatory activity of the ARE [118]. Testosterone stimulation increased the expression of GDNF mRNA and protein in cultures of enriched mouse PTM cells and testosterone stimulation of co-cultures of PTM cells and undifferentiated spermatogonial increased the proliferation of the spermatogonia. Finally, co-culture of undifferentiated spermatogonia with PTM

cells in the presence of testosterone increased the number and size of germ cell colonies observed after transplantation into recipient mice. Together, these data suggest that in the absence of AR expression in PTM cells, production of GDNF (and/or other factors) is decreased and insufficient GDNF signaling activity is present for stem cell self-renewal, which could explain the progressive loss of spermatogonia [112, 113]. In contrast to the mouse studies, androgen stimulation of PTM cells isolated from human testes did not increase GDNF mRNA or protein levels but expression of AR and a marker of differentiated smooth muscle cells were elevated [119].

Testosterone signaling in Sertoli cells can indirectly regulate gene expression in spermatogonia. The introduction of adenovirus vectors expressing AR in Sertoli cells of SCARKO mice resulted in the activation of the ZBTB16 (PLZF) and c-kit genes that in testis are only expressed in spermatogonia and are associated with the expansion of undifferentiated and differentiated spermatogonia, respectively [32]. It also was found that the non-classical and classical pathways provide different signals to spermatogonia, because only activation of the classical pathway permitted the reestablishment of PLZF transcription; whereas, activation of classical or non-classical signaling resulted in increased expression of the c-Kit gene.

Because the number of spermatogonia is similar in SCARKO and wild type mice [56], it appears that AR-mediated actions in Sertoli cells do not have a large effect on the maintenance of spermatogonia stem germ cells or their conversion to spermatogonia. One potential explanation for this finding is that in Sertoli cells, there is no evidence yet that *Gdnf* expression is regulated by androgen. Instead follicle-stimulating hormone (FSH) is the major known hormonal stimulator of *Gdnf* RNA expression in these cells [118, 120].

Androgen and AR Are Required to Complete Meiosis

The reduction of intratesticular testosterone levels in rodents by hypophysectomy, EDS or GnRH antagonist treatments as well as in *hpg* mouse models results in the significant loss of spermatocytes and spermatids. The loss of these germ cells can be reversed under these conditions by testosterone treatment (reviewed in [121]). Similarly, spermatocyte numbers are reduced and very few round spermatids are produced in ARKO and *Tfm* mice indicating that AR is required for testosterone-mediated support of meiosis [55, 56, 58]. In addition to the previously mentioned AR actions in PTM cells that contribute to maintaining the number of spermatogonia, AR activity is required in Sertoli cells to permit completion of meiosis as evidenced by the progressive loss of primary spermatocytes during stages VI-VII in SCARKO mice [122]. The stage-specific loss of germ cells in the absence of testosterone signaling matches the stages in which AR expression levels normally peak in Sertoli cells, implicating AR-mediated signals from Sertoli cells as being essential for maintaining meiosis. In support of this idea, SCARKO mice have meiosis

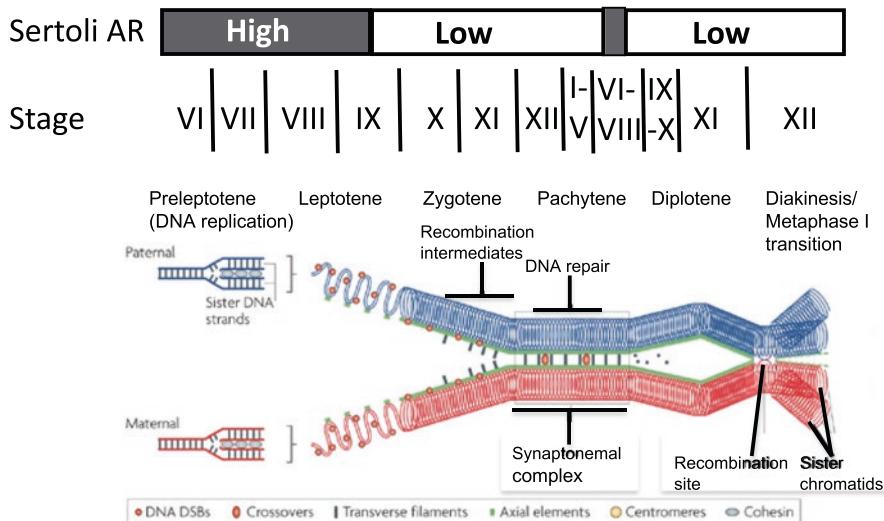


Fig. 2 AR expression levels in the Sertoli cell in relation to the major events of meiosis. The relative levels of AR expression in Sertoli cells (shaded = high expression, clear = low expression) are shown during the mouse stages of the cycle of the seminiferous epithelium (Stages I-XII) in relation to the steps of meiosis from preleptotene through the diakinesis/metaphase transition. Shown are the replication of the maternal and paternal DNA strands during preleptotene, the incorporation of DNA double strand breaks (DSBs) during leptotene, and the binding of SYCP2–3 axial, lateral element proteins, SYCP, SYCE1–3 and Tex12 central element proteins to unsynapsed chromatin during leptotene and zygotene that are required for formation of the synaptonemal complex. The formation of recombination intermediates during zygotene and DNA repair during pachytene are indicated. The formation of the synaptonemal complex and DNA repair that occur during pachytene and the formation of recombination sites during diplotene are also displayed

arrested in the pachytene or diplotene stage of prophase I such that cell division does not occur, and few haploid spermatids are produced [104, 122].

Male germ cells enter into meiosis I normally in the absence of testosterone signaling (Fig. 2). They progress through the leptotene and zygotene stages of meiosis producing double strand DNA breaks that are required for later recombination events and the formation of chiasmata that are needed for separation of the chromosomes. Pairing of the chromosomes (synapsis) is initiated but not completed during pachytene and double strand breaks are not repaired, which inhibits recombination and desynapsis such that 85% of spermatocytes had univalent chromosomes [123]. These events result in the induction of cell cycle checkpoint responses that lead to cell cycle delay or arrest, the apoptosis of the germ cells and the halting of meiosis during pachytene.

The absence of testosterone signaling is associated with the loss of proteins that are essential for the completion of meiosis. In spermatocytes of SCARKO mice,

expression was decreased for RAD51 and DMC1 proteins that are required for the resolution of double strand DNA breaks, recombination and chromosome separation. There is also decreased expression of other proteins (TEX15, BRCA1, BRCA2 and PALB2) that regulate the loading of RAD51 and DMC1 onto the sites of double strand breaks [123]. The effects of the lack of testosterone signaling in Sertoli cells have focused on the morphologically visible halting of meiosis in pachytene. However, it is reasonable to propose that the first and perhaps most significant targets of AR-mediated actions are present in preleptotene and leptotene spermatocytes when proteins normally are produced that are required for chromosome synapsis and to allow passage through cell cycle checkpoints during pachytene. Interestingly, the preleptotene and leptotene stages of meiosis coincide with the peak expression of AR in Sertoli cells during stages VI-VIII providing support for the idea that AR-mediated production of Sertoli cell-derived products are required for chromosome synapsis.

Thus far, the mechanisms by which testosterone and AR-mediated signals from Sertoli cells are delivered to the meiotic cells are not well understood. However, increased signaling by members of the epidermal growth factor (EGF) family of growth factors in the testis was detected in SCARKO mice and may be one mechanism by which decreased AR signaling may alter signals to germ cells [123]. It is possible that testosterone may support meiosis by limiting EGF activity derived from Sertoli cells to specific stages of the cycle of the seminiferous epithelium. This idea is bolstered by the finding that mice constitutively overexpressing the EGF receptor have spermatogenesis blocked during meiosis with a phenotype similar to that of SCARKO mice [123]. Although information regarding the germ cell targets of testosterone signaling from Sertoli cells is beginning to become available, further studies are required to identify products secreted from Sertoli cells in response to testosterone signaling that permit the completion of meiosis.

Androgen Is Required for the Attachment and Release of Elongated Spermatids

Most SCARKO mouse models do not provide information about testosterone support of round and elongated spermatid development because spermatogenesis is arrested in meiosis. However, a hypomorph SCARKO model from Braun's group that displays partial AR activity provided important information about testosterone-regulated events during spermatid maturation. In the Sertoli cell AR hypomorph model, round spermatids are released prematurely due to the reduction in AR activity. Also, terminal differentiation and release of elongated spermatids from Sertoli cells was blocked. The unreleased elongated spermatids were observed to be degenerating between Sertoli cells or were phagocytized by Sertoli cells and transported to the basement membrane for recycling [18, 124, 125]. Similar premature

detachment of step 8 round spermatids was observed in earlier testosterone suppression models [19, 126].

Changes in the Sertoli-spermatid adhesion complexes that occur during the transition to elongated spermatids may explain the premature release of spermatids in the absence of testosterone signaling. Before round spermatids initiate their elongation process in stage VIII, the adhesion complexes between Sertoli cells and germ cells consist of predominately desmosome type connections [127]. During the development of elongated spermatids beginning in stage VIII, a new adhesion complex is formed called the ectoplasmic specialization (ES) that are unique to the testis [128]. It is possible that the expression or localization of proteins needed for the formation of the ES and elongated spermatid attachment may be dependent upon AR signaling in Sertoli cells because suppression of intratesticular testosterone decreases the levels of protein components of ES junctions including $\beta 1$ -integrin, p-FAK and c-Src via the ERK signaling pathway [129].

The paradox of how testosterone can be essential for both the formation of the ES needed for the attachment of elongating spermatids and the release of mature elongated spermatids (sperm) appears to be resolved. During stage VII nearly 24 h prior to the release of sperm, the ES linking the Sertoli cell and elongating spermatid is disassembled and the components are recycled for use at new spermatid adhesion sites [130]. Thus, the ES is no longer present where the sperm are released. After dissolution of the ES, adhesion of elongated spermatids to Sertoli cells is maintained via the tubulobulbar and focal adhesion-related disengagement complexes. Testosterone signaling supports the disengagement of sperm from these complexes [124]. The O'Donnell group provided evidence that only the disengagement complex remains associated with sperm that fail to be released in the absence of testosterone. This complex retains $\alpha 6\beta 1$ integrin and tyrosine-phosphorylated FAK from the ES [124]. Notably, FAK and Src kinase can be phosphorylated in response to non-classical testosterone signaling. Furthermore, inhibition of Src kinase activity resulted in lower levels of sperm release [131]. The idea that components of the disengagement complex could be phosphorylated via a testosterone-mediated pathway is consistent with findings that activation of non-classical signaling targets Src and ERK kinases occurs at the time and location of sperm release in cultured seminiferous tubules [132].

Identification of Androgen-Regulated Genes Using Mouse Models

Reviews of the regulation of gene expression by androgen in the testis were published recently [1, 2]. A summary of gene expression surveys from various rodent models of generalized or cell-specific absences of testosterone signaling is provided here.

Altered testosterone signaling and SCARKO models: The expression of mRNAs isolated from whole testes were studied in neonatal wild type or adult *hpg* mice injected with testosterone propionate for 4–24 h [86, 133, 134], normal vs *tfm* mice [135], AR hypomorph mice [136] and 4 other SCARKO models [17, 83, 84, 137]. One common theme for nearly all of the models was the finding that a greater percentage of genes were down-regulated by AR and testosterone. Nevertheless, the *hpg* and *tfm* mouse model studies determined that testosterone signaling increased the expression of genes encoding the BTB proteins claudin 3 and claudin11 [133], genes associated with vitamin A metabolism, solute transport and cytoskeletal function [135] as well as metabolic processes and signal transduction [138]. The SCARKO mouse models identified AR-repressed genes associated with MAP kinase and serine endoprotease activity and up-regulated genes needed for signal transduction, cell adhesion, Ca^{2+} binding, Ca^{2+} dependent phospholipid binding and serine protease inhibition [83]. Also, there was differential expression of genes encoding proteases, cell adhesion proteins, cytoskeletal elements, extracellular matrix components [17], tight junction proteins, basement membrane components, tissue remodeling factors, paracrine factors for the support of germ cells, transport proteins [84] as well as cell adhesion, nuclear localization and meiosis [137].

SPARKI knock in mice: The SPARKI (Specificity-affecting AR knock in) mouse model expresses a mutant AR that can bind to classical AREs that also can be bound by other steroid hormone receptors. However, AR from SPARKI mice do not bind to selective AREs that are bound only by AR. Expression of genes known to be regulated via AR in Sertoli cells including *Rhox5*, *Tsx* and *Drd4* were dramatically down-regulated in SPARKI mice; whereas, other known AR-regulated genes were not affected [139]. These gene expression studies indicate that some testis genes are regulated via classical AREs, and other genes are regulated through selective AREs or another mechanism, but both mechanisms are required for full fertility.

Ribo-Tag Mouse Models: An important advance in the identification of cell-specific changes in AR-regulated mRNAs is the use of transgenic mice expressing a RiboTag that allows for immunoprecipitation of ribosomes and any associated RNA. Conditional expression of the RiboTag using a cell-specific Cre recombinase allows the isolation of the population of RNAs in the process of translation from individual cell types (the translome). The first use of the RiboTag strategy combined with microarray analysis identified surprisingly few Sertoli cell-specific mRNAs after increasing testicular testosterone levels for four hours [140].

A second RiboTag approach was used in which mRNA expression was compared in 10 day-old mice expressing the RiboTag in an otherwise wild type background (SCRIBO mice) versus a SCARKO background (SCARIBO mice) [141]. This analysis of the ribosome-associated RNAs by RNA-seq yielded the most extensive list of AR-regulated genes in the testis to date. Without first isolating mRNAs from ribosomes of specific cell types, RNA-seq analysis identified 938 AR-regulated genes from all testis cells with slightly more genes being stimulated than inhibited by AR. About 20% (187) of the genes were differentially expressed by two-fold or more. Isolation of mRNAs associated with the ribosomes of Sertoli

cells identified 1090 translated mRNAs that were differentially expressed in response to the loss of AR. The AR-regulated mRNAs enriched in Sertoli cells included those that encoded proteins associated with calcium ion, actin or cytoskeletal protein binding, proteins active at the plasma membrane, adhesion functions including cell-cell junction and anchoring junctions as well as proteins located at the stereocilium. AR was found to be more likely to repress (59%) than activate (41%) Sertoli cell-enriched gene expression. The RiboTag strategy was more sensitive for identifying Sertoli cell-specific AR regulated mRNAs as 55% of the 1090 Sertoli cell-enriched AR-regulated genes found using the RiboTag strategy escaped detection in the less specific whole testis SCARKO vs wild type (SCARIBO vs SCRIBO). Among the AR-regulated genes were several that were not essential but were known to encode proteins that contribute to processes important for male fertility including *Tgfb2* which is involved in SC barrier formation and maintenance; *Nr5a2*, an activator of steroidogenic enzymes and *Timp1*, an inhibitor of matrix metalloproteinases.

The RiboTag approach also found 445 genes that are regulated in other cell types by AR actions in Sertoli cells. These non-Sertoli cell genes encoded proteins involved in binding to adenyl ribonucleotides, adenyl nucleotides, nucleosides, and ATP, which may suggest that the results of androgen signaling in SCs instructs other testicular cells to alter their nucleic acid and energy metabolism [141]. The use of similar RiboTag strategies could be used in the future to identify genes in germ cells at specific stages of development that are regulated by testosterone and AR actions in Sertoli and other somatic cells of the testis.

Although many of the identified AR-regulated genes contribute to maintaining processes that are essential for spermatogenesis and fertility, few are known to be essential. It is possible that the combined effects of AR-regulated gene expression may be required to support specific stages of spermatogenesis. However, further studies are required to identify the mechanisms by which testosterone and AR support specific processes required for spermatogenesis.

Synergism and Antagonism of Androgen and FSH Signaling

The use of the higher primate rhesus monkey model has provided much needed information regarding the initiation of testosterone signaling during testicular development and cross talk between testosterone and FSH signaling pathways. For example, the question of why spermatogenesis does not occur in male infant primates during “mini puberty” three to 6 months after birth when androgen and FSH levels are temporarily elevated has been at least partly explained by the lack of androgen and FSH responsivity in Sertoli cells [142]. It is only during puberty when the serum concentrations of testosterone and FSH rise again and the responsivity of Sertoli cells to androgen and FSH increases that spermatogenesis can be initiated.

Further studies of rhesus monkeys show that androgen treatment can inhibit the expression of some FSH-stimulated genes including *inhibin- β* B and *AMH* in

cultured pubertal but not infant Sertoli cells. In contrast, genes involved in mediating FSH signaling including *FSHR*, *GNAS*, and *RIC8* were induced by androgens in pubertal monkey Sertoli cells (but not infant cells) and androgens further induced expression of the FSH signaling genes as well as genes that regulate germ cell differentiation (*SCF*, *GDNF*) and genes encoding carrier proteins (*ABP*, *TF*) after stimulation with FSH [143]. These results indicate that androgens can perform some of the same gene regulation duties as FSH in Sertoli cells and that androgens and FSH can act in synergy to maximize the expression of some Sertoli cell genes that are required to support spermatogenesis. It has been suggested that androgen-mediated signaling may contribute to the activation of FSH-mediated processes in Sertoli cells that are required for efficient germ cell differentiation [143]. Presently, there are no reports of androgen response elements near the promoters of the genes induced by both androgen and FSH, raising the possibility that the non-classical pathway may be responsible for the androgen-mediated stimulation of gene expression [143].

The ability of androgens to activate some downstream targets of FSH signaling may explain how, after initiation, spermatogenesis can be maintained in the absence of FSH or FSHR. Also, new findings demonstrated that overexpression of a constitutively activated FSHR variant protein in Sertoli cells permits the maintenance of spermatogenesis in the absence of AR [144]. These results may be explained by continuous FSH signaling compensating for the activation of kinases in the non-classical testosterone signaling pathway and/or expression of AR target genes.

Although androgens and FSH can act in synergy to regulate downstream targets in cultured Sertoli cells [143], it is possible that during the cycle of the seminiferous epithelium *in vivo* there is little opportunity for synergism because the expression peaks and nadirs for FSHR and AR occur at opposing stages of the cycle [145, 146]. Instead, the complimentary expression patterns of AR and FSHR during the cycle of the seminiferous epithelium may allow for the continuous expression of certain genes that are essential to maintain spermatogenesis.

Summary and Future Directions

Decades of research have brought us to an understanding of the spermatogenesis processes that are regulated by testosterone. For example, androgen is required for the testis descent into the scrotum. Androgen also is needed for maintenance of the BTB, completion of meiosis, attachment of spermatids and the release of mature elongated spermatids require testosterone signaling in Sertoli cells. In addition, there is also evidence that AR-mediated expression of GDNF in PTM cells contributes to the maintenance of spermatogonial stem cells.

We also know now that in the testis androgen-mediated signals from somatic cells expressing AR are important for establishing the appropriate numbers, maturation state and gene expression profiles of other somatic cells that are required to maintain spermatogenesis. This concept of androgen-mediated interdependence of

the somatic cells is different from older ideas that each somatic cell provided its support to germ cell development in isolation from the other cells. In addition to the testosterone signaling providing for the production of GDNF, BTB maintenance, fluid production and germ cell adhesion, it is not yet clear what other contributions testosterone provides to the niches for the spermatogonial stem cells and the developing germ cells. For example, we do not know whether disruption of the BTB in the absence of testosterone signaling is the major factor responsible for the halting of meiosis or whether the testosterone-mediated expression of secreted factors by Sertoli cells or factors on their plasma membranes are required to ensure that meiotic cells can produce the proteins that permit the progression through all the stages of meiosis.

Thus far, attempts to produce sperm from stem cells in culture have all required a “testis-like” environment including some type of testis tissue to progress through meiosis. These results suggest that either the structure of the testis or factors produced by somatic cells are required to complete meiosis, which increases the relevance of niche factors that are produced in response to testosterone signaling. Therefore, to provide a tissue free culture environment that allows the production of sperm it will be necessary to identify the essential factors produced in response to testosterone signaling. In addition, targeting these essential factors for meiosis or other niche enhancing factors may allow the creation of strategies for the elimination of sperm production *in vivo* and new ideas for male contraceptives.

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Use of Molecular Modeling to Study Spermatogenesis: An Overview Using Proteins in Sertoli Cells



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Spermatogenesis

Spermatogenesis is a controlled organization of cellular events precisely regulated by several endocrine factors such as testosterone, FSH, LH and estrogen, taking place in a well facilitated niche called seminiferous tubule that compromises Sertoli cells and germ cells. Spermatogenesis is essential for production of mature fertile sperm which in turn depends on the careful regulation of varied events such as the formation and restructuring of BTB [Blood-Testis-Barrier], apical ES [Ectoplasmic specialization], and on the coordination of actin and microtubule filaments for the proper transport of germ cells across seminiferous epithelium [SE] towards the lumen of the seminiferous tubule. The protein components of BTB, apical ES, and varied other proteins such as actins, microtubules, kinases are crucial drug targets for inducing alterations in sperm production and also they act as suitable models for understanding the processes in regulating spermatogenesis. The mechanisms of BTB, apical ES restructuring, coordination of cytoskeletons and protein interactions have been identified and extensively studied. Nevertheless, there are several gaps in the functionality of these processes and it is incomplete with respect to the knowledge of structural details, though varied detailed reports directly or indirectly highlight the structural details of these proteins. In the present chapter structural and

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functional details of a few selected proteins that play significant role in sperm production are discussed.

Sertoli Cell Proteins: An Overview

The protein components of BTB, apical ES, actin filaments, microtubules and intermediate filaments play crucial role in the coordinated cascade of events in spermatogenesis. In the course of spermatogenesis, the developing germ cells are transported across the seminiferous epithelium from basal into the adluminal compartment and released as mature spermatozoa into the lumen of seminiferous tubule. The cross sections of seminiferous epithelium display primary germ cells, such as spermatogonia and preleptotene spermatocytes residing in basal part, and developing primary, secondary spermatocytes and spermatids residing in the adluminal part of SE. The basal and the adluminal part is segregated by the specialized blood-testis-barrier [BTB]. BTB is formed by the synchronized components of basal ES, tight junctions [TJ], desmosomes and gap junctions [GJs] that are located in the space between adjacent Sertoli cells. The spermatocytes, after passing through meiosis II division, enter into a process known as spermiogenesis wherein they remain associated with Sertoli cells through the apical ES and stage-specific restructuring of apical ES. It is essential for the release of mature spermatozoa into the lumen. Proteins, such as N-cadherin, nectins of non-actin based adherens junctions [AJ], CAR, JAM-A of TJs, connexin-43 of GJs, desmoglein-2 of desmosomes, $\alpha 6\beta 1$ -integrin and laminin- $\alpha 3\beta 3\gamma 3$ chains of focal adhesion complexes are a few of the well-studied proteins that are involved in the restructuring of BTB and apical ES [1, 2]. In addition, several kinases such as FAK, c-Yes, c-Src form an integral part of BTB and apical ES restructuring [3, 4]. Besides, actin binding proteins such as Eps8 (epidermal growth factor receptor pathway substrate 8) and Arp3 (actin related protein 3) also play crucial role in modulating actin dynamics [5]. Actin acts as the major player in transporting cargo such as spermatocytes and spermatids [6]. Actin filaments form the major part of the apical ES that transport the developing spermatocytes along the apical ES-BTB axis and continued association of actin filaments with the microtubule is essential for regulating spermatogenesis. Microtubule regulating proteins such as MARK4 also play a vital role in maintaining the polarity of round and elongating spermatids and in the restructuring of apical ES [7, 8]. Besides, drug transporters such as P-glycoprotein, BCRP are expressed by developing germ cells and involve in conferring protection to developing sperm by selective influx and efflux of various drugs [9].

Although the functional importance for various proteins expressed in Sertoli cells and germ cells have been elucidated, their structural details remain scarce. Although the structural details were described for a few proteins such as FAK, N-cadherin elsewhere, the structure function co-relation with respect to spermatogenesis has not been addressed effectively. Recently, for a few proteins like MARK4, BCRP, P-glycoprotein, c-Src, c-Yes, the structure and functional mechanisms in

relation to their role in spermatogenesis have been defined using computational structure prediction and molecular docking analysis.

Homology Modelling, Molecular Docking Analysis of Proteins in Understanding Sertoli Cell Function and Spermatogenesis

Computational structure prediction and molecular docking analysis have been used for a long time in understanding the structure-function correlation of proteins. Owing to the difficulties in obtaining the three-dimensional structure by means of experimental methods like crystallography, and NMR, computational methods have come into practice. Also, identifying a suitable inhibitor for a specific protein using experimental methods requires several screening procedures using millions of chemical compounds which are expensive and time consuming. Both computational structure prediction and molecular docking studies have drastically shortened the time span taken for the initial shortlisting of compounds and have proved advantageous in understanding protein structure and in designing counteracting drugs. In understanding spermatogenesis, inhibitor based model systems play a major role in elucidating several cellular processes. Molecular modeling techniques play a crucial and supportive role in understanding structure-function relationships of many important proteins and their correlation with BTB, apical ES, actin filaments and microtubules.

Many such experimental model systems have been proposed using inhibitors such as Bisphenol A [BPA], cadmium, and the male contraceptive, Adjudin. Changes in the spatiotemporal expression of crucial proteins of BTB, apical ES, actin and microtubule were evaluated to understand their role in modulating restructuring of BTB, apical ES, branching/bundling of actin and dynamic stability of microtubules during spermatogenesis. Along with the functional insights offered by these model systems, computational structural prediction and molecular docking studies add valuable information over the mechanism that underlies the inhibition of these proteins by BPA, cadmium and adjudin.

Computational structure prediction and molecular docking analysis in understanding the inhibition mechanism of Adjudin and its insights towards development of male contraceptives.

Adjudin, 1-(2,4-dichlorobenzyl)-1H-indazole-3-carbohydrazide, is a potential non-hormonal male contraceptive and it has been used as the model system to identify several proteins in Sertoli cells that can be used as cellular targets for designing novel male contraceptive agents [10–15]. Of various proteins studied, MARK4, P-glycoprotein and BCRP were a few of them that were identified as important targets for development of novel contraceptives [8, 16, 17].

MARK4 [MAP/microtubule affinity-regulating kinase 4], a752 aa long testis specific isoform was identified as a regulator of microtubule dynamics in Sertoli cells [7, 8]. Microtubules are hollow cylindrical structures that has 13

protofilaments which are constituted by heterodimers of $\alpha\beta$ -tubulins [18]. Besides, γ -tubulin forms the third isoform of tubulin which primarily forms the microtubule organization center [MTOC] from which microtubule protofilaments radiate [19]. Microtubules form specialized organelles that are essential for conferring cell polarization and separation of sister chromatids during mitosis and meiosis and are crucial for intracellular transport. In Sertoli cells, numerous environmental toxicant based model studies elucidate that microtubules along with apical ES involve in transport of cellular cargo that facilitate germ cell division, maturation and also in conferring polarity to the elongating and elongated spermatids [20, 21]. Specifically the studies conducted using 2,5-hexanedione, showed that disruption in microtubule dynamics affects the microtubule mediated transport in Sertoli cells [22]. Furthermore, microtubules also play a vital role in spermatid transport and sperm head shaping. As part of the maturation process, spermatid nuclei are embedded in crypts between adjacent Sertoli cells and their translocation towards luminal edge occurs in a specific manner, during which microtubule is associated with motor proteins such as Dynein [23–25].

The structure of microtubule protofilaments and the effect of toxicants that disrupts the dynamic instability of microtubules are well explained using structural and functional studies of tubulin isoforms α and β which has a common domain organization. They have an N-terminal nucleotide binding domain, followed by the intermediate domain and a terminal tail region. Various studies elucidate the structural organization of tubulin heterodimers and explain how the dynamics of microtubules is affected in presence of toxicants. The crystal structure of taxol bound tubulin structure [PDB ID:1JFF] elucidates the mechanism of a plant based anti-cancer agent Taxol which suppressed dynamics of microtubules [26]. Taxol binds near the conserved M-loop region in the intermediate domain of β -tubulin, wherein it arrests the flexibility of nucleotide binding loops thereby preventing the exchange of GDP for GTP, which substantially affects the elongation phase of protofilament. Another remarkable feature is that Taxol renders the straight conformation in protofilaments instead of curved architecture which is usually seen in presence of GDP in β -tubulin capped filaments. This report sufficiently explains how the dynamic instability of microtubule can be affected by drugs like Taxol which, in turn, disrupt the microtubule-mediated transport of cargos such as cellular organelles and growth factors. This affects germ cell development/maturation and spermatids that are bound to the apical ES of the seminiferous epithelium.

Several microtubule associated proteins [MAPs] have been identified to affect the dynamic instability nature of microtubules [27, 28]. Computational studies are used to understand how other cellular proteins, such as MARK4, which lacks complete structural data can directly associate with microtubules. The binding of intrinsically disordered gamma synuclein can affect resistance against taxol and can help in restoring dynamics of microtubules. The entire mechanism of resistance was elucidated with the help of computational structure prediction, *in silico* protein-protein docking and molecular dynamics simulation studies. It was found that gamma synuclein binds with the C terminal tail region of tubulin heterodimer complex, which

has been proved to be the region for protein-protein interaction between microtubule binding proteins and microtubule [29]. The binding of gamma synuclein to tubulin heterodimer brings changes in the conformation of M-loop of tubulin that eventually affects binding of taxol. Hence, in the presence of gamma synuclein, the restrictive effects imposed by taxol on flexibility of nucleotide binding loops of nucleotide binding domain in both α and β -tubulin is reversed thus restoring the dynamics of microtubule.

In addition to the motor proteins, several microtubule regulating kinases have been identified to bind with microtubule associated proteins and alter the dynamics of microtubules. One such crucial protein is MARK4 which is a novel member of MARK family that exerts direct binding with microtubule [30]. The adjudin based model system has evaluated the importance of MARK4 in spermatogenesis. The results highlight that MARK4 inhibition by adjudin affects its spatio-temporal expression also it resulted in the loss of polarity at spermatid head which leads to premature release of sperm into the lumen of the SE [8]. These studies illustrate that MARK4 can be used as a potential drug target against spermatogenesis to develop potential contraceptives.

One such application of molecular modelling studies involves MARK4 wherein *in silico* modelling was used to study the key structural features of MARK4. It was analyzed and pharmacophore based screening of novel inhibiting molecules was used to identify lead drug molecules against MARK4 [31]. MARK4 belongs to the Microtubule Affinity Regulating Kinases, that is classified as a member of ser/thr kinase family and are homologs of (partition-defective 1) PAR-1proteins [32]. MARK family has four members including MARK4 and they share a similar domain organization. It has a N-terminal kinase domain that holds N and C lobes with the ATP and substrate binding sites at the interface, followed by the conserved CD motif which is shown to be essential for mediating interaction with MARK partners. A charged linker region that connects to the UBA domain, which is by itself unique in terms of exhibiting auto-regulatory effect on kinase domain. It is followed by a long spacer region which is disordered in nature and finally ends in the C-terminal kinase-associated domain [KA1 domain] [33]. The MARK4 was primarily identified as a neuronal protein, whose kinase activity and its modifications were related with Tau associated neuronal disorders [34]. Although MARK1-3 phosphorylates microtubule associated proteins on their tubulin binding domains [35], MARK4 remains unique in exhibiting direct association with microtubules. Experimental studies have shown that MARK4 co-localizes with microtubules and its overexpression effects microtubule bundling. It was, therefore, proposed to be involved in regulating microtubule dynamics [30]. MARK4 is also observed to co-localize with vimentin and regulate cytoskeletal network and cell cycle progression [36]. On other hand, owing to its crucial role in regulating the microtubule dynamics and predominant expression in testis, the role of MARK4 in spermatogenesis was studied [8]. The results revealed that MARK4 exhibits stage specific expression wherein in the apical ES, it is expressed in the stages IV-VI tubules surrounding the entire head of spermatids. In the stage VII tubules it gets translocated towards the concave side of the spermatid head, later in stage VIII it gets dispersed towards the

tip of the spermatid tip and gradually its expression gets decreased. The adjudin model based evaluation of MARK4 revealed that alterations induced in the expression/ function of MARK4 affects the polarity of the elongating spermatids resulting in the mis-orientation of elongating spermatids causing them to get detached from the apical-ES, resulting in premature release of spermatids into the lumen of SE [8]. Thus, MARK4 modulates microtubule dynamics and also participates in cross talk between the cytoskeletons in Sertoli cells, therein identifying itself as a potent target for the development of male contraceptives.

In addition, understanding the structure of MARK4 can also provide insights on how it binds to and regulates microtubule. Computational modeling was employed to predict the kinase-UBA region of MARK4 in the inactive state [31], which is marked by the inward folding of T-loop and the outward displacement of the core helix α C in catalytic kinase domain. Homology based molecular modeling has been crucial in identifying the presence of unique DFG-in Asp-in/ α C helix-outconformation along with the absence of an additional hydrophobic pocket adjacent to the ATP binding site in its inactive state which is highly unusual in the inactive conformation of kinases. It leads to further computational investigation to evaluate the ability of ATP to bind to the catalytic cleft in the inactive state. As expected, the unique conformation of MARK4 catalytic domain has facilitated ATP binding into the cleft with the favorable interactions made by the unique DFG-in motif. The presence of a unique structural feature in MARK4 is a valuable insight that is favorable in the development of MARK4 specific inhibitors. Ligand based pharmacophore search was used over broad spectrum kinase inhibitors to identify key functional groups in the compounds that specifically target MARK4. Molecular docking and high throughput virtual screening [HTVS] methods were used to screen compounds based on their binding efficiency and molecular dynamics simulation studies used to determine the stability of the molecular interactions between MARK4 residues and the screened compounds. Comparison of modeling profile between adjudin and ATP in binding with MARK4 in inactive state has provided useful insights into the mechanism of MARK4 drug interaction. Adjudin has a similar binding profile to ATP and induces similar structural changes (data not published). These studies using computational techniques illustrate that adjudin and newly identified lead compounds can act as potent ATP competitors which unlike other type I and type II kinase inhibitors can be effectively used to inhibit both active and inactive states of MARK4. Thus, computational modeling studies provide insight into the structure and inhibition mechanism of MARK4, whose inhibition hinders the maturation of elongating spermatids and aids in the release of immature spermatids into the lumen leading to disruption in spermatogenesis.

Three dimensional structure of MARK4 is also used to understand how it associates with mammalian target of rapamycin complex 1' mTORC1. Recent studies have identified both mTORC1 and mTORC2 as regulators of BTB dynamics. The downstream signaling molecule of mTORC1, known as rp6 (ribosomal protein 33), activate sp-rpS6-Akt1/2-MMP-9 pathway, which, in turn, activates Arp2, that together with Arp3 forms Arp2/3 complex. It is known to cause de-bundling and truncation of actin microfilaments by mis-localization of TJ proteins (e.g., occludin

and claudin-11) at the Sertoli cell-cell interface, thereby perturbing the TJ-barrier function in BTB [37, 38]. Interestingly, MARK4 is identified to be the negative regulator of mTORC1, wherein MARK4 is observed to negatively regulate the expression of mTORC1 by activation of Rag GTPases, therein identifying MARK4 as an upstream regulator of mTORC1 [39]. This observation is a key finding, since the expression of mTORC1 is essential to cause restructuring of BTB which is achieved by inducing de-bundling of actin bundles to allow the transit of spermato-tids, whereas the integrity of BTB is restored by the negative regulation of MARK4 on mTORC1 activation. Hence an irreversible inhibition of MARK4 can cause the cell to lose control over the restructuring of BTB which could lead to the continued disruption of BTB causing adverse effects that could entirely unsettle the spermatogenesis process. Thus, the predicted structure of MARK4 and insights into its inhibition mechanism can be advantageous to help understand the regulation of Microtubule dynamics, cytoskeleton communication, mTORC1 regulation by MARK4 and for development of inhibitors that could affect these processes for reversible and temporary arrest of sperm production to be used as potential non hormonal male contraceptives.

Computational structure prediction has also facilitated the understanding of adju-din interactions with the drug transporters in the Sertoli cell. Studies reported that the drug transporters like P-glycoprotein and BCRP alter the availability of adju-din. *In silico* interaction studies highlight the interaction mechanism of adjudin with these proteins which can be helpful in designing compounds that could bypass drug transporters and possess better bioavailability to target the intended proteins [9]. P-glycoprotein is observed in the BTB and its expression is observed to be diminished during the stages where BTB restructuring occurs [17]. Indeed, P-glycoprotein is also observed to have structural association with FAK, which is a key regulator of integrin based signaling [40]. Both P-glycoprotein and BCRP are prominent ABC drug transporters located in BTB that act as drug efflux pumps that prevent the transport of drugs such as adjudin into adluminal compartment in the seminiferous epithelium of testis.

The tertiary structure for P-glycoprotein was predicted using the computational homology modelling technique which used the mouse P-glycoprotein as a template [41]. The predicted structure was observed to have homologous monomers attached to each other through specific linkage. They exhibit the inward facing conformation with the cavity being open towards the cytoplasmic surface, with the nucleotide [ATP] binding domain of two monomers located at the C-terminal side which is located on the cytoplasmic surface. Interpolating this to the orientation of P-glycoprotein on BTB, the C terminal side is located towards the basal compartment and the extracellular region facing towards the adluminal compartment. Molecular docking studies show that adjudin can effectively be trapped in the membrane spanning region of P-glycoprotein. Since these drug transporters are selective in transport of molecules across the membrane, BTB in case of Sertoli cell, it can be postulated that the substrate specific ATPase activity is not initiated for adjudin. Hence the trapped adjudin is effluxed towards the basal compartment thereby preventing its entry into the adluminal compartment. Pharmacokinetic studies using

the RNAi against P-glycoprotein has also verified this observation stating that RNAi affected the flux. Thus under normal conditions P-glycoprotein can effectively prevent the entry of adjudin from basal to adluminal compartment, whereas under the condition of RNAi mediated suppression of P-glycoprotein, it cannot eliminate adjudin that has already translocated to the adluminal compartment. Thus in this study, computational modeling and docking studies have effectively identified the morphological and structural conformation of P-glycoprotein and identified key set of residues that are essential for the binding and efflux of adjudin away from the adluminal compartment in SE of Sertoli cells.

In a similar study, the bioavailability of adjudin has been evaluated using another member of ABC drug transporter, BCRP [16, 42]. BCRP, unlike P-glycoprotein whose expression is confined to BTB, is unique in expressing near apical ES confining to the Sertoli cell-spermatid interface. It suggests that in addition to P-glycoprotein, BCRP can act as the second level protector of germ cells from the action of drug molecules. Structurally, BCRP differs from the rest of the members of ABC drug transporters by the organization of its domains. Its N-terminal region begins with the nucleotide binding domain, which is usually located in the C-terminal region of other drug transporters including P-glycoprotein. BCRP also forms a homodimer unit, along with five other units form a single functional hexamer unit in the interface of Sertoli cell-spermatid interface. Structure prediction and *in silico* modelling was used to predict monomer unit as well the hexamer unit of BCRP in both inward and outward conformations using computational structure prediction methods. BCRP adopts a characteristic ‘V’ shaped conformation at its inward facing conformation while adopting ‘X’ shaped conformation at its outward conformation. The switch between the conformations helps in traversing or repelling out the drug molecules away from the apical ES. Results of molecular docking studies with adjudin show that adjudin forms strong hydrogen bonds, Pi-Pi interactions, electrostatic and van der Waals interactions with residues of BCRP. Interaction profile analysis demonstrates how adjudin switches between the interaction with catalytic R465 and R482 in accordance with inward and outward conformations respectively. In both cases, the 3-carbohydrazide moiety of adjudin helps in anchoring adjudin with BCRP, thus suitable replacement of this moiety can help adjudin to evade these interactions for traversing into adluminal compartment. As in P-glycoprotein, the inward facing conformation is observed to efflux adjudin away from reaching the spermatid and other proteins like MARK4 that are located near the spermatid head.

Computational structure prediction and modelling has a profound application in filling the gaps regarding protein structure and to understand how tertiary structure of proteins correlate with their functionality. It also projects important inter domain movements like domain rearrangement and conformational changes during the activation of proteins. Molecular docking and molecular dynamics simulation studies have proven to be instrumental in providing insights into the interaction mechanism within residues as well as between proteins and drug molecules and to establish an interaction profile based on each drug molecule. Understanding the structure of MARK4, BCRP, P-glycoprotein and their interactions with existing drug molecules using computational methods provides a base for further investigations on their

effect in spermatogenesis and towards the design and development of more specific inhibitors.

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Destruction or Reconstruction: A Subtle Liaison between the Proteolytic and Signaling Role of Protein Ubiquitination in Spermatogenesis



Giovanna Berruti

Background

Any cells of an organism have developed mechanisms to sense and respond to their environment. Post-translational protein modifications have evolved as the universal tool that cells have developed to switch on/off dynamic processes. The post-translational protein modification known as protein ubiquitination determines the half-life, stabilization, refolding, translocation and subcellular sorting of proteins crucial for cell physiology [1–7]. Moreover, modification by ubiquitination is critical for the down-regulation of steroid hormone receptors, plasma membrane receptors and transporters, and ion channels [7–14]. Cumulatively, the ubiquitin system functions as the key regulator of proteostasis, i.e., the maintenance of a healthy proteome essential for cell metabolism, cell proliferation and differentiation, organelle biogenesis, and stress adaptation [15].

The ubiquitin (Ub) system is complex existing different hierarchical relationships among its various components [16, 17]. The signaling molecule resides in the 76-amino acid, 8.5 kDa Ub moiety covalently ligated to the selected protein through a series of actions that require the involvement of ubiquitin-activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s), and ubiquitin protein ligases (E3s) [18–20]. E1s, E2s, and E3s work cooperatively to hallmark the protein in question by catalyzing the formation of an isopeptide bond between the C-terminal glycine of Ub and the amino group on the side chain of a lysine residue in the tagged protein (Fig. 1). Recent evidence has shown that Ub can also be attached to other residues, including cysteines, serines, threonines, and the N-terminus of the polypeptide backbone [21–25]. Further, the Ub tag in the substrate protein could consist in

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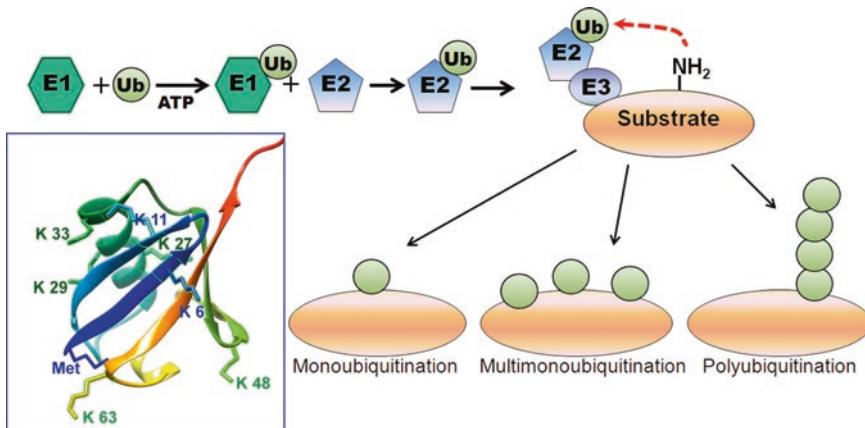


Fig. 1 Patterns of protein ubiquitination. Upper half of the cartoon, The addition of Ub units at specific lysine residues on the target substrate occurs through a series of actions that require the involvement of ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin protein ligase (E3); Lower half, Different topologies of ubiquitination: mono-ubiquitination occurs at a defined lysine residue while there is multi-mono-ubiquitination when multiple lysine residues are modified with one ubiquitin each. Poly-ubiquitination occurs when a polymeric chain of a substrate-attached Ub is just added to the substrate. These chains can be short and contain only two Ub molecules or long and incorporate more than ten Ub moieties. Here, it is illustrated the chain of four Ub molecules, i.e., the canonical tag for proteasomal degradation. In the boxed area, structure of Ub showing the seven lysine (K) residues and the methionine (Met) at the amino-terminus

mono-, multi- and poly-ubiquitination. The lysine residues present in Ub are seven, namely, Lys6, Lys11, Lys27, Lys29, Lys33, Lys48 and Lys63 (Figs. 1 and 2). It follows that in polyubiquitination, additional Ub moieties can be added through conjugation to the same internal Lys residue as in the previously bound Ub moiety; this topology gives rise to homotypic Ub chains (Fig. 2). It has to be highlighted, however, that in addition to homotypic chains: (a) mixed Ub chains can be generated if different linkages alternate at succeeding positions of the chain (Fig. 2); (b) certain E3 ligases can generate branched Ub structures via Ub lysines 6, 27, and 48 through autoubiquitination [26] (Fig. 2). The different Ub-chain topologies thus resulting dictate different physiological consequences [23–25, 27] (Fig. 2). For instance, it was proposed that Lys48-based polyubiquitination serves as a signal for degradation by the proteasome [16], while monoubiquitination is rather considered a signal for non-proteolytic functions such as histone modification involved in epigenetic control of gene expression [28], budding of viruses [29], and cytoskeleton arrangement [30]. To add to the complexity, recent studies, however, have shown that mono- and multi-monoubiquitination could serve as degradation signal for some tagged proteins [27].

Eukaryotic cells dispose of two major degradation systems, the ubiquitin–proteasome system (UPS) and the autophagy–lysosome pathway (ALP). The UPS is the canonical proteolytic route for cytosolic damaged, misfolded, and/or short-lived

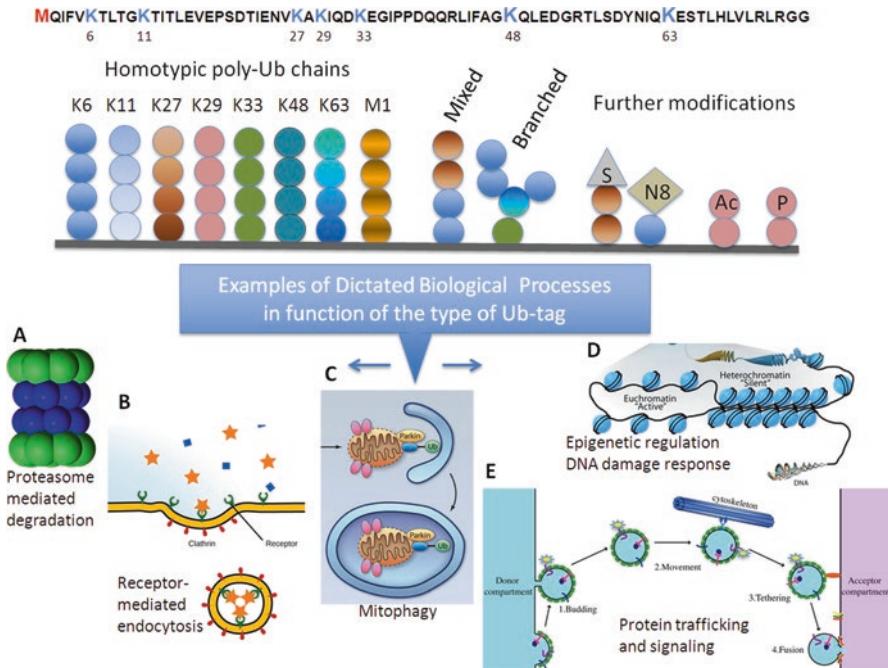


Fig. 2 Complexity of the Ub code. Upper half of the cartoon, The amino-acid sequence of Ub is reported; the lysine residues (K), evidenced in blue, are numbered starting from the N-terminus methionine 1 (M, red). Below, schematic representation of the increasing complexity of the poly-ubiquitin modification. Mono-ubiquitin can be extended on K-residues or the N-terminal M, thus giving rise to eight homotypic poly-ubiquitin chains. In addition to homotypic chains, there are heterotypic chains that are generated with more than one type of linkage in mixed or branched polymers. Further complexity rises from the cross talk between Ub and Ub-like proteins, such as SUMO (S) and Nedd8 (N8), or other post-translational modifications like acetylation (Ac) and phosphorylation (P). Lower half, Physiological roles associated with different types of Ub tags. (A). Proteasome-mediated degradation has been traditionally associated to poly-ubiquitination via K48; (B). Receptor-mediated endocytosis is often associated to multi-mono-ubiquitination as well as mono-ubiquitination; (C). K6- and K63-linked Ub chains are related to mitophagy while K27-linked poly-ubiquitin is involved in autophagy; (D). Mono-ubiquitination is associated to DNA damage while K63-linked chains are related to nuclear protein interactions as well as sumoylation is related to chromatin/heterochromatin modifications; (E). Intracellular protein trafficking and signaling (earmarked by yellow star) are associated individually to specific types of poly-ubiquitination (K63-linked, M1-linked, K11/K63 mixed and defined branched chains) as well as to mono-ubiquitination

proteins [15, 16, 31]. The ALP functions predominantly in the degradation of bio-macromolecules delivered by way of endocytosis, phagocytosis, autophagy, or bio-synthetic transport [15, 31, 32], including the removal of dysfunctional or superfluous cellular organelles. Albeit apparently functionally separated, the two systems intersect and communicate; the turnover of proteasomes (proteaphagy) is, in fact, mediated by autophagy [33] and how the UPS and ALP collaborate has been recently dissected and elucidated by studying the control of ribosome recycling and turnover

[34]. At the present state of knowledge with canonical certainties put in discussion (for example, no essentials of Lys48-based poly-ubiquitin chains for proteasome targeting), a not yet clarified enigma remains, i.e., which is/are the typology/ies of Ub signature, if any, necessary for substrate targeting to the either UPS or ALP systems. UPS and ALP, more recently, have been the object of extensive studies and documentation. For this reason, from a general point of view, I remind to refer to the excellent reviews, some of which reported here, already available in the literature to avoid redundant repetitions.

Protein ubiquitination, however, regulates also non-proteolytic events, including membrane protein trafficking, vesicular-based transport, synaptic plasticity, protein kinase activation, DNA repair, and chromatin dynamic [5, 11, 17, 20, 35–37]. To date, several reports have shown how ubiquitination is critical for proper protein localization and recognition by signaling and regulatory complexes, thus affecting from a general point of view cellular signaling and/or homeostasis [6, 24, 25, 38, 39]. In such a context, Lys63-linked Ub chains were assumed to have a non-degradative role in cellular signaling and intracellular trafficking [24, 40] (Fig. 2).

Ubiquitination of target proteins is a reversible modification. The enzymes that oppose the function of E3 ligases are known as deubiquitinases (DUBs) [41–47]. Protein deubiquitination is important for several reasons. When it occurs before the commitment of a substrate to either UPS or ALP proteolysis, it negatively regulates protein degradation. To this regards, it has been suggested a kind of proofreading mechanism wherein ubiquitin is removed from proteins inappropriately targeted to the proteolysis [41]. On the other hand, surely some DUBs work as parts of the proteasome itself by removing Ub moieties from proteins committed to degradation; thereby these DUBs allows the recycling of free Ub within the cell and keep the proteasome free of unanchored Ub chains that can compete with ubiquitinated substrates for Ub-binding sites [43]. Evidence has also accumulated about the different ways through which DUBs recruit their protein substrates. There are DUBs that bind directly to an Ub signal that they just then cleave without any direct relation with the protein substrate (a kind of aspecific housekeeping enzyme) [41–43], and there are DUBs that target either selected types of Ub chains [46–48] or selected proteins [47], thus influencing specifically peculiar cellular pathways and/or processes. These DUBs, besides the canonical catalytic domain, possess additional protein-protein interaction domains. In brief, DUBs are far from being uniform in structure and function and this feature may explain because progress in understanding DUB function has lagged behind that of the Ub conjugation machinery. The human genome encodes about 100 DUBs while about 20 DUBs exist in *Saccharomyces cerevisiae*. At the present, six structurally distinct DUB families have been described: Ub-C-terminal hydrolases (UCHs; 4 members in humans), Ub-specific proteases (USPs; 54 human members), ovarian tumour proteases (OTUs; 16 human members), the Josephin family (4 human members), the motif interacting with ubiquitin (MIU)- containing novel DUB family (MINDYs; 4 human members) and a family of Zn-dependent JAB1/MPN/MOV34 metalloenzymes (16 human members) [49].

Spermatogenesis is the process that leads to the production, starting from a pool of diploid stem cells named spermatogonia, of highly differentiated haploid spermatozoa. The complexity of spermatogenesis relies collectively on the coexistence of mitosis, meiosis, and cell differentiation in a unique process. The Ub system is essential for the proteostasis of each eukaryotic cell; there are however a number of properties that appear to be peculiarly related to the processes that articulate spermatogenesis. Albeit not intended to be an exhaustive review, some recently emerged aspects of Ub system in spermatogenesis that are discussed here can serve as a guide for tackling future studies on this topic.

Proteolytic Functions of the Ub System along Spermatogenesis

Spermatogonial Development

To date, the role of the UPS in protein turnover in spermatogonial stem cells (SSCs)/spermatogonia (SPGs) has been rather unexplored. In the testis while some SSCs self-renewal, other SSCs differentiate; this occurs for the entire adult life of the male. In vitro, SSCs undergo asymmetric divisions; thereby, it was proposed that SSC asymmetric divisions contribute to their self-renewal and differentiation [50]. One of the first members of the Ub system discovered to be present in the testis was the deubiquitinating enzyme Uchl1, otherwise known as PGP 9.5, [51]. SSCs/SPGs express Uchl1 that regenerates monoubiquitin from ubiquitinated proteins targeted to the proteasome. Interestingly, Uchl1 was found to segregate asymmetrically to the two daughter cells from asymmetric division and, more specifically, the daughter cell with a high Uchl1 level resulted to be positive to the undifferentiated spermatogonial marker Plzf; conversely, the one with a low Uchl1 level expressed the differentiated spermatogonial marker c-Kit [52]. Remarkably, the authors succeeded to show asymmetric segregation of Uchl1 and Plzf not only with cultured SSCs, but also *in situ* in seminiferous tubules [52]. This suggests that a full functionality of Uchl1 is consistent with maintaining the undifferentiated state of SSCs. It is however fair to remember that there is no general agreement about the possibility of asymmetric division of SSCs *in vivo* in mammals [53]. A recent, sophisticated study suggests a different and generic mechanism underlying the fate of SSCs: these stem cells appear not to rely on asymmetric division, but SSCs with higher level of SHISA6, a cell-autonomous Wnt inhibitor, remain in the undifferentiated cell pool, while those with lower levels of the inhibitor are inclined to differentiate [54].

The HR23B gene encodes a mammalian homolog of *Saccharomyces cerevisiae* RAD23, a nuclear protein containing an ubiquitin-like domain involved in nucleotide excision repair (NER). Defective NER is associated with three clinically and genetically heterogeneous human syndromes: xeroderma pigmentosum (XP), Cockayne syndrome (CS), and trichothiodystrophy (TTD) [55]. HR23B gene has been validated as a sensitivity determinant for histone deacetylase inhibitor

(HDACI)-induced apoptosis so that HR23B^{-/-} mice were generated to assay its biological relevance in vivo [56]. Unexpectedly, HR23B deficiency did not result in a NER defect, but HR23B^{-/-} mice showed impaired embryonic development and, if survived, in addition to a retarded growth, male mice resulted to be sterile. This sterility is consequence of an early failure of spermatogenesis since HR23B knock-out yields a phenotype like that known as Sertoli cell-only syndrome [56], with the total absence of male germ cells. Recently, HR23B/RAD23 have been shown to interact with the proteasome to which it delivers multi-ubiquitinated proteins and to play a proteolytic role involved not only in NER, but also in stress response, transcription and ER-associated protein degradation [57].

By gene expression analysis using microarrays, numerous genes codifying for gene products related to the Ub system have been identified in SSCs/SPGs. Among these there are the E1 enzymes Uba1 and Uba6, E2 enzyme Ube2, E3 enzymes Huwe1, Trim47, and Rnf149 [58]. A part the evidence of their expression, to date these UPS genes are still waiting for elucidation of their specific functions in SSC/SPG biology. An exception could be Huwe1. Despite the fact that Huwe1 has a broad range of tissue expression with a cytoplasmic protein distribution, in neurons and spermatogonia/pachytene spermatocytes the E3 ubiquitin ligase is present in the nucleus [59]. This aspect, together with the fact that *in vitro* experiments have shown that Huwe1 binds to the E2 enzyme UBC4 thus ubiquitinating the histones H2A and H2B [60], has led to propose that Huwe1 plays an important role in the proliferation and differentiation of spermatogonia, as well as in the regulation of sex chromosomes inactivation during meiosis. Collectively, these roles have been experimentally confirmed by inactivation of the Hewel gene at key stages during spermatogenesis [61].

Meiosis

The importance of the Ub system during meiosis (and mitosis) emerged outstanding since the discovery (late 1980s/early 1990s), through studies of cell division in frog and clam oocytes [62, 63], that it is the cyclin-Cdk complex to drive cells into M phase and that the concentration of cyclin fluctuates, rising gradually during interphase to then falling rapidly to zero at the end of M prophase.

Meiosis is a highly regulated cell division process subdivided in sequential phases such as meiotic entry, genetic recombination of homologous chromosomes, meiotic sex chromosome inactivation (MSCI), double cell division, and meiotic exit. Despite temporal differences in their meiotic programs, both sexes require a fully functional Ub system, like various studies exploiting gene-targeting technologies have shown. Given the complexity of the epigenetic regulation, including that relies on the ubiquitin code, of meiosis in mammals, I suggest here to refer to published reviews [64–66]. Below, a few representative examples related to UPS in spermatogenesis are considered.

Meiotic Entry

Fizzy-related 1 (FZR1) is an activator of the Anaphase promoting complex/cyclosome (APC/C), an E3 ubiquitin ligase that promotes the metaphase-anaphase transition in mitosis [67]. Recently, it is emerging a role for APC/C^{FZR1} activity in oocyte meiosis [68] while loss of APC/C^{FZR1} activity in the male germline leads to infertility due to the absence of mature spermatozoa [69]. As to the male infertility, spermatogonia of APC/C^{FZR1-/-} undergo abnormal proliferation and show delayed entry into meiosis. Although early recombination events could initiate, the developing spermatocytes fail to progress beyond zygotene and undergo apoptosis. The authors [69] proposed that the requirement for APC/C^{FZR1}-mediated cyclin B1 degradation is crucial in early meiosis because cyclin B1 must be kept in check during early prophase I so to allow the completion of recombination events.

Meiotic Progression

There are a number of UPS genes that have been described to be potentially involved at various time-points of meiotic progression, like the components of APC/C complex cited above, (for a review, see ref. [58]); for most of them, however, the effective function of their gene products during spermatogenesis has yet to be elucidated. Proteolysis is rightly thought to be essential in regulating the cell cycle also in meiosis; checkpoint protein requirements, however, are emerged to be different between somatic cell mitosis and germ cell meiosis. Accurate chromosome segregation during spermatogenesis, for instance, is highly dependent on BubR1, but not Mad2, Bub3, Rae1 and Nup98 that are all components of the mitotic spindle checkpoint, the stability of which is down-regulated through the UPS [70]. Therefore it may be that the control of meiotic cell cycle is, at least partially, under the surveillance of non-conventional and germ cell signal-responsive variants of the Ub system. This topic deserves to be better defined in the future.

Spermiogenesis

Spermiogenesis is an extraordinary differentiation process through which haploid round spermatids metamorphose to become streamlined spermatozoa with an extreme cell polarization and a flagellum conferring the capacity to move. In higher eukaryotes spermatozoa are in fact the only cells that leave their organism to exert out of the confines of the body their basic function, fertilization. The differentiation steps include the morphological changes occurring as consequence of the nuclear chromatin compaction, acrosome formation, axoneme development, mitochondria reorganization, head elongation, tail assembling and, last but not least, removal of most cytoplasm and cellular structures/organelles now acting only as a rubbish.

Chromatin Condensation

Chromatin remodeling is a gradual process continuing from spermatogonia proliferation, through meiosis, to the formation of mature sperm. To acquire its stream-like shape and simultaneously protect the genetic material during the long trip to the egg, sperm have evolved a peculiar chromatin organization. Briefly, nucleosomes are disassembled, transition proteins replace somatic and testis-specific histones and, in turn, protamines replace transition proteins. As a consequence, sperm acquires a highly condensed and inaccessible organization of chromatin with haploid DNA efficiently packaged into peculiar, small donut-shaped, organizational units known as toroids [71]. Interest in studying the regulation of sperm chromatin organization is multiple, because any alterations in protein composition or integrity of the chromatin may contribute to male infertility.

Moreover, many proteins that function only temporarily are formed during replacement of nucleoproteins [72, 73]. An example is the H2A histone variant H2A.Lap1. H2A.Lap1 dynamically loads onto the inactive X chromosome in later stages of round spermatid, thus enabling the transcriptional activation of genes that were previously repressed, i.e., the MSCI (meiotic sex chromosome inactivation) silenced genes [74]. As parenthetic clause, it is here noticed that the activation of sex-linked genes required for male reproduction is briefly resumed successively in the section of this minireview devoted to non-proteolytic functions of the Ub system along spermatogenesis. The histone-to-protamine transition is however essential to produce fertile spermatozoa; consequently, histones as well as the temporarily functioning/transition proteins must be eliminated. The presence of efficient degradation machinery, possibly residing within the nucleus of spermatids themselves, is therefore mandatory. The UPS provides, at least in part, this machinery. Histones are in fact targets of multiple dynamic post-translational modifications, particularly on their C-terminal tails. The major ones are acetylation, methylation, phosphorylation, sumoylation, and, last but not least, ubiquitination [73, 75]. A testis-specific isoform of the E2 enzyme UBC4, namely UBC4-testis, is induced in round spermatids and early elongated spermatids [76]. UBC4-testis interacts with the testis-specific E3 ligase E3Histone/LASU1 in ubiquitinating histones H1, H2A, H2B, H3 and H4 at early spermiogenesis [60]. The poly-ubiquitinated histones are then targeted to the proteasome for degradation. Evidence of the presence of poly-ubiquitinated proteins as well as of proteasomes in the nuclei of both rodent and human spermatids has been provided [77]. Lastly, it could be worth of mention to remember that studies in *Drosophila melanogaster* have shown that 12 of the 33 subunits of the 26 S proteasome are represented by paralogous genes [78] and, in each case, one of the paralogs is testis-specific [79]. Knock out of one of these paralogs, namely a6T, is resulted in abnormal nuclear morphology and maturation yielding a male sterile phenotype [80] suggesting a role of sperm proteasome in chromatin remodeling.

Acrosome Biogenesis and Axoneme Development

Acrosome formation has been described to be coupled to an increase in anti-ubiquitin labeling [81]; further, proteasomes have been found to mark the surface of the developing acrosome [82] and an E3 ligase, UBR7, has been identified inside the acrosome [83]. A direct involvement of the proteolytic function of UPS in the biogenesis of the vacuole, however, has still to be found. Parallelly, E3 Ub ligases like MARCH10 [84] and MARCH7 [85] as well as proteasomes [82] have been detected in the mammalian sperm flagellum. Interestingly, spermatozoa from male mice deficient for the Ub ligase HERC4 are resulted to be affected with a 50% reduction in tail motility, although HERC4 substrate targets remain elusive [86]. To date it is, however, not still defined a role of the UPS-mediated degradation in the axoneme formation.

Mitochondria Reorganization

During spermatogenesis the number of mitochondria undergoes spatiotemporal variation. Mammalian spermatogonia have about 2000–3000 mitochondria/cell whereas a mammalian sperm has about 70–80 mitochondria, all confined in the mitochondrial sheath that is the final element of the sperm tail to be formed. Remarkably, the number of mitochondria decreases dramatically, concomitantly with their peculiar redistribution, in the haploid phase of spermatogenesis. Thereby, spermiogenesis implies a massive mitochondrial reorganization that requires, from one hand, removal/degradation of most organelles and, from the other hand, the microtubule-mediated transport of mitochondria to the forming tail midpiece [87]. It is to decipher and clarify on the basis of which mechanism most mitochondria are lost but a number survives going to relocate at the tail mitochondrial sheath. This aspect has so far received minimal attention. There could be a signal that hallmarks the mitochondria that have to be eliminated or, vice versa, that have to be saved. Alternatively, the two different fates, i.e., survival or elimination, could reside in a merely casual passive selection. Undoubtedly, elongating spermatids to transform into elongated spermatids loss most their cytoplasm, where the majority of mitochondria reside, and this occurs in the form of the cytoplasmic lobe/residual body that is then resorbed mostly by Sertoli cells [88]. Such phenomenon exhibits the feature and fate of a phagosome englobed in the host cell; in other words, sperm mitochondria are not eliminated through endogenous mitophagy, i.e., the autophagy-mediated destruction of damaged mitochondria. On the other hand, mitochondria in both spermatids and mature sperm are ubiquitinated [89]. Although the exact mechanism that supervises such (global or partial?) ubiquitination is still vague, this indicates that the mitochondria destined to the tail mid-piece, the only to be present in mature sperm, are ubiquitinated. This argument will be further considered in the section “**Passing of the baton between Non-Proteolytic functions and Proteolytic functions of the Ub system in sperm biology**”.

The other crucial aspect, in addition to their massive removal, of mitochondria reorganization during spermiogenesis is the formation of the tail mitochondrial sheath, a sperm-specific structure wrapping both the axonemal complex and nine outer dense fibers in a left-handed double helical array. It is evident that becoming part of an ‘ex novo’ developed organelle that highly polarizes the differentiated sperm, the flagellum, requires unique structural-remodeling capacities and a well-functioning cytoskeleton with its ability to transport protein complexes, vesicles, and organelle over long distances. As to this topic I suggest to read the review [87], albeit the written ‘codes’ that determine which cargos bind to a protein adaptor or motor protein, differently from somatic cells, remain virtually unknown.

Non-proteolytic Functions of the Ub System along Spermatogenesis

Ubiquitination could also regulate non-proteolytic events such as protein activity, protein interactions and subcellular localization [25], transport of newly synthesized mature proteins to the membranes [3, 13], DNA damage repair and DNA replication mechanisms [90], and cellular signaling by recruiting proteins to concur to particular signaling pathways like inflammatory signaling and apoptotic cell death [91]. As already said, the complexity of spermatogenesis relies on the coexistence of mitosis, meiosis, and cell differentiation in a unique process where the Ub system plays direct role also in non-proteolytic (NP) control mechanisms. For instance, modifications in chromatin organization are related not only to the histone-to-protamine transition with related histone degradation, but also to peculiar phenomena like the meiotic synapsis and desynapsis of chromosomes, homologous recombination, and meiotic sex chromosome inactivation (MSCI). Parallelly, the metamorphosis of haploid spermatids into spermatozoa is accomplished also through non-proteolytic functions by components of the Ub system. For space constraints and convenience of clarity for the readers, here I highlight only few aspects as representative of the topic that heads this paragraph.

NP-Ub System and Meiotic DNA

Histone ubiquitination can both stimulate and repress various cellular processes [92]. For example, ubiquitination of H2A at gene promoter regions suppresses gene transcription [93–95], while intragenic ubiquitination of H2B facilitates transcription elongation [96–99]. Histone ubiquitination is also associated with DNA damage responses where H2A and H2B ubiquitination are enriched at sites of DNA damage [100–103]. These post-translation chromatin modifications can occur both

in somatic and germ cells and they are not further discussed here. Below, events characterized to be spermatogenic-specific are reported.

During mammalian male meiosis, the heteromorphic sex chromosomes (X and Y) condense in a separate chromatin domain known as the XY or sex body [104]. X and Y chromosomes are largely heterologous and show homologous synapsis only at the small pseudoautosomal region (PAR). BRCA1, a multifunctional DNA repair protein that possesses transcriptional, ubiquitin ligase (E3), and heterochromatin-related gene silencing activity [105], detects the non-synapsis regions of chromosomal axial elements and promotes the localization of ATR, a member of the PI3-like kinase family, to the X, Y chromosomes; this will lead to the phosphorylation of histone H2AX at serine 139 [106]. Hence, i.e., from its formation on, the XY body is positive for phosphorylated H2AX [107]. Phosphorylation of H2AX initiates repression of genes on the sex chromosomes [108], a process called ‘meiotic sex chromosome inactivation’ (MSCI). Remarkably, because all this process can occur, H2AX needs to be previously ubiquitinated; the polycomb repressive complexes PRC1 and PRC2 catalyse the mono-ubiquitination of H2AX at lysine 119 [75], giving thus the start to the process of MSCI. Moreover, the E2 enzyme HR6B, which is enriched in the XY body of pachytene spermatocytes, functions in the maintenance of X chromosome silencing in both spermatocytes and spermatids. Conversely, *Hrb6*-KO mice show derepression of X-chromosomal gene activity that leads to abnormal global upregulation of gene transcription from the X chromosome [109].

RNF8 possesses a RING domain at its C-terminus and is counted among the E3 Ub ligases. RNF8 also participates in the DNA damage response and ubiquitinates histones, promoting the recruitment of downstream DNA damage response factors, such as 53BP1, BRCA1 and Rad51 [100, 110, 111]. It was suggested that RNF8 could play an important role in the chromatin remodeling occurring during MSCI. A study carried out to check such a hypothesis led to the generation of *RNF8*-deficient mice uncovering, however, a surprising result. Absence of H2A ubiquitination by RNF8 in spermatocytes of *RNF8*-KO mice did not affect XY body formation, MSCI, or meiotic progression, but its absence in elongating spermatids invalidated nucleosome removal at the histone-to-protamine transition; consequently, this results in a defective spermiogenesis and infertility [95]. Moreover, a further study [112] has shown that ubiquitination-deficient mutations in MIWI protein, a *Piwi* family member - Piwi proteins are essentials for gametogenesis in animals [113] - cause male infertility. The Authors [112] have identified the culprit; MIWI binds, sequestering it, RNF8 in the cytoplasm of early spermatids and MIWI degradation by APC/C in late spermatids is required for nuclear translocation of RNF8. However, ubiquitination-deficient mutations in MIWI prevent MIWI from degradation so that RNF8 cannot translocate into the nucleus to catalyze histone ubiquitination and trigger histone removal.

As last mention to illustrate the ubiquitin regulatory network that is at the head of the meiotic sex-genes silencing/postmeiotic sex-genes activation, I report the intriguing model proposed by Adams and co-workers [114]. According to the authors “regulation of ubiquitin leads to the organization of poised enhancers and promoters during meiosis, which induce subsequent gene activation from the otherwise silent sex chromosomes in postmeiotic spermatids”. Due to MSCI, silenced sex-genes must escape silencing for activation in spermatids thereby ensuring their function for male reproduction. Adams et al. [114] succeeded in demonstrating that RNF8 and SCML2, a germ-line specific Polycomb protein, cooperate to regulate histone ubiquitination during meiosis to establish active histone modifications for subsequent gene activation. For example, the authors show that SCML2 deubiquitinates RNF8-independent H2AK119ub but does not deubiquitinate RNF8-dependent polyubiquitination. RNF8-dependent polyubiquitination is required for the establishment of H3K27 acetylation, a marker of active enhancers, while persistent H2AK119ub inhibits establishment of H3K27 acetylation.

As concluding remark, I make a general recall to the epigenetic of the male gamete. Major epigenetic signatures include DNA methylation, histone modifications/localizations, and expression profiles of non-coding RNAs. These marks drive gene expression patterns in the cell and have a profound impact in the early phases of embryo development. Fertile mammalian sperm have epigenetic modifications consistent with gene ‘poising’ at the promoters of genes involved in development; these epigenetic signatures include the localization of retained histones that are not removed at the histone-to protamine transition and are signed by ubiquitin modifications. The field of epigenetics is burgeoning, thus I invite the readers to give a glance at this topic [114–117].

NP-Ub System and Spermiogenesis

The post-genomic era has provided insight into the complexity of the Ub system [7]; comprehensive proteomics studies have identified tens-of-thousands of ubiquitination sites on thousands of proteins [118]. As reported above, proteins can be mono-, multi-, poly-ubiquitinated while Ub molecule has seven Lys residues all of which can be ubiquitinated; moreover, new emerging findings reveal that Ub can be not only ubiquitinated, but also modified by other modifications. These last include SUMOylation, i.e., the addition of the Ub-like modifier protein SUMO that is conceptually similar to polyubiquitin chain formation [119], phosphorylation [120, 121] and acetylation [122]. Altogether, these complex patterns constitute a ‘ubiquitin code’, which is read by hundreds of proteins that incorporate ubiquitin-binding domains. Thus the more recent experimental evidence has shaken the long-standing dogma according to which Ub constitutes a targeting signal for protein degradation only. As to the Ub system and spermiogenesis, however, it remains a consistent part of work to be still done; most studies in the literature, in fact, have been devoted to sperm ubiquitination as ‘degradative’ signal only.

NP-Ub System and Activation of Silenced Genes

As to this topic, I send back to the works just discussed above dealing with the male epigenome, in particular to the silencing/activation of sex-linked genes.

NP-Ub System and Acrosome Biogenesis

Protein ubiquitination functions in protein trafficking in both endocytic and secretory pathways [32]. One of the cytomorphogenic events that hallmark spermiogenesis is the biogenesis of the acrosome. The acrosome is a unique membranous organelle located over the anterior part of the sperm nucleus, rich of hydrolytic enzymes and considered to be indispensable for fertilization [123]. Originally described as a modified lysosome, it was then proposed as a direct Golgi-derived secretory vesicle and, more recently, as a lysosome-related organelle (LRO) [124, 125]. According to the experimental evidence that both the endocytic and biosynthetic machineries concur to acrosome biogenesis [123–128] and that acrosome functionality reflects the modular LRO-like structural organization [123, 128], the notion that the acrosome is a LRO is now currently accepted. The endosomal system constitutes a network of progressively maturing vesicles characterized by modular organization, high spatial regulation and interconnectivity with the biosynthetic route. The ubiquitin-specific protease USP8, originally named UBP_y [129], participates in the endosomal sorting of transmembrane proteins both in somatic and male germ cells [127, 128, 130–133] where it interacts with the proline-rich SH3 domain of the signal-transducing adaptor Hbp/STAM2, a component of the endosomal sorting complex ESCRT-0 [124, 132]. USP8 deubiquitinates both cargo proteins, typically signaling molecules as transmembrane tyrosine kinase receptors [129–131, 133], and ESCRT-0 proteins [132] thus modulating both the function of the signaling molecules and the stability of components of the endosomal trafficking machinery (for a recent review see [134]). The proteolytic cleavage of USP8 increases the DUB enzymatic activity whereas USP8 phosphorylation-dependent association with 14–3–3 proteins inhibits the deubiquitinating activity [135]. Somatic mutations of human USP8 cause Cushing's disease [136, 137] and defects in the down-regulation of USP8 are found more and more to be related to tumorigenesis [134, 138]. As to germ cells, human *USP8* has been identified as candidate gene for male fertility traits [139, 140]. Consistently with the above findings, USP8 is resulted to cooperate in acrosome biogenesis by regulating the trafficking of vesicular cargoes destined to the forming LRO; one of such cargos has been identified in a molecular variant of the tyrosine kinase membrane receptor MET [127]. As further remark, it is to remember that USP8 possesses a MIT (microtubule interacting and trafficking/transport) domain at its aminus-terminus, which could provide a direct link between the sorted vesicular cargo and microtubules [124]. Spermatids are characterized to exhibit peculiar microtubule arrays, such as the cortical microtubule network (early spermatids), the manchette (elongating spermatids), the axonemal microtubules (elongated spermatids); all these cytoskeletal structures function as tracks during

spermiogenesis [87, 124, 127]. Like the cortical microtubule array supplies the tracks along which USP8-signed cargo is trafficked to the acrosome, it might be that USP8 is involved also in the manchette-mediated transport (USP8 locates on the manchette both at light [124] and electron microscopy [128] level) and/or intraflagellar transport. These two hypotheses are still to be explored.

In conclusion, acrosomogenesis is a clear example of a non-proteolytic involvement of the Ub system. It remains, however, to decipher which are the Ub marks (Lys63-, Lys11-, Lys27-, Lys33-, Lys48- linked or other types of linkage) and ubiquitin chain architectures that sign the targeted cargos and how these signatures are “translated” by the spermatid protein transport systems to build the acrosome.

Passing of the Baton Between Non-Proteolytic Functions and Proteolytic Functions of the Ub System in Sperm Biology

As already noticed, the number of mitochondria decreases notably during spermiogenesis and the mitochondria located at the sperm tail mid-piece are ubiquitinated. It remains as an unsolved question to understand the physiological meaning of such ubiquitination (signal for degradation or signal for regulation of organelle localization or both? In other words, may be that in order to be successively degraded these organelles must be recognized and, therefore, signaled?). More E3 Ub ligases have been coupled to spermatid/sperm tail suggesting a potential involvement for each of these in the construction of the flagellum [83–85]. To date, however, these assertions remain generic.

In *Drosophila* spermiogenesis the *pink1-parkin* pathway has been shown to play a critical role in regulating mitochondrial morphology and function [141]. PINK1 encodes a putative serine/threonine kinase with a mitochondrial targeting sequence, while PARKIN is an E3 Ub ligase responsible for directing the autophagic clearance of defective mitochondria [142]. In human *PARKIN* mutations are responsible for a familiar form of autosomal recessive juvenile parkinsonism [143]. At present, it is not known if there is a direct involvement of PARKIN in mammalian spermiogenesis/tail morphogenesis. It has been, however, demonstrated that the murine PACRG (*Parkin* co-regulated gene) protein interacts with MEIG1 (meiosis-expressed gene 1) that migrates to the manchette in elongating spermatids; together, PACRG/MEIG1 form a complex in the manchette that is necessary to transport cargos, such as SPAG16L, to build the sperm tail [144]. As said, USP8 too locates at the spermatid manchette and, to complete the localization of USP8 in haploid mouse male germ cells, USP8 localizes also at the centrosome and principal piece of the flagellum [82]. In somatic cells USP8 is known to play a critical role in the control of mitochondrial quality; it is, in fact, required for the efficient recruitment of PARKIN to depolarized mitochondria so to trigger subsequent mitophagy [145]. Such a direct interaction between a DUB and E3 ligase is not surprising; E3 ligases are often regulated by DUBs [24] and in the case of PARKIN, USP8 deubiquitinates

directly the ligase by acting on non-canonical Lys6-linked Ub chains [145]. Thus, when levels of USP8 are reduced or its activity is inhibited, an accumulation of Lys6-linked Ub conjugates on PARKIN delays its overall activity in mitochondrial quality control. Consequently, USP8 activity is crucial for Parkinson's pathogenesis [145] as indicated also by further proof. Another USP8 protein target is alpha-synuclein. USP8 removes Lys63-linked Ub chains on alpha-synuclein, thus contributing to the accumulation of misfolded alpha-synuclein into Lewy bodies, cellular inclusions characteristic of the Parkinson disease [146]. Upon the experimental evidence on USP8 capacity of hydrolyzing different Lys-linked Ub chains [147] with repercussions on mitochondrial quality, it could be worthy of consideration to investigate about a potential involvement of USP8 in tailoring the ubiquitin signature (and fate) of mitochondria during sperm tail development. A recall to the wobbler mice might be useful. The Wobbler mouse is a spontaneous mutant used as model of motor neuron degeneration [148] associated to male infertility (wobbler sperm are acrosomeless and immotile, with a tail mid-piece characterized by a disorganized and mislocalized mitochondrial sheath, see [128, 149]). Relevant USP8 upregulation has been found in wobbler cells (in particular, spinal cord oligodendrocytes and spermatids) selectively affected by the wobbler mutation [150, 151]. This suggests that increased levels of USP8 could reflect the induction of USP8-mediated (rescuing or maladaptive) responses to the disorder.

A statement that is widely accepted among reproductive/developmental biologists and geneticists is that almost all eukaryotic animals inherit their mitochondria from the maternal parent. Until some decades ago, the prevailing explanation for such a phenomenon has been a passive model of simple dilution of the few paternal mitochondria by an excess copy number of the oocyte mitochondria [152]. Recent studies in *Caenorhabditis elegans*, which produces non-flagellated amoeboid sperm with mitochondria of canonical morphology, have brought to the light the involvement of autophagy in degradation of paternal mitochondria after fertilization [153]. Further research towards such a direction in the mouse has shown the presence of the autophagy receptor p62 and the ubiquitin-like modifier of autophagy LC3 in the sperm tail [154]. A successive study carried out always on mouse yields contrasting results [155]. The Authors employed embryos obtained by transgenic oocytes, expressing GFP-tagged autophagosome LC3, which were fertilized with transgenic spermatozoa bearing red fluorescent protein (RFP) labeled-mitochondria. It was thus provided evidence against sperm mitophagy; the authors stated in fact that maternal inheritance of mtDNA is not an active process of sperm mitochondria elimination achieved through autophagy, but may be a passive process as a result of pre-fertilization sperm mtDNA elimination [155]. Successively Politi and coworkers [156], by investigating the fate of *Drosophila* paternal mitochondria after fertilization, have found that paternal mitochondrial destruction is mediated by a common endocytic and autophagic pathway that implies a divergence from the classic autophagic pathway of damaged mitochondria. Still more recently, a study from Sutovsky's group [157] has established that sperm mitophagy, at least in higher mammals, occurs post-fertilization and relies on a combined action of both ALP and UPS systems employing an unconventional, ubiquitin-recognizing autophagic

pathway independent of canonical autophagy receptors such as LC3. Since propagation of paternal mitochondrial genes results in heteroplasmy, which could be potentially detrimental for embryo development [158], it would be desirable that this topic could be clarified, at least in human, in consideration of the diffused employment of assisted reproductive technologies.

As conclusive mention, I want to reserve a hint for another sperm organelle that, at a first glance, could appear to be out of place in such a context, the centrosome. Generally speaking, centrosome is a non-membrane bound organelle composed of two centrioles surrounded by pericentriolar material (PCM) where γ -tubulin nucleates microtubules as a part of the centrosomal γ -tubulin ring complex (γ -TuRC) [159]. Centrosomes are in fact major microtubule-organizing center of the cell (MTOC). Contrary to mitochondria, at fertilization sperm centrosome is inherited as first embryonic MTOC indispensable for early embryogenesis [160]. As known the oocyte's centrosome is reduced and casts off into the first and second polar body during oogenesis in mammals [161]; so, once a sperm enters the oocyte's cytoplasm at fertilization, the male gamete provides the essential centrosome in the form of the distal centriole that previously functioned as basal body for the development of the sperm tail. Centrosomes, however, contain also proteasomes [82, 162] that are thought to regulate the degradation of local ubiquitin-conjugates. Indeed, more molecular species referable to components of the Ub system have been found to reside at the centrosome and results have been obtained indicating that these selected components participate in the ubiquitin-dependent regulation of centrosome architecture [163]. Going back to the assembly of the first centrosome upon fertilization in mammals and, more generally, in vertebrates, it is now widely shared, with the exception of mice and some other murine animals, that the zygote forms its centrosome from the paternal distal centriole and from a pool of maternal factors that have been characterized to some extent but non been comprehensively identified [164]. Clearly, the enrichment in the research on centrioles and PCM during fertilization and embryonic development could improve our understanding of fertilization and aid in more efficient diagnoses of human infertility.

Concluding Remark

In this review, I have attempted to provide an overview of how the Ub system in its integrity is not only involved, but dictates the biological progressing of spermatogenesis. In comparison to other post-translational modifications like phosphorylation, the temporal, spatial, and substrate context of ubiquitination is extremely intricate. The complexity of the Ub signal has recently become even more evident with the discovery that Ub itself can be post-translationally modified, including its phosphorylation [25]. Here, I have deliberately avoided dealing this last topic because it is emerging at present and no research in this connection has been so far addressed to spermatogenesis. Being not this an exhaustive review, my point of view has been to supply sufficient information to the readers to recognize that Ub

system is not only as a blunt tool used during spermatogenesis to degrade proteins via the proteasome. This view has been, generally speaking, superseded in the last years with the impressive acquisition of data on the plethora of cellular functions that the Ub system governs; this has to be applied to spermatogenesis too. What may become the next frontier in ‘Ub system and male germ cell differentiation/function’ can be the NP-roles of Ub system during spermatogenesis. The idea that ‘signaling’ pools of ubiquitinated proteins could compete with the ‘proteasome-targeted’ pools provides a challenge to identifying and studying the roles of the Ub chain types that characterize such signaling pools. Here, some proposals for further investigation are put forward. To recall only some examples: (a) Which Ub modification does include the localization of retained histones that are not removed at the histone-to protamine transition?; (b) Which are the Ub sorting signals that, like topogenic sequences, address selected proteins to an unconventional organelle such as the acrosome is?; (c) Given that Ub chains are formed as structurally distinct polymers via different linkages, which is/are the Ub chain/s that promote/s protein trafficking at the manchette and centrosome/basal body?; (d) Through which sorting signal, if any, are spermatid mitochondria selected for their removal by Sertoli cell’s phagocytosis and/or destination to the tail mitochondrial sheath? Which Ub chain topology does mark the sperm tail mitochondria?; and still other, going on, unsolved questions.

The improved understanding of Ub-system biology during spermatogenesis could be hopefully employed in the exploration of human reproduction/fertility and, given the strong increase in the use of Assisted Reproductive Technologies, pre-implanted ART offspring.

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Spermiation: Insights from Studies on the Adjudin Model



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Introduction

Spermatogenesis is defined as a series of biological processes that result in the generation of sperm. It includes the self-renewal of spermatogonial stem cells, proliferation of spermatogonia by mitosis, differentiation of spermatogonia and spermatocytes, generation of haploid spermatids by meiosis I/II, and spermiogenesis [1, 2]. Spermiogenesis is characterized by a series of morphological events in which spermatids travel across the apical compartment of the seminiferous epithelium while maturing into spermatozoa [3]. After the mature elongated spermatids reach and line up along the luminal edge of the seminiferous epithelium in an orderly fashion, they undergo the final process, spermiation, in which the residual cytoplasmic components are shed and spermatozoa are released into the seminiferous tubule lumen [4]. Thus, spermiation can be disrupted by defects in spermatid

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orientation, phagocytosis of the cytoplasm and cytoplasmic components by Sertoli cells (the epithelial cells in the seminiferous epithelium), premature spermatid release and abnormal spermatid retention.

The precise timing of spermiation safeguards the daily output of millions of sperm by the testis in rodents and humans, thereby ensuring that sufficient sperm enter the epididymis [5]. Thus, it is important to understand how spermiation is regulated. Our current understanding of spermiation involves a collaborative effort among various cellular structures and processes with a strong emphasis on a testis-specific cell junction known as the apical ectoplasmic specialization (apical ES). The apical ES is an atypical adherens junction that connects elongating/elongated spermatids to Sertoli cells which are the only somatic cell type in the seminiferous epithelium, so that spermatids remain embedded within the seminiferous epithelium until sperm release [6]. It is typified by a unique arrangement of discrete actin bundles between cisternae of endoplasmic reticulum and the apposing plasma membrane of a Sertoli cell and an elongating/elongated spermatid (namely step 8-19 spermatids in the rat testis) [6, 7]. The adhesion complex that underlies the function of the apical ES, the $\alpha 6\beta 1$ -integrin-laminin $\alpha 3\beta 3\gamma 3$ complex, connects spermatids to adjacent Sertoli cells [8]. It has also been reported that the apical ES associates with microtubules (MTs) [6, 9]. In terms of function, the apical ES assembles and disassembles continuously throughout the epithelial cycle to correlate with i) the transport of developing spermatids across the apical compartment of the seminiferous epithelium [3, 10]; ii) the proper orientation of polarized spermatids [11, 12] and iii) the final release of spermatozoa [10]. Thus, understanding the regulation of the apical ES is likely to further our understanding of the underlying mechanism of spermiation.

In despite of advances in understanding the regulation of spermiation during the past few decades, many unanswered questions remain, partly due to the lack of a suitable *in vivo* model. Adjudin, 1-(2,4-dichlorobenzyl)-1*H*-indazole-3-carbohydrazide, is a small molecule with a molecular weight (Mr) of 335.19 that exerts its effects by primarily targeting apical ES [13, 14]. At a single dose of 50 mg/kg b.w. (by oral gavage), this compound can induce premature spermatid release from the seminiferous epithelium, thereby leading to infertility [13, 15, 16]. After examining testes treated with Adjudin (a single dose of 50 mg/kg b.w.) at different time points, we have obtained new insights on the regulation of spermiation [17–23]. In this review, we summarize these findings and contend that rats treated with Adjudin represent a valid model for the study of spermatogenesis in particular the biology of spermiation.

Adjudin Targets both Actin- and Microtubule (MT)-Based Cytoskeletons

We first identified the targets of Adjudin by analyzing the gene profiling data (untreated rats *vs.* rats treated with Adjudin at a single dose of 50 mg/kg b.w. for 4 days) [24] using gene set enrichment analysis (GSEA) [25]. Twenty-eight out of 133 actin-associated gene sets were shown to be significantly enriched at a false

discovery rate (FDR) of $q < 25\%$, which included gene sets associating with the regulation of actin filament length and actin binding (Fig. 1a). Interestingly, multiple Arp2/3 complex-related genes and pertinent regulating proteins/genes were among the genes that were enriched in the gene sets (Tables 1 and 2). The expression profiles of these genes showed trends of up-regulation following Adjudin treatment (Fig. 1b). To verify the results of GSEA, we performed gene ontology (GO) analysis of genes showing changes greater than two-fold at 4 days after Adjudin treatment. Gene ontology categories, such as the regulation of actin cytoskeleton organization, were enriched (Fig. 1c), further confirming the finding that the organization of the actin cytoskeleton was closely associated with the Adjudin phenotype. We also performed GSEA on MT-associated gene sets. Eighteen out of 58 MT-associated gene sets were shown to be significantly enriched at a FDR of $q < 25\%$, which included gene sets associating with the regulation of MT polymerization or depolymerization and other MT-based processes (Fig. 2a). Interestingly, multiple MT motor proteins, such as kinesin family proteins and dynein (light chain), as well as the MT severing protein katanin, were among the genes that were enriched in the gene sets (Table 3). These genes showed significant down-regulation in their levels following Adjudin treatment (Fig. 2b), suggesting a severe disruption in MT organization in the testis. In stark contrast to the down-regulation of the aforementioned genes, the expression levels of two enriched MT-associated genes [microtubule associated protein, RP/EB family, member 1 (also known as EB1) and echinoderm microtubule associated protein like 2 (EML2)] that are known to stabilize MTs were significantly up-regulated (Fig. 2b), which was indicative of either an off-balance in the stability of MTs or a negative feedback loop in the organization of MTs following Adjudin treatment. Taken together, these analyses indicate that both actin and MT-based cytoskeletons are the targets of Adjudin. Indeed, the general organization of both actin- and MT-based cytoskeletons was disrupted in a time-dependent manner when Adjudin-treated rat testes were examined [17]. Given that both actin filaments and MTs are required for spermiation, we contend that rats treated with Adjudin represent a good model to study spermiation. A detailed discussion of this point is provided in the following sections.

Rats Treated with Adjudin as a Model for Studying Sperm Release

In rat testes, sperm release takes place at stage VIII of the seminiferous epithelial cycle, facilitated by the disassembly of the apical ES [5, 26]. Given that Adjudin targets primarily the apical ES in the seminiferous epithelium to cause premature spermatid release, we hypothesized that testes treated with Adjudin can be used as a model to study the biology of sperm release at spermiation. Sperm release has been shown to associate with the dendritic assembly of a membrane-related actin network in the region devoid of the apical ES, specifically at the concave side of

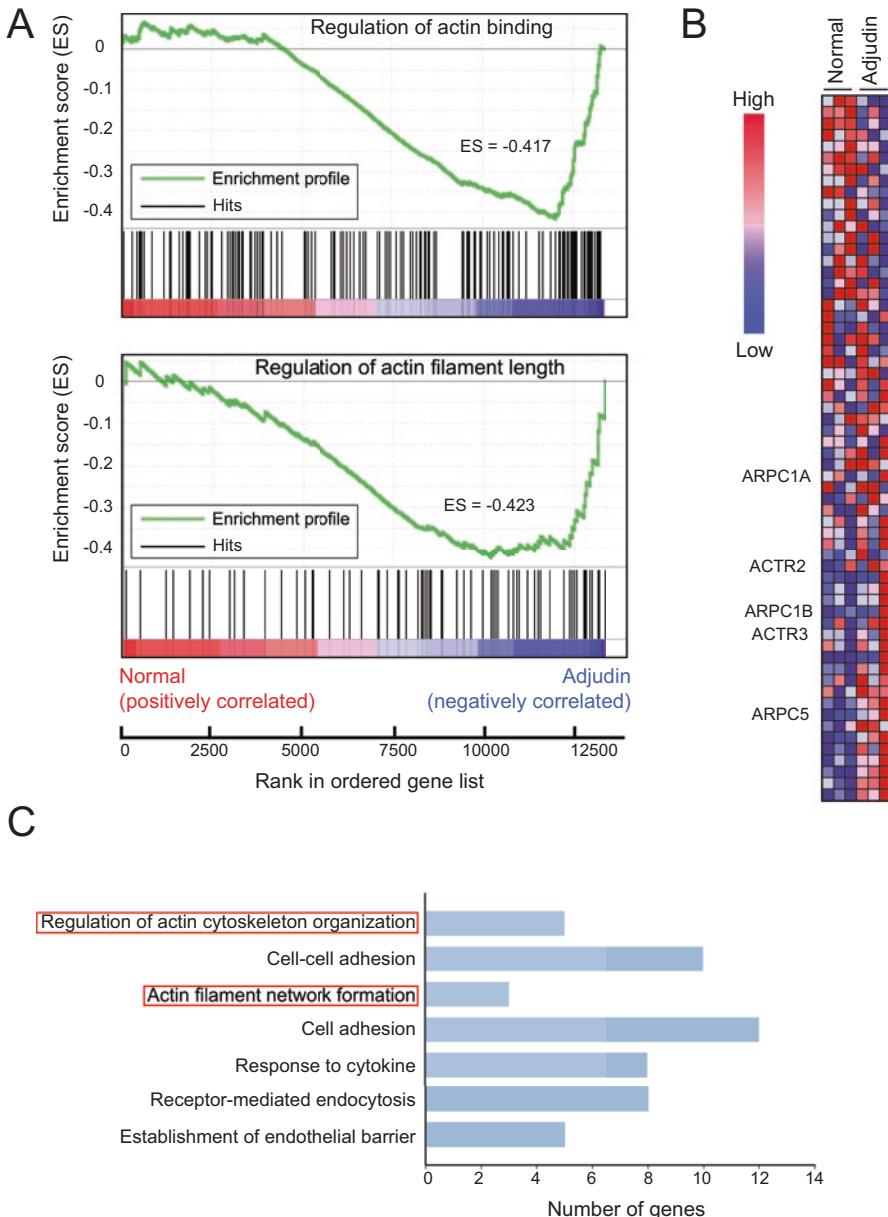


Fig. 1 (a) GSEA (gene set enrichment analysis) of the gene expression profiles from untreated rats vs. rats treated with Adjuvin (one dose at 50 mg/kg b.w, 4 days). Gene expression profiling data were obtained from the GEO database (GSE5131) [24]. The raw .cel files generated by the Affymetrix Rat Genome 230 2.0 Array were combined and converted into a single .gct file using the GEO Importer tool at the GenePattern website (<http://software.broadinstitute.org/cancer/software/genepattern#>). A total of 133 gene sets comprised of genes potentially involved in the regulation of the actin cytoskeleton were selected and downloaded from the Molecular Signatures Database(v5.2) (<http://software.broadinstitute.org/gsea/msigdb>). GSEA was performed as previously described [25, 47]. In brief, the javaGSEA desktop application was downloaded from the

Table 1 Profiles of actin-related proteins with core enrichment in the gene sets

Protein symbol	Protein name	Rank in gene list	Rank metric score
ARPC1A	Actin-related protein 2/3 complex, subunit 1A	10,198	-0.254
PFN1	Profilin 1	11,192	-0.363
TPM4	Tropomyosin 4	12,012	-0.500
SEPT9	Septin 9	12,013	-0.500
DAG1	Dystrophin-associated glycoprotein 1	12,113	-0.524
ACTN1	Actinin, alpha 1	12,155	-0.533
ARPC1B	Actin-related protein 2/3 complex, subunit 1B	12,206	-0.544
TLN1	Talin 1	12,215	-0.545
ABRA	Actin-binding Rho activating protein	12,260	-0.555
PLDN	Palladin homolog (mouse)	12,349	-0.578
ACTR3	Actin-related protein 3 homolog (yeast)	12,422	-0.598
MARCKS	Myristoylated alanine-rich protein kinase C substrate	12,433	-0.600
MYH9	Myosin, heavy chain 9, non-muscle	12,459	-0.609
CAPZA2	Capping protein (actin filament) muscle Z-line, alpha 2	12,490	-0.618
PDLIM5	PDZ and LIM domain 5	12,548	-0.632
AVIL	Adillin	12,802	-0.752
TPM1	Tropomyosin 1 (alpha)	12,830	-0.765
ARPC5	Actin-related protein 2/3 complex, subunit 5	12,934	-0.847
ABLIM2	Actin-binding LIM protein family, member 2	12,961	-0.847
LASP1	LIM and SH3 protein 1	13,012	-0.885
BIN1	Bridging integrator 1	13,157	-1.038
CORO1A	Coronin (actin-binding protein), 1A	13,170	-1.067
TNNT2	Troponin T type 2 (cardiac)	13,208	-1.160
MYL3	Myosin, light chain 3	13,233	-1.213
SORBS2	Sorbin and SH3 domain containing 2	13,283	-1.419
ICAM1	Intercellular adhesion molecule 1	13,329	-2.280

◀ **Fig. 1** (continued) GSEA website (<http://software.broadinstitute.org/gsea/index.jsp>). Gene sets containing between 5 and 500 genes were considered, and statistical significance was assessed using 1000 gene set permutation. Genes were ranked by “Signal2Noise” metric. Pathways enriched with a FDR < 0.25 were considered significant. Relative expression was rank-ordered by signal-to-noise ratios of triplicate normal samples vs. triplicate Adjudin samples. Genes associating with both actin-binding (upper panel) and the regulation of actin filament length (lower panel) were significantly correlated with the Adjudin phenotype (norm $p < 0.001$; FDR $q < 0.25$). (b) Heat map depicting changes in gene expression comparing Adjudin-treated rats with normal rats. Arp2/3 complex-related genes are highlighted. (c) Gene ontology analysis of differentially expressed genes between untreated and Adjudin-treated (4 days) groups, as performed using DAVID software (v6.8) [48]. A total of 436 differentially expressed genes ($>2\times$) (for a detailed list of the genes, see Supplementary Table 1 in [24]) were included in the analysis. Functional pathways representative of each gene signature were analyzed for enrichment in gene categories from the Gene Ontology Biological Processes database (GO-BP) within DAVID software (<https://david.ncifcrf.gov/>) [49]. Representative GO-BP categories with at least three genes and a $p < 0.05$ were identified and selected. The representative enriched biological processes related to the actin cytoskeleton (red rectangle), cell adhesion and cytokines are shown. The vertical axis represents the gene ontology categories, and the horizontal axis indicates the number of genes in each ontology category

Table 2 Gene members in each gene ontology category

Gene ontology category	Gene names (symbols)
Regulation of actin cytoskeleton organization	Peptidylglycine alpha-amidating monooxygenase (<i>Pam</i>); Ezrin (<i>Ezr</i>); glycoprotein m6b (<i>Gpm6b</i>); protein kinase C, delta (<i>Prkcd</i>); LDL receptor related protein 1 (<i>Lrp1</i>)
Cell-cell adhesion	Annexin A2 (<i>Anxa2</i>); signal transducer and activator of transcription 1 (<i>Stat1</i>); C-X-C motif chemokine ligand 12 (<i>Cxcl12</i>); vasodilator-stimulated phosphoprotein (<i>Vasp</i>); Calpastatin (<i>Cast</i>); Calponin 3 (<i>Cnn3</i>); Transgelin 2 (<i>Tagln2</i>); LRR binding FLII interacting protein 1 (<i>Lrrkip1</i>); N-myc downstream regulated 1 (<i>Ndr1</i>); myosin VI (<i>Myo6</i>)
Actin filament network formation	Actinin, alpha 1 (<i>Actn1</i>); lymphocyte cytosolic protein 1 (<i>Lcp1</i>); actin-related protein 2/3 complex, subunit 5 (<i>Arpc5</i>)
Cell adhesion	Flotillin 2 (<i>Flot2</i>); glycoprotein nmb (<i>Gpnmb</i>); laminin subunit gamma 1 (<i>Lamc1</i>); adhesion G protein-coupled receptor G1 (<i>Adgrg1</i>); cluster of differentiation 47 also known as integrin-associated protein (IAP) (<i>Cd47</i>); collagen type XV alpha-1 chain-like (<i>Col15a1-L</i>); ATPase Na+/K+ transporting subunit beta 2 (<i>Atpb2</i>); integrin subunit alpha 9 (<i>Itga9</i>); Sorbin and SH3 domain containing 2 (<i>Sorbs2</i>); integrin subunit beta 1 (<i>Itgb1</i>); galectin 3 binding protein (<i>Lgals3bp</i>); collagen type XVIII alpha 1 chain (<i>Col18a1</i>)
Response to cytokine	Cathepsin B (<i>Ctsb</i>); TIMP metallopeptidase inhibitor 1 (<i>Timp1</i>); Serin (or cysteine) peptidase inhibitor, clade A, member 3 N (<i>Serpina3n</i>); signal transducer and activator of transcription 1 (<i>Stat1</i>); collagen type III alpha 1 chain (<i>Col3a1</i>); nitric oxide synthase 3 (<i>Nos3</i>); signal transducer and activator of transcription 3 (<i>Stat3</i>); interleukin 6 signal transducer (<i>Il6st</i>)
Receptor-mediated endocytosis	Scavenger receptor class B, member 1 (<i>Scarb1</i>); Cortactin (<i>Ctn</i>); transferrin receptor 2 (<i>Tfr2</i>); phospholipase A2 receptor 1 (<i>Pla2r1</i>); scavenger receptor class a, member 5 (<i>ScarA5</i>); galectin 3 binding protein (<i>Lgals3bp</i>); LDL receptor related protein 1 (<i>Lrp1</i>); LDL receptor related protein 8 (<i>Lrp8</i>)
Establishment of endothelial barrier	Ezrin (<i>Ezr</i>); Moesin (<i>Msn</i>); rap guanine nucleotide exchange factor 2 (<i>Rapgef2</i>); SRY box 18 (<i>Sox18</i>); myeloid-associated differentiation marker (<i>Myadm</i>)

elongated spermatids [5, 27]. The loss of the apical ES and the changes in actin organization can be observed during the stage VII-to-VIII transition in the rat testis by staining the F-actin cytoskeleton and apical ES adhesion complexes [28, 29]. Interestingly, treatment of rats with Adjudin at a single dose of 50 mg/kg b.w. disrupted the F-actin bundles of pre-existing apical ESs associating with elongating/elongated spermatids [17]. Furthermore, analysis of gene profiling data revealed that the Arp2/3 protein complex is highly influenced by Adjudin (Table 1). Arp3 is an actin barbed end nucleation protein (i.e., by inducing branched actin polymerization in linear actin filaments, which in turn causes unbundling of a bundled actin filaments to become a branched actin network) showing a stage-specific expression pattern in the testis. It is predominantly expressed at the concave side of elongating spermatids at stage VII of the seminiferous epithelial cycle but its expression quickly diminishes at stage VIII, coinciding with sperm release [28]. It is believed that Arp3

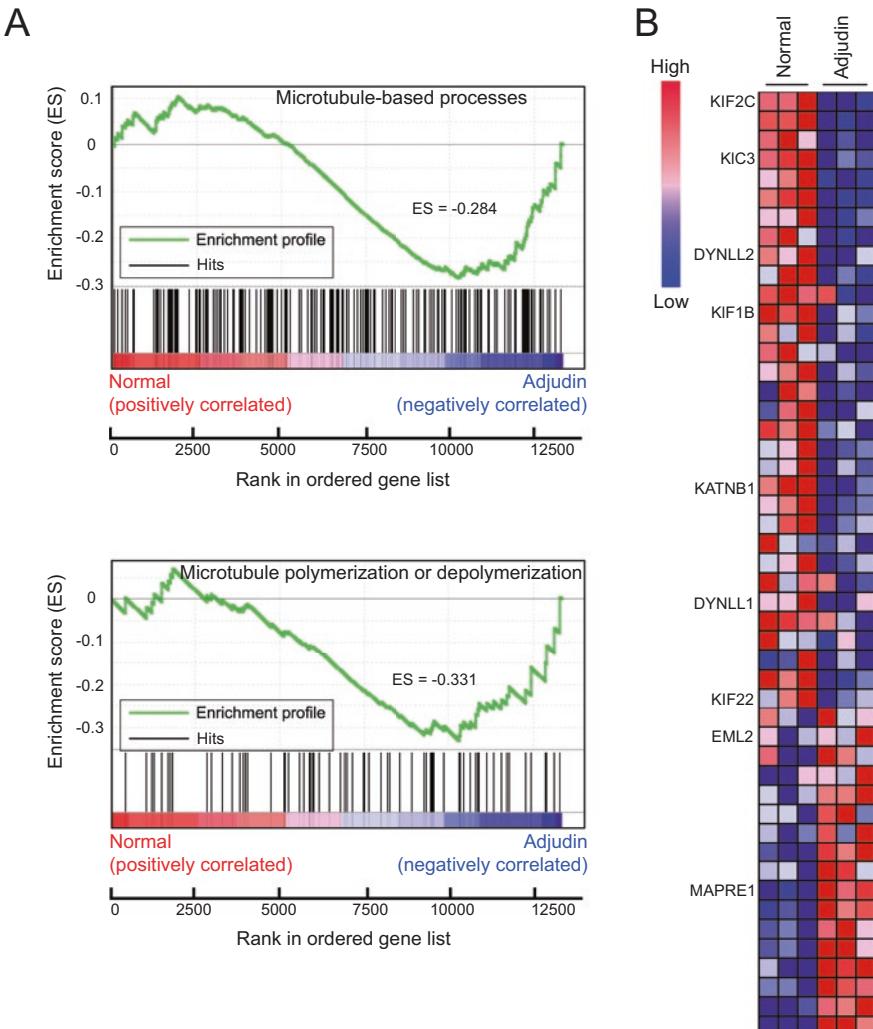


Fig. 2 (a) GSEA of the gene expression profiles for untreated rats vs. rats treated with Adjudin (one dose at 50 mg/kg b.w., 4 days). Fifty-eight gene sets associating with MT regulation were selected and downloaded from the Molecular Signatures Database (v5.2). Relative expression was rank-ordered using signal-to-noise ratios of triplicate normal samples vs. triplicate Adjudin samples. Genes associating with both MT-based processes (upper panel) and MT polymerization or depolymerization (lower panel) were significantly correlated with the Adjudin phenotype ($\text{norm } p < 0.001$; FDR $q < 0.25$). (b) Heat map depicting changes in gene expression comparing Adjudin-treated rats with untreated rats. The MT motor proteins kinesin and dynein (light chain), MT severing protein katamin, as well as MT-associated proteins [microtubule-associated proteins (MAPs), namely RP/EB family, member 1 (MAPRE1, also known as EB1) and echinoderm microtubule associated protein like 2(EML2)] are highlighted

Table 3 Profiles of microtubule-related proteins with core enrichment in the gene sets

Protein symbol	Protein name	Rank in gene list	Rank metric score
DYNC1LI1	Dynein, cytoplasmic 1, light intermediate chain 1	10,451	-0.477
RAB21	RAB21, member RAS oncogene family	10,468	-0.480
AP3M1	Adaptor-related protein complex 3, mu 1 subunit	10,593	-0.507
RHOT2	Ras homolog gene family, member T2	10,726	-0.530
ROCK2	Rho-associated, coiled-coil containing protein kinase 2	10,831	-0.554
TBCE	Tubulin-specific chaperone e	10,853	-0.557
DYNC1LI2	Dynein, cytoplasmic 1, light intermediate chain 2	11,632	-0.768
TUBB3	Tubulin, beta 3	11,913	-0.877
KIF1A	Kinesin family member 1A	11,920	-0.881
KIF4A	Kinesin family member 4A	12,421	-1.143
DYNLRB1	Dynein, light chain, roadlock-type 1	12,689	-1.355
TUBB	Tubulin, beta	12,890	-1.550
TUBB6	Tubulin, beta 6	13,283	-2.835
EML2	2	10,633	-513
MAPRE1	Microtubule-associated protein, RP/EB family, member 1	11,771	-0.823
KIF4A	Kinesin family member 4A	12,421	-1.143
MYH9	Myosin, heavy chain 9, non-muscle	12,459	-0.609

converts F-actin bundles at the apical ES into a branched/dendritic actin network during stage VII of the seminiferous epithelial cycle [28, 30]. This helps to destabilize the apical ES in preparation for sperm release at stage VIII. In testes treated with Adjudin, the spatial expression of Arp3 was altered as early as 6 hrs following a single dose of Adjudin at 50 mg/kg b.w., in which the expression of Arp3 was no longer restricted to the concave side of spermatids at stage VII of the seminiferous epithelial cycle [17]. This disruption in Arp3 expression was exacerbated with increasing time after Adjudin treatment. After 1 day of Adjudin treatment, only small areas of Arp3-positive staining associated with spermatids, and the overall fluorescence intensity of Arp3 significantly decreased [17, 28]. Furthermore, the association of Arp3 with F-actin was enhanced 12 hr after Adjudin treatment as revealed by co-immunoprecipitation (co-IP) experiments [18], confirming that Adjudin utilizes Arp3 to affect the organization of the actin cytoskeleton, probably by enhancing F-actin bundle dissolution at the apical ES. These alterations in F-actin organization and Arp3 expression, as well as the interactions between the two proteins, following Adjudin treatment correlated with the onset of premature spermatid release. Taken together, this information demonstrates that Adjudin-induced spermatid exfoliation mimics the initial steps of normal sperm release during spermiation.

Rats Treated with Adjudin as a Model for Studying Elimination of the Spermatid Cytoplasm

During spermiation, most cytoplasmic components are stripped away from elongated spermatids [31]. The removed cytoplasm, known as the residual body, is subsequently engulfed by Sertoli cells, transported towards the base of the seminiferous tubule and progressively degraded by phagocytosis and lysosomal degradation. By stage X of the seminiferous epithelial cycle, most residual bodies are inconspicuous. A previous study using taxol (paclitaxel), an anti-tumor drug known to stabilize MTs and inhibit MT-related processes, reported a disruption in the elimination of the cytoplasm in elongated spermatids, in which residual bodies were not transported to the base of the seminiferous tubule but remained at, or near, the luminal edge [32]. This study revealed the important role of the MT-based cytoskeleton in the elimination of the cytoplasm from spermatids. Interestingly, rats treated with a single dose of Adjudin at 50 mg/kg b.w. by oral gavage showed similar defects in residual body transport [17], in which residual bodies failed to move towards the base of the seminiferous tubule. This was probably due to the fact that Adjudin targets the MT network in the testis. As revealed by the results of the gene profiling analysis, multiple MT-associated proteins (MAPs) were affected by Adjudin treatment, including the MT plus-end binding protein EB1 (Table 3). EB1 has been shown to regulate not only MTs but also F-actin filaments in Sertoli cells [33]. Following a single dose of Adjudin at 50 mg/kg b.w., the spatial expression of EB1 started to shift as early as 6 hrs after treatment, with the truncation of EB1-positive track-like structures [17]. This alteration in EB1 distribution coincided with the disruption in MT organization in the testis, in which MT track-like structures were also truncated following Adjudin treatment [17]. At 6 and 12 hrs after Adjudin treatment, however, the binding of EB1 to MTs was enhanced as demonstrated by co-IP experiments [18]. These results are indicative of a rescuing effect, in which EB1 possibly maintains the stability of MTs. However, 1 day after Adjudin treatment, this association between proteins was largely abolished, and the disassociation between EB1 and MTs persisted for 4 days after treatment [18]. Therefore, as revealed by the Adjudin model, residual body transport may be regulated, at least in part, by the expression of EB1 and its association with MTs in the testis.

In this context, it is interesting to note that the defects in MT organization in Adjudin-treated testes also negatively affected spermatid transport across the seminiferous epithelium. Abnormal spermatid retention could be observed as early as 6 h after Adjudin treatment [17]. It has been speculated that Adjudin disrupts spermatid transport via a mechanism that is similar to that described for defective residual body transport [9, 17].

Rats Treated with Adjudin as a Model for Studying Spermatid Orientation

The proper arrangement of elongated spermatids along the luminal edge of the seminiferous tubule represents the initial step of spermiation. This arrangement includes both the polarization of spermatid heads along the apical-basal axis and the collective orientation of spermatid heads across the entire plane of the seminiferous epithelium [11, 12, 34]. It is interesting to note that Adjudin perturbed spermatid polarity as early as 6–12 h after treatment, in which spermatid heads no longer pointed towards the base of the seminiferous tubule [35, 36]. This is understandable given that Adjudin primarily targets the apical ES, and the apical ES is the major structure involved in the orientation of spermatids [4]. Furthermore, spermatids lacking polarity in Adjudin-treated testes have been shown to associate with a marked decrease in the expression of actin-binding protein drebrin E [23], cell polarity complex component Par6 [22] and microtubule affinity-regulating kinase MARK4 [36] at the apical ES. Drebrin E can stabilize F-actin filaments at the apical ES via direct binding [23, 37]. The Adjudin-induced decrease in drebrin E expression thus facilitates the breakdown of the F-actin bundles at the apical ES. Par6 belongs to the classic partitioning-defective polarity complex, which has been shown to regulate epithelial apical–basal polarity in many cellular contexts [38, 39]. It has also been shown to regulate spermatid polarity [22]. Therefore, it is plausible that the Adjudin-induced decrease in Par6 expression disrupts spermatid polarity. MARK4 prevents the binding of tau and other MT-associated proteins to MTs by phosphorylating these proteins at Ser residues [40]. A down-regulation of MARK4 expression caused by Adjudin was shown to impede the phosphorylation of MT-associated proteins, subsequently stabilizing MTs and inhibiting MT-related processes. Taken together, this information indicates that spermatid polarity is under the control of both actin- and MT-based cytoskeletons, and Adjudin-treated testes represent a valid model to systematically identify the important players that regulate spermatid orientation.

Concluding Remarks

In this review, we have discussed the idea that testes treated with Adjudin are a good model for understanding spermiation. We showed that the Adjudin model is suitable for investigating sperm release, residual body removal and spermatid orientation during spermiation (Fig. 3). We are aware that the information obtained from the Adjudin model cannot recapitulate the entire process of spermiation. For example, the current study on spermatid polarity and apical ES dissolution using the Adjudin model focuses mainly on the early phases of spermiation and not the late phases, which involve apical ES removal and spermatid disengagement. Furthermore, a study on residual body transport using the Adjudin model can only provide a partial

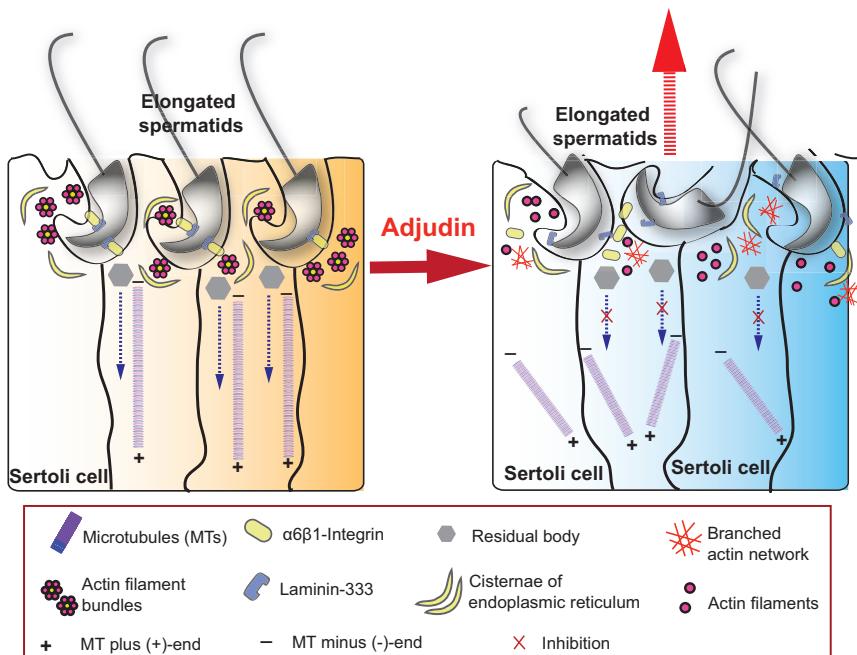


Fig. 3 Schematic illustration depicting the mechanism of action of Adjudin. During stage VIII of the epithelial cycle, elongated spermatids line up along the luminal edge of the seminiferous tubule, which is facilitated by the actin-rich apical ES. Adhesion complexes, such as the $\alpha 6\beta 1$ -integrin-laminin $\alpha 3\beta 3\gamma 3$ complex, anchor spermatids onto Sertoli cells. Residual bodies are removed from the spermatids, phagocytosed by Sertoli cells and transported towards the base of the seminiferous tubule via the MT network for their eventual lysosomal degradation. Adjudin targets the apical ES between spermatids and Sertoli cells, thereby converting actin bundles at the apical ES into a branched/dendritic actin network, perturbing spermatid polarity and inducing premature spermatid release. The MT network is also disrupted, leading to a failure of residual body transport

picture of spermatid cytoplasm elimination, as it cannot explain how the cytoplasm is disassociated from the spermatid in the first place. A more detailed examination of the Adjudin model with new advances in super resolution microscopy [41–43] and next generation sequencing [44–46] may be valuable to understanding the entire process of spermiation.

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Regulation of Human Spermatogenesis



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Introduction

Human spermatogenesis (HS) is an intricate network of sequential processes responsible for the production of the male gamete, the spermatozoon. These processes take place in the seminiferous tubules (ST) of the testis, which are small tubular structures considered the functional units of the testes. Each human testicle contains approximately 600–1200 STs [1], and are capable of producing up to 275 million spermatozoa per day [2].

The processes involved in HS are common to other mammals, and include spermatogonial proliferation and differentiation into spermatocytes, meiosis of spermatocytes, spermiogenesis, and spermiation. The cell types that participate in human spermatogenesis (i.e. Sertoli cells, Leydig cells, germ cells, and peritubular cells) are also found in and share similarities with other mammals, Fig. 1.

Furthermore, the ultrastructure of the human ST follows the same pattern of other mammals. It is composed of a basal membrane that supports a layer of Sertoli cells (SC). The germ cells (GC) are distributed through the ST in a very organized fashion, with the less matured cells occupying the basal compartment and

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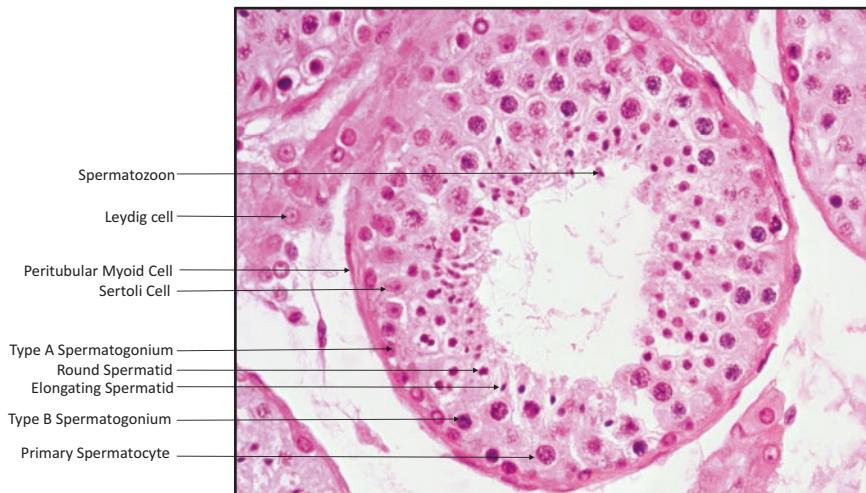


Fig. 1 Cross section of a human testis seminiferous tubule stained with Hematoxylin and Eosin. Visualized interstitial cells include Leydig cells in this figure; additional interstitial cells not visualized in the section include macrophages and other immune cells. Peritubular myoid cells line the seminiferous tubule which contain Sertoli cells, type A (dark and pale) spermatogonia, type B spermatogonia, primary spermatocytes, secondary spermatocytes, round spermatids, elongating spermatids, and spermatozoa

progressing to the adluminal compartment as they differentiate [3]. The peritubular cells (PTC), together with fibrocyte-like adventitial cells and a collagen matrix surround the ST, forming a peritubular tissue [4]. The SC, in conjunction with the basal membrane, the peritubular tissue, and endothelial cells from the testes capillaries, constitute the blood testicular barrier (BTB), a functional wall that regulates the para-cellular transit of molecules, creating an ideal environment for the GC proliferation and differentiation [5]. All of the cells that participate in spermatogenesis are regulated by a myriad of factors, such as hormones, neuropeptides, paracrine, autocrine, and physical factors. This results in a tightly controlled process that can be influenced by several conditions.

Despite all similarities, human spermatogenesis has some striking differences when compared with other animal models, specifically rat and mouse models. In rat and other rodents, the PTC create a continuous layer around the ST, while among humans, the PTC are separated by gaps, suggesting that they might play a secondary role in the BTB function [4]. Another difference is regarding the seminiferous epithelium cycle. Germs cells differentiate in synchronized and consistent cellular associations, called stages, at a certain location in the ST, and whereas there are 12–14 stages in rodents [6], among humans, only 6 stages exist [3, 7]. In addition, in most mammals, these stages progress in “waves” along the length of the STs [8], but several studies have failed to find such organization in human ST, and some authors define the arrangement of stages in humans as a random event [2]. Furthermore, spermatogenesis is notably less efficient in humans than other

mammals [9]. This is probably due to the longer duration of the entire process and to the high rate of apoptosis [10–12]. These differences should be noted and taken into account when animal data is extrapolated to humans.

Leydig Cells

The Leydig cells are fundamental for sperm production because they secrete the principal spermatogenesis regulator: testosterone (T). Located outside the ST, these cells have all the ultrastructural features of a steroidogenic cell, such as prominent nucleolus, vast meshwork of agranular endoplasmic reticulum, numerous tubovesicular mitochondria and lipid inclusions [13]. Thus, the testes are the main source of testosterone (T) after puberty, capable of producing up to 10 mg of T per day.

Testosterone is synthetized from cholesterol in a multistep process, involving several organelles and enzymes. The initial step is the transport of cholesterol from the cytoplasmic storage site to the mitochondrial membrane. This transport is the rate-determining step and is mediated by two key factors, the translocator protein (TSPO), which acts as a transport channel, and the steroidogenic acute regulatory protein (StAR) [14, 15]. The mechanism of action of StAR is still unknown, but it seems that this protein is the main determinant of the testosterone synthesis rate, and is the target of several factors that regulate the steroidogenic function of LC [16]. In the mitochondrial membrane, the cholesterol is metabolized by the CYP11A enzyme into pregnenolone, which is transferred to the smooth endoplasmic reticulum and transformed into testosterone through a series of enzymatic reactions [17].

Leydig cells are also an important source of estradiol, another key regulator of spermatogenesis [18]. Estradiol is synthetized from testosterone via the microsomal P450 aromatase enzyme, which determines the testosterone-to-estradiol rate [19]. The T/E rate is a main driver of spermatogenesis, and, when abnormal (<10), it is associated to impaired semen parameters [20].

The luteinizing hormone (LH) is the main regulator of LC function. Acting on the LH/HCG receptor [21], the LH starts two parallel chain of events, one acute and another chronic. Activated by the coupled G protein - cAMP mechanism, the acute response includes the synthesis of StAR and cytochrome P450 enzymes, and results in an immediate increase of testosterone and estradiol production within 24–72 h [22, 23]. Conversely, the chronic response is mediated through the ERK1/2 and Akt pathways, and consists in the inhibition of LC apoptosis [24, 25]. Additional factors known to stimulate Leydig cells include: gonadotropin-releasing hormone, melatonin, epidermal growth factor, insulin-like growth factor 1, atrial natriuretic peptide, ghrelin, and gamma-aminobutyric acid (GABA) [26–33].

In addition to the hypothalamic/pituitary negative feedback, testosterone also acts as an autocrine factor, binding the androgen receptor (AR) in the LC [34]. Short-term and long-term suppression occurs, the latter is mediated by the androgen receptor pathway and the former is mediated by competitive inhibition of the 3β hydroxysteroid dehydrogenase enzyme activity [35].

The gonadotropin-releasing hormone (GnRH) is also believed to regulate LC activity. The exact mechanism of this regulation is still to be elucidated, but a paracrine via seems to be implicated. As in other vertebrates, GnRH receptor has been found in human LC, and it is supposed to be activated by GnRH or GnRH-like molecules produced probably by Sertoli cells [26, 27, 36, 37]. Melatonin, another neurohormone, has drawn some attention recently. It is supposed to activate a corticotrophin-releasing hormone pathway, decreasing StAR activity and steroidogenesis [28]. Some non-hormonal factors, such as epidermal growth factor, insulin-like growth factor 1, atrial natriuretic peptide, and ghrelin have been shown to affect human LC activity in some degree, while a number of others have been studied in animal models, but lack human data [31, 38–41].

Another evolving field is the role of neuronal signaling in the testis function. Even though most studies in this area were made using animal models, there is compelling evidence that neurotransmitters may be involved in some conditions causing male infertility. The most studied molecule in this setting is the gamma-aminobutyric acid (GABA), whose receptors and synthesis enzymes were found in the human LC. Apart from being a tropic molecule involved in the growth and differentiation of these cells, GABA may also modulate their steroidogenic function, in the same way it does within neuronal cells [32]. Despite the great amount of information and insights that were given through animal models, humans studies are needed to complete elucidate the complex LC regulation system.

Sertoli Cells

The Sertoli cells (SC) are the main conductors of spermatogenesis, being responsible for the nutrition, transport, and protection of germ cells. Located in the ST and occupying approximately 40% of the seminiferous epithelium of adult men [42]. They display a polarized alignment, with the base resting over the basal membrane and the apex pointing to the ST lumen. Up to 50 GC at different stages of development can be supported by a single SC at a time, and to do so, their morphology is constantly changing, with numerous cytoplasmic ramifications involving the GC [43]. Its complex cytoskeleton is composed of actin filaments, tubulobulbar complexes, ectoplasmic specializations, intermediate filaments, and microtubules, each one with its own ultrastructure and function. This intricate structure is an important component of the blood-testicular barrier (BTB), and essential for GC development [44]. In addition, SC have a central role in the testis embryology. The SRY gene product starts a complex network of transcription factors that induces the differentiation of SC from progenitor cells of mesenchymal origin, the trigger of the male gonadal development [45].

Sertoli cells secrete several factors that regulate virtually every step of spermatogenesis. Androgen-binding protein (ABP) is one of such products, functioning as a carrier protein that binds testosterone and dihydrotestosterone with high affinity, limiting the bioavailability of androgens in the extracellular space of the testis and

epididymis [46, 47]. The ABP functions are unclear, but they might include the maintenance of the extremely high androgen concentration in the testicular parenchyma and epididymal lumen, the regulation of spermatogenesis and sperm maturation, and the controlled release of the hormones into the circulation [48–50]. The importance of ABP for HS is highlighted by the finding that dysregulation of its production is associated to some male infertility conditions such as non-obstructive azoospermia (NOA) [51].

Inhibin B is another SC-produced regulation factor. It is glycoprotein hormone composed of two subunits (α and β) that regulates the production and release of FSH from the anterior pituitary gland [52]. In adult men, the subunit α is located predominantly in the SC, while the subunit β is located predominantly in spermatocytes and spermatids, suggesting that some GC differentiation is needed for inhibin B production [53]. Details of the mechanism by which inhibin B acts are still to be elucidated, but a pathway using specific membrane receptors have been suggested [54]. Some in vitro studies have shown that inhibin B can also act as potential paracrine and autocrine regulators of LC and SC, modulating their proliferation, differentiation, and steroidogenesis [55–57].

Animal models and in vitro studies have also found that several other Sertoli cell-derived products, such as transferrin, ions and vitamin transporters, lactate, acetate, extracellular matrix components, proteases and protease inhibitors, TGF-a, TGF-b, IL-1, IL-6, SCF, GNDf, and MIF also contribute to regulate the microenvironment in which the germ cells mature, but their mechanisms of action are still unclear and further studies are necessary to clarify them and to give us more tools to manage male infertility [58–62] (Figs. 2 and 3).

Sertoli cells function as a regulatory hub, receiving inputs from other cells and organs via several pathways such as hormonal, non-hormonal, paracrine, and even autocrine factors. The combined action of all these factors will modulate SC's functions, and, eventually, modulate differentiation of GCs. Foremost in this regulatory network is follicle-stimulating hormone (FSH), a glycoprotein hormone produced by the pituitary gland under the stimuli of GnRH. The FSH receptor is a transmembrane G-couple receptor located mainly in SC [63–65]. Despite the lack of human studies, animal models have shown that, when bound to FSH, the receptor activates several pathways (i.e adenylate cyclase/protein kinase A, MAP kinase, calcium influx, phosphatidylinositol 3-kinase, Phospholipase A2) [64]. The final net effect of FSH action on SC is the general up-regulation of GC supporting SC functions, including metabolism, secretory function, and maintenance of cells junctions [64, 66]. Notwithstanding the pivotal role of FSH, it is not essential to initiation or maintenance of spermatogenesis, since clinical data from men with mutations in the FSH or FSH receptor genes combined with animal models have shown that spermatogenesis is heavily affected but not extinguished without its actions [67, 68]. Despite its secondary role, disturbances in FSH action can hamper spermatogenesis. For example, several genetic polymorphism that affect the FSH gene in humans have been reported, and some can negatively impact spermatogenesis, and even modify the outcomes of some treatments [69, 70]. In addition, a clinical study with men under a combined contraceptive regimen of T plus progesterone revealed that FSH

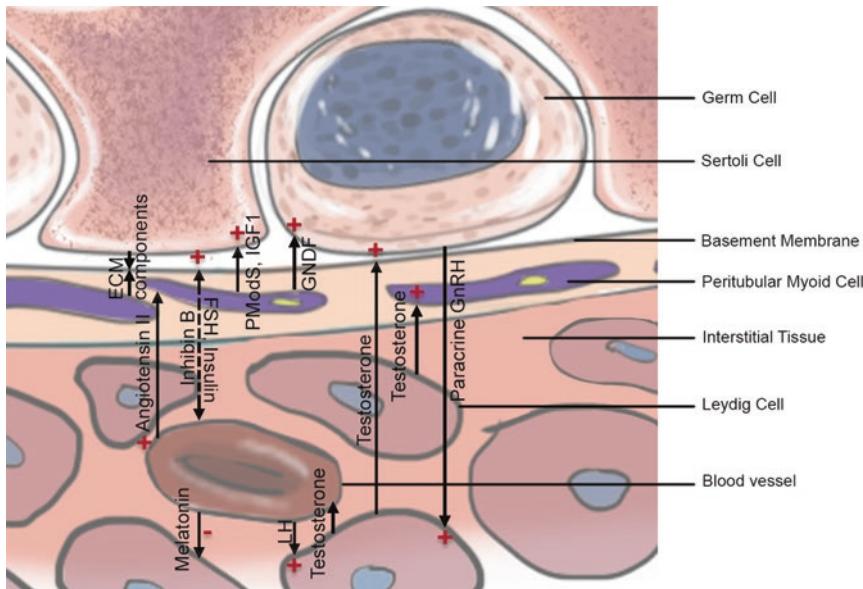


Fig. 2 Schematic demonstrating the endocrine and paracrine regulation between the blood and testis. Lutenizing hormone (LH) positively stimulates Leydig cells to produce testosterone and positively regulate Sertoli cells to support spermatogenesis. Follicle stimulating hormone (FSH) and insulin positively stimulates Sertoli cells to support spermatogenesis as well. Angiotensin II positively stimulates pertitubular myoid cells; peritubular myoid cells release Peritubular Modifying Substance (PModS), Insulin-like Growth Factor-1 (IGF1), and Basic Fibroblast Growth Factor (bFGF) among other molecules to regulate Sertoli cells as well as Glial Derived Neurotrophic Factor (GDNF) to positively regulate germ cell development. Peritubular cells contribute extracellular matrix (ECM) to help create the basement membrane. Tight junctions can be observed between the Sertoli cells creating the blood-testis-barrier. Inhibin is released from the Sertoli cells that are supporting spermatogenesis as a negative feed-back mechanism to temper central FSH production. Testosterone production also negatively feeds back centrally to modulate LH production and maintain gonadal homeostasis

supplementation was more effective than LH supplementation in supporting primary spermatocytes [71].

Androgen signaling, on the other hand, is indispensable to spermatogenesis, and is also a main regulator of SCs. The androgen receptor is a steroid nuclear receptor coded in a single copy gene located on the X chromosome [72]. Androgen receptors are found in SC, LC, and PTC, but GC don't express them, hence, all the androgen regulatory functions are performed indirectly [73]. After the AR is combined to its ligands (T or DHT), it translocates to the nucleus and modulates the transcription of several genes by binding to specific DNA sequences called androgen response elements [64]. Unfortunately, few consistent SC target genes have been identified using animal models, and no studies have been done using human SC [74, 75]. Alternative signaling pathways for androgen action such as binding to G-protein coupled receptor, activation of Ca^{2+} -dependent mechanisms, and MAP kinase

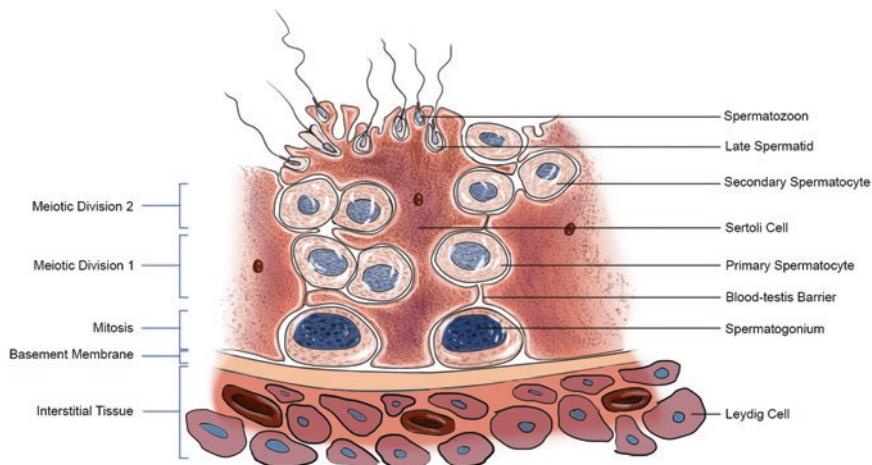


Fig. 3 Illustration demonstrating the regulatory role of Sertoli cells on differing stages of germ cell development. Sertoli cell release of Glial Derived Neurotrophic Factor (GDNF), basic Fibroblast Growth Factor (bFGF) and Leukemia Inhibitory Factor (LIF) promote self-renewal, while B-cell Lymphoma 2 (Bcl-2) promotes apoptosis and Retinoic Acid (RA), Stem Cell Factor (SCF) and Notch promote differentiation among spermatogonia. Among spermatocytes, continued growth and differentiation requires lactate production among Sertoli cells

pathways have been proposed, but, again, there is also paucity of data about them and no humans studies have been conducted [76–78]. The importance of androgens for spermatogenesis is evidenced by knock-out animal models and the clinical presentation of men who carry AR gene mutations and polymorphisms. These studies revealed that meiotic progression and spermatid maturation are especially sensitive to SC androgen signaling [72]. In addition, the number of CAG repeats on the AR gene has been shown to be inversely associated with the receptor activity and with sperm production in men [79, 80].

The carbohydrate metabolism of SCs are critical for GC differentiation, because spermatocytes and spermatids are unable to metabolize glucose, relying dependent upon SC-derived lactate to produce ATP [81]. Therefore, regulation of SC carbohydrate metabolism has a direct effect on GC maturation. Insulin is the most important regulator of SC metabolism and acts via a tyrosine kinase receptor, increasing the density of GLUT4 glucose transporters in the plasma membrane. Insulin-deprived SCs showed decreased lactate production, partially explaining the association between diabetes mellitus (DM) and male infertility [82]. In addition, drugs used for DM treatment were reported to improve SC metabolism and might offer a treatment option for men with insulin deficiency/resistance and infertility [83, 84].

Recently, several studies using animal models and *in vitro* human SC have provided valuable information regarding other potential SC regulation pathways. Substances such as androgens, estradiol, thyroid hormones, melatonin, basic fibroblast growth factor, growth hormone, PModS (peritubular modifies Sertoli), interleukins, and the mTOR pathway have been implicated in the modulation of SC

functions but further studies are needed to investigate the clinical significance of their action [85–94].

Peritubular Cells

Peritubular cells are large and flat cells with numerous cytoplasmic processes. They form two to six discontinuous cell layers around the ST [95]. The PTC exhibit features of both connective tissue cells and smooth muscle cells, playing several important functions such as the propulsion of testicular fluid containing immobile spermatozoa towards the rete testis, production of components of the extracellular matrix, and regulation of the inflammatory response in the testis [96]. PTMCs also secrete IGF-1, bFGF which in return regulate SCs [90, 97]. Furthermore, PTCs help maintain the BTB via secretion of fibronectin, collagen 4, proteoglycans, and entactin [90, 97].

Recent studies using cultures of human PTC have highlighted their secretory function, identifying several paracrine factors that regulate SC, LC and GC functions [98]. PModS is a factor secreted by PTC that modulates the secretion of transferrin, inhibin and androgen-binding protein by Sertoli cells [90, 91]. Glial cell line-derived neurotrophic factor (GDNF) and CXCL12, are two important GC regulators, and are also produced by PTC, underlining the importance of these cells for GC development [98, 99].

The maintenance of the BTB is also a function of PTC. This is evidenced by their ability to secrete components of the ECM, such as fibronectin, collagens, proteoglycans and entactin [98, 100, 101]. In addition, the avascular nature of the ST, is at least, partially maintained by the PTC-secreted pigment epithelium-derived factor (PEDF), a molecule that has a potent anti-vasculogenic action [102].

Testicular inflammation is often seen in infertile men, and reflected by immune cell infiltration, high levels of reactive oxygen species, remodeling of the ST, and impaired contraction of PTC [101, 103–106]. Some authors have suggested that PTC are an active player in this mechanism since they can secrete pro-inflammatory mediators under certain stimuli. In vitro studies using human PTC have shown that prostaglandins, epinephrine, angiotensin II, ATP, TNF- α have direct action on PTC, increasing the secretion of pro-inflammatory molecules [107–111].

The regulation of human PTC has been the main objective of several studies. Receptors for FSH and LH are not present among PTCs [112]. Conversely, androgen receptors are abundant, and animal models demonstrate that virtually all PTC functions as well as spermatogenesis are severely impaired in the absence of PTC androgen signaling [113]. The presence of the membrane-associated G protein-coupled estrogen receptor (GPER) in PTC suggest that estrogens have a role in their regulation [114]. In addition, prostaglandin E2 seems to be another modulation factor of PTC secretory function, acting in an autocrine fashion and stimulating the synthesis of GDF [115].

Immune Cells and Inflammatory Regulation

The testis is uniquely immune-privileged. This is achieved through the blood testis barrier (BTB) specifically giving privilege to the adluminal compartment of the seminiferous tubule; however, the remainder of the testis also demonstrates immune-privilege. The fluid of the seminiferous tubules contains immunosuppression activity, dendritic immune cells of the interstitium do not present auto-antigens among germ cells outside of the BTB, and testis macrophages exhibit anti-inflammatory cytokine production and poorly stimulate T cell proliferation [116, 117]. The BTB is created by an intermix of SC junctions such as tight junctions, basal gap junctions and adherens junctions [118]. The BTB separates the basal and adluminal compartments of the seminiferous tubule; the resulting adluminal compartment housing GCs including spermatocytes, spermatids and spermatozoa are immunologically privileged. Spermatogonia remain on the basal side of the ST, while preleptotene and leptotene spermatocytes migrate through the BTB from the basal to adluminal compartment [119]. The BTB develops at the time of puberty when spermatogenesis initiates and functions to protect haploid GCs as they develop new antigens not recognized by interstitial immune cells [119, 120]. Beyond the BTB, somatic cells such as Sertoli cells are thought to mediate immunologic protection for the developing germ cells [121]. Interestingly, the Sertoli cells share many properties of macrophages and immune cells; these similarities include production of inflammatory mediators, response to immunologically pathologic molecules such as lipopolysaccharide (LPS) and their cyclical ability to phagocytose nearly 75% of germ cells during normal spermatogenesis [122–127].

Within the testis, several different subtypes of immune cells exist in the interstitial space. These include macrophages, T cells, mast cells, natural killer cells, and rarer dendritic cells and B cells [119]. These cells contribute to both innate and adaptive immune responses. Macrophages are among the most dominant cell type in the testis interstitium and are thought to contribute to the regulation of spermatogenesis. Macrophages are thought to exist in a classically activated state (M1) or alternatively activated state (M2). M1 macrophages are CD68 positive while M2 macrophages are CD68 and CD163 positive [128]. M1 macrophages are stimulated by noxious stimulus and secrete pro-inflammatory cytokines such as interleukin-6, tumor necrosis factor (TNF α) and interleukin 12 (IL12) and are involved in clearance of pathogens and generation of reactive oxygen species [129, 130]. M2 macrophages on the other hand produce anti-inflammatory cytokines such as interleukin 10, and transforming growth factor β , and only low levels of pro-inflammatory cytokines [129]. Resident macrophages of the testis interstitium through lineage tracing have been shown to be derived from fetal liver, yolk sac and hematopoietic stem cells of the bone marrow [131, 132]. These resident macrophages are typically M2 in subtype (80%) and have been shown to produce cytokines associated with down-regulating inflammation including interleukin 10 (IL10), and transforming growth factor β (TGF β) when stimulated [133–136]. These macrophages have also been shown to be generally refractory to production on pro-inflammatory cytokines

with noxious stimuli compared to macrophages among other tissues, and is thought to occur via down regulation of toll like receptor 4 (TLR4), central blockade of NF- κ B signaling via lack of polyubiquitination of I κ B α , and paradoxical high expression of anti-inflammatory cytokines with noxious lipopolysaccharide stimulation [116, 137–139]. In addition to the resident macrophages, monocyte-like macrophages derived from the circulation also migrate to the testis interstitium and are thought to make up the M1 proportion of testis macrophages [128, 140, 141]. The resident M2 macrophages are thought to temper the balance between pro- and anti-inflammatory states, many of the products such as cytokines, play intricate roles in spermatogenesis [122, 142–144]. Macrophages are thought to exert their action in the testis directly through phagocytosis or indirectly through paracrine signaling. Macrophages have been shown to exert their effects on both Leydig cells and Sertoli cells, but it is largely unknown what effects they have on germ cells and spermatogenesis [145–147]. Macrophages are thought to be required for normal testis function, since ablation of macrophages using a transgenic mouse model (csfmop/csfm^{op}) resulted in both decreased testosterone production and spermatogenesis [148, 149]. A subset of macrophages have also been identified to be closely localized to spermatogonial stem cells and have been demonstrated to produce factors that facilitate spermatogonial proliferation and differentiation, including colony stimulating factor 1 (CSF1), as well as retinoic acid biosynthesis enzymes [150]. Abnormally high numbers of macrophages however, may be implicated in male infertility. Increased numbers of CD-68 macrophages were identified in testes among men with sertoli cell only syndrome and hypospermatogenesis; the location of these cells were typically in the interstitium among normal testis biopsies but also localize to the seminiferous tubule membrane and in the adluminal compartment among the infertile testis biopsies [104].

Molecules typically to be identified among those involved in inflammation such as interleukin 1 α (IL1 α), IL6, activin A, tumor necrosis factor (TNF α) and nitric oxide (NO) have been shown to play key mediatory roles in spermatogenesis and interestingly, produced by non-immune cells within the testis. For instance, IL1 α is produced by Sertoli cells, most greatly in response to phagocytosis of residual bodies during spermiation [126], as well as pachytene spermatocytes and round spermatids [151, 152]; IL1 β however, has been shown to be produced by Leydig cells and a sub-population of monocyte-like macrophages [133, 153–155]. Similarly, IL6 has been shown to be produced by both Leydig cells and Sertoli cells in a coordinated fashion during spermatogenesis [60, 122–124, 156]. TNF α is thought to be produced by testis macrophages as well as pachytene spermatocytes and round spermatids [157–159]; TNF α production is thought to inhibit Sertoli cell production of occluding, a key cell-cell tight junction protein [90]. Within the seminiferous tubule, TNF α results in increased lactate production among Sertoli cells required for later stages of spermatogenesis and less germ cell degradation [160–164], while in the interstitium, TNF α downregulates testosterone production among Leydig cells [165]. Activin A production is thought to occur predominately in peritubular myoid cells and Sertoli cells but has also been visualized among spermatogonia, spermatocytes and spermatids, and is stimulated by IL1 [127, 166–168]. Activin A

has been shown to stimulate DNA synthesis among spermatogonia and preleptotene spermatocytes [169]. Nitric Oxide (NO) functions as a regulatory molecule at low concentrations, but induces oxidative stress at high concentrations [170, 171]. At higher concentrations, NO has been demonstrated to inhibit Leydig cell steroidogenesis [172–175].

Germ Cells

The germ cells are a group of cells that undergo several differentiation steps to become the spermatozoa, being the only human cell type capable of meiosis. Their location is restricted to the interior of the ST, where they are distributed in a highly organized manner, with the less matured cells occupying the basal compartment and progressing to the adluminal compartment as they differentiate [2, 3]. The GC are classified in 4 broad subtypes; spermatogonia (SPG), primary spermatocytes (PSC), secondary spermatocytes (SSC), and spermatids (SPT). Even though their differentiation is a continuum and the exact boundaries between them are difficult to establish, each subtype has specific characteristics regarding morphology, localization, metabolism, and regulation [7]. For didactic purposes, we will address the regulation of each germ cell subtype separately.

Spermatogonia

Using conventional microscopy and staining techniques, it is possible to identify 3 subtypes of human SPG, Apale, Adark, and B types, based on their nuclei characteristics [3, 7]. In humans, there is some debate regarding the spermatogonial differentiation. In some models, Adark are the SPG types who are able to self-renew, maintaining the pool of SPG stem cells, and the Apale SPG are only able to produce B SPG [176]. An alternative model states that the Apale SPG are considered the active stem germ cell, that are able to undergo mitosis to either self-renew or to produce type B SPG, whereas the Adark SPG are the quiescent or reserve stem germ cells, that can be recruited if the Apale population is lost [177]. On the other hand, both models assume that the type B SPG are committed to divide via mitosis to produce PSCs.

A recent study using immunofluorescence techniques and several markers of spermatogonial differentiation showed that Apale and Adark SPG are probably the same cell type, and that their different morphologies are associated with different stages of the cell cycle (i.e., Adark are not in the active cell cycle, while Apale are undergoing cellular division). In addition, the same study found that a single A type SPG differentiates into a single B type SPG, and that B type SPG undergo 3 divisions to produce PSC [178]. This new model for spermatogonial renewal and differentiation is interesting but needs further validation.

The mechanism by which human SPGs are directed to self-renew, differentiate, or undergo apoptosis is of paramount importance in the understanding of how several conditions can affect spermatogenesis. Animal models have shown that GDNF, a paracrine factor produced by SC and PTC¹⁸⁰¹⁰⁰, is a primary driver of spermatogonial self-renew [180, 181]. Human Adark as well as Apale SPG express GFR α -1, a GDNF co-receptor, and its signaling has been demonstrated to promote spermatogonial self-renewal in *in vitro* studies with human SPG [182–184]. This pathway may be impaired among some infertile men with NOA [179]. Leukemia inhibitory factor (LIF) and basic fibroblast growth factor (bFGF) are SC derived factors that have demonstrated an ability to induce human SPG proliferation *in vitro*, but their *in vivo* roles are unclear [185, 186]. Self-renewal pathways remain to be further delineated; however, key regulatory genes identified from animal and human studies noted to be expressed among SPG stem cells include: GFR α -1, B cell CLL/Lymphoma 6B (BCL6B), fibroblast growth factor receptor 3 (FGFR3), inhibitor of DNA binding 4 (ID4), LIM homeobox 1 (LHX1), spalt like transcription factor 4 (SALL4), POU class 3 homeobox 1 (POU3F1), and ETS variant 5 (ETV5) [186–192].

The pathways for human spermatogonial differentiation and commitment to meiosis are even less explored. Although retinoic acid (RA) is highlighted as the main player in mammal SPG differentiation [122–125, 134, 135, 157, 158, 182, 183, 188, 189, 193, 194], few studies addressed the effects of action of RA on human GC, most using fetal GC and showing conflicting results [122, 123, 195–198]. Only one study using adult GC from cryptorchid men demonstrated that RA in conjunction with stem cell factor (SCF) were able to induce GC differentiation [199]. In addition, reports have suggested that the Notch1/Jagged2 signaling as well as bone morphogenic protein 4 (BMP4) signaling from SCs also stimulates GC differentiation [200, 201]. Animal studies have identified key regulatory genes found to be expressed in differentiating SPGs and include: KIT proto-oncogene receptor tyrosine kinase (KIT), spermatogenesis and oogenesis specific basic helix loop helix containing protein 1 and 2 (SOHLH1/2), synaptonemal complex central element protein 3 (SYCE3), SSX family member 3 (SSX3), synaptonemal complex protein 3 (SYCP3) and nuclear receptor subfamily 6 group A member 1 (NR6A1) [186–192].

Spermatogonial apoptosis is directed by SC as a manner of controlling the number of GC and maintaining the proportion SC/GC constant. In fact, up to 70% of the developing GC are discarded during spermatogenesis [202]. The signal to SPGs to enter into apoptosis is given by SCs through the intrinsic apoptotic pathway, which involves the Bcl-2 family proteins, but how this signal is transduced from SCs to SPGs remains unknown [203]. Furthermore, the transcription factor nuclear factor (NF)- κ B is also involved, but instead of working directly on SPG, it induces the transcription of SC genes encoding factors involved in the regulation of GC death [204, 205].

Spermatocytes

The spermatocytes are produced by mitotic division of B PSG and committed to undergo meiosis. They are also the first GC within the BTB to the adluminal compartment, where they are immunologically privileged and are able to proceed through meiosis. The spermatocytes are classified based on the stages of meiosis. The primary spermatocytes are in meiosis I and are subclassified according to the substages of prophase I; preleptotene (R), leptotene (L), zygotene (z), and pachytene (p), whereas the secondary spermatocytes are in meiosis II and are not subclassified [3, 7].

The exchange of genetic material between two homologous chromosomes (chromosomal crossover) occurs during the pachytene stage in a very organized manner, and it is crucial to human evolution and to the correct chromosome segregation [206, 207]. Checkpoints during meiosis I ensure that GCs with altered chromosomal segregation are eliminated via apoptosis, avoiding the production of aneuploid sperm that could bring harm to the offspring [208–210]. The apoptosis of spermatocytes are likely directed by SCs and executed via extrinsic FAS-FAS ligand mechanism [203, 211]. Failure of recombination caused by several mechanisms is frequently found at higher rates in patients with NOA and may explain a proportion of “idiopathic non-obstructive azoospermia infertility” cases [212–215]. Therefore, the offspring of these men, who were successfully treated with microsurgical testicular sperm extraction (microTESE) coupled with intracytoplasmic sperm injection (ICSI), have higher risk of chromosomal abnormality.

Spermatocytes rely on lactate as their principal energy source [216]. Since the SPC are inside the BTB, SCs are the only source of lactate, which is transferred to the SPC via monocarboxylate transporters. This process is stimulated by FSH, androgens, insulin and some paracrine factors, and its disruption may lead to arrest of spermatogenesis and subsequent infertility [81, 83, 84, 94]. Furthermore, SC-derived lactate is also an inhibitor of spermatocyte apoptosis by blocking the FAS receptor pathway, in a mechanism not related to energy production [217]. Further studies might reveal drugs that could be used to improve spermatogenesis in men with lactate production disarrangements.

Spermatids

After completion of meiosis II, the n haploid cells generated are called spermatids. From this point onward, no further cell division occurs, and these cells undergo a complex maturation process by which they eventually become spermatozoa. During this stage, called spermiogenesis, the SPT, which are initially round, gradually elongate and a series of cytoplasmic changes take place. The Golgi apparatus fuses to form the acrosome, the sperm tail arises from the centriole and is reinforced by two other structures, the outer dense fibers and the fibrous sheath, the mitochondria

increase in number and move to a position around the tail basis forming the mid-piece, and the excess of cytoplasm containing the remnants of Golgi apparatus and other organelles are discarded by the extrusion of the residual body. At the end of spermiogenesis, almost all organelles suffered some kind of modification [218, 219].

In the meantime, the nuclear structure is also modified, becoming more condensed and migrating to an eccentric position. In addition, around 85% of the nuclear histone content is replaced by protamines, around which the DNA is neatly packed in supercoiled structures called toroids, increasing its protection and becoming in most part transcriptionally silent. In this setting of highly condensed DNA, protein synthesis relies on RNA stored in the chromatoid body, thus regulation of spermiogenesis should involve pathways that act on RNA translation onward (i.e. epigenetic) [220–223]. Defects in protamination and DNA packing can impair embryo development, causing a negative impact on natural and assisted reproductive techniques outcomes [224–226].

After all these changes, the fully matured SPD detaches from SC and is released into the tubule lumen as spermatozoa. This detachment is called spermiation and comprises the controlled release of intercellular junctions. The mechanism of human spermiation is not completely understood, but it seems that the detachment of the ectoplasmic specializations progress from the tail to the head, and that the last point of contact is maintained by small tubulobulbar complexes [227, 228]. This stage is tightly regulated by SCs, and is under the control of FSH and testosterone signaling. Even though defects in spermiation alone have not been found to cause human male infertility, they are likely to contribute to infertility or sub-fertility in humans [71, 229–231].

Genes and Non-Coding RNA

Over 1000 genes have been described to regulate spermatogenesis, many of which are highly conserved among species. Some examples of highly conserved genes include: bone morphogenetic protein (BMP), SMAD, Deleted in azoospermia (DAZ)/BOULE, Nanos family genes, P-element induced wimpy testis in drosophila (Piwi) family, deadbox helicase 4 (Ddx4)/vasa, maelstrom, Tudor family, Musashi family, Pumilio family [232]. These genes simply do not turn on and off however. Increasingly, findings are demonstrating the importance and frequency of spatio-temporal gene expression and translation. This may occur under transcriptional or post-transcriptional control. Transcriptional control may occur through many modalities including epigenetic changes to the chromatin, and DNA, presence or absence of transcription factors and promoters. However, the importance of post-transcriptional regulation has more recently come into light. These processes include RNA modifications, RNA-binding proteins, long non-coding RNAs, and small non-coding RNAs [233]. Post-transcriptional regulation is thought to largely occur in the chromatoid body which is a germ cell-specific RNA processing center, thought to play a role in mRNA sequestration and translational repression [233],

[234]. We will discuss some of the latest research delving into the role of these non-coding RNA in spermatogenesis.

Three classes of small RNA's exist including microRNAs (miRNAs), PIWI-interacting RNAs (piRNAs), and small interfering RNAs (siRNAs) [235]. Small interfering RNAs have been shown to be present among spermatogonial stem cells [236], and other germ cells during spermatogenesis [237]. piRNAs play an important role during spermatogenesis, and are upregulated during 2 stages of spermato-genic germ cells. First, among primordial germ cell development, i.e. pre-pachytene piRNAs; second, in meiotic prophase 1, i.e. pachytene piRNAs [238]. PiRNAs are thought to protect germ cells through 3 modalities. PiRNAs present among pre-pachytene germ cells are thought to play a protective role through repression of transposon activity via de-novo methylation of transposon-encoding genes [239–241]. PiRNAs present among pachytene germ cells are also thought to repress transposons by post-transcriptional cleavage of the target transposon transcript [242, 243], and through elimination of mRNAs during later stages of spermatogenesis, i.e. spermatid formation [244]. PiRNAs are also capable of facilitating epigenetic modification via guiding DNA methylation for transposon-encoding gene silencing among germ cells [245]. Studies have also shown that heterochromatin protein 1 (HP1) and H3K9me3 combine transport to complexes of piwi and piRNA-targeted sequences, resulting in chromatin inactivation and resultant heterochromatin formation [246, 247].

The critical importance of small RNAs is perhaps best illustrated by the significant disruption of spermatogenesis with conditional knock out (cKO) mouse models of the machinery required to produce small RNAs, DiGeorge Syndrome Critical Region 8 (DGCR8), DROSHA, and DICER [248]. These studies have revealed that Argonaute (AGO)-bound small RNAs play a critical role throughout spermatogenesis, particularly among primordial germ cell proliferation, meiotic prophase I, and spermatid elongation as evidenced by studies to date [249–252]. Roles of specific miRNA in spermatogenesis are presently being investigated. To date, several of the following microRNAs have demonstrated important roles during spermatogenesis. miRNA-34 family of 6 miRNAs may play an important role during meiosis, where their expression is significantly upregulated. Furthermore, deletion of the miR-34 family using mouse models results in infertility, with aberrant spermatogenesis during the pachytene phase and spermatid elongation phase of spermiogenesis [253–255]. In a combined in-vitro and in vivo experiment, authors determined that miR-34c targets and interacts with activating transcription factor 1 (Atf1), which codes a protein implicated in germ cell apoptosis and is intimately related with B-cell lymphoma 2 (Bcl-2) and Bcl-2 associated X protein (Bax) expression [256]. Conversely, experiments assessing overexpression of miR34c among HeLa cells, leads to downregulation of TG-interacting factor 2 (TGIF2), and neurogenic locus notch homolog protein 2 (NOTCH2) which are involved in transcriptional regulation and developmental cell fate. Specifically, TGIF2 inhibits TGF- β expression which is essential in late meiosis and NOTCH2 is involved in germ cell self-renewal [257]. A conditional germline knockout of miR-17-92 cluster containing 4 miRNA families: miR-17, miR-18, miR-19, and miR-25 result in heterogeneous tubules

some containing sertoli cell only (SCO), devoid of germ cells; however, these miRNAs have paralogs elsewhere in the genome which become compensatorily upregulated in response to the conditional knockout [258]. This cluster of miRNAs are highly expressed among undifferentiated spermatogonial stem cells, and play a role in promoting proliferation, i.e. stem cell self-renewal and subsequently inhibiting differentiation. These miRNAs have predicted mRNA targets that fit this narrative including Bcl2111, Kit and suppressor of cytokine signaling 3 (Socs3) [258]. MiR-21 also promotes self-renewal of spermatogonial stem cells (SSCs) via anti-apoptotic mechanisms [260]. MiR-18 from the oncomir-1/miR-17-92/Mirc1 is highly expressed in spermatocytes, and targets heat shock transcription factor 2 (Hsf2) which is a transcription factor and is differentially active at varying stages of spermatogenesis [259]. MiR-383 is expressed in spermatogonia and primary spermatocytes and downregulates interferon regulatory factor 1 (IRF1) and subsequent cell cycle proteins: Cyclin D1, cyclin dependent kinase 219 (CDK219), p21 and resultantly downregulates mitosis [261]. MiR-15a also effects cell cycle regulation by downregulating Cyclin T2; miR-15a is found in lower levels among spermatogonia and spermatocytes and facilitates differentiation [262]. MiR-184 is preferentially expressed in spermatogonia and round spermatids, and serves to downregulate nuclear receptor corepressor 2 (Ncor2) [263]. Other miRNAs found to be highly expressed in purified spermatogenic, stage-specific murine cells are summarized in and excellent review by Hilz *et al.* 2016 [248]. MiR-let-7 family and miRNA clusters 1 and 3 comprising miR-17-92, miR-106b-25/Mirc3 play a role in spermatogonial differentiation [258, 264]; each of these miRNA interact with the retinoic acid signaling pathway which is essential for spermatogonial differentiation and entry into meiosis [265], let-7 family achieves this through regulating the insulin growth factor 1 (IGF1) signaling pathway [266]. Mir-146 also targets a co-regulator of the retinoid receptors mediator complex subunit1 (Med1) that is necessary for spermatogonial differentiation [265]. MiR-221 and miR-222 downregulates expression of KIT, thus, working against differentiation and thus likely supporting spermatogonial self-renewal [267]. Mir-20 and miR-106a promote SSC renewal through signal transducer and activator of transcription 3 (STAT3) and Ccnd1 regulation [268]. The miR-18 family has been identified to be involved during latter stages of spermatogenesis; it's targets encode heat shock factor 2 (HSF2) which works to condense spermatid chromatin [259], and the protein ataxia-telangiectasia mutated (ATM) which repairs damaged DNA [269]. Also important during later stages of spermatogenesis, miR-122a cleaves transition protein 2 (Tnp2) which encodes a nuclear transition protein which functions to substitute histones and promote chromatin condensation following meiosis [270]. Small RNAs are also thought to be involved in transcriptional silencing which is necessary during and following meiosis. AGO4 knockouts demonstrate a lack of meiotic silencing and subsequent subfertility [271]. Also of note, 11, of 28 miRNAs that are preferentially or specifically expressed in the testes localize to the X chromosome [272]. Many miRNAs are being investigated in their potential role in male infertility and are summarized in [273].

Long non-coding RNA (lncRNA) also appear to play an important role in the regulation of spermatogenesis, but are fraught with a limited understanding at

present. LncRNAs interact with chromatin binding proteins, RNA binding proteins (RBPs) and may function to activate or repress translation, mRNA protection or degradation, and act as a miRNA decoy [232]. Profiling testicular LncRNAs has led to the identification of 241 lncRNAs exhibiting SSC specific expression, 3639 limited to type A spermatogonia, 98 to pachytene spermatocytes, and 166 to round spermatids [274]. Further evidence to support the importance of lncRNAs during spermatogenesis is derived from a study of SSCs in culture that demonstrated a significant change in response to introduction and removal of glial derived neurotrophic factor (GDNF) with 1154 upregulated lncRNAs and 1473 downregulated in response to GDNF. Here, lncRNA033862 is an antisense transcript of the GDNF receptor alpha1 (GFR α 1) and positively regulates Gfr α 1 expression promoting SSC self-renewal [275]. lncRNA-HSVIII localizes to the cytosol and nuclei of germ cells as well as Leydig cells; expression initiates among early pachytene spermatocytes, increases in late pachytene spermatocytes, and tapers off in spermatids. lncRNA-Tcam1 has been identified in mice with an expression pattern correlated to Tcam1 mRNA and localizes to the nuclei of germ cells. However, the function is unknown [276]. Circular RNA is another classification of lncRNA. They are highly expressed in numerous tissues, and are second most abundant in testis tissue, behind brain [277]. Very little knowledge surrounding circRNA is presently available, however some signs of their regulatory role in the testis are emerging. During mammalian sex determination, the testis-determining gene, Sry, regulates Sox9, activating a cascade of events for male sex development. Sry circular RNA (circSry), is generated by an inverted splicing event and functions to positively regulate male differentiation via serving as a microRNA sponge competitively binding miR-138 [278, 279].

Conclusion

Spermatogenesis relies on a series of highly temporo-spatially coordinated genetic and molecular events. Through a complex interplay of intracellular programs and intercellular communication, the somatic cells and the germ cells interact and facilitate mitotic divisions among spermatogonia for self-renewal and differentiation, meiosis I and II through spermatocytes, and spermiogenesis and spermiation to complete the development of a spermatozoa capable of fertilizing an oocyte. Many of the molecular events and processes are yet to be described. The field is working to understand these events and significant efforts are being made to understand the dynamic regulatory events for cell renewal, and differentiation. The role of non-coding RNAs appears to be important as an additional level of post transcriptional regulation and further research is needed.

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Testicular Torsion and Spermatogenesis



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Introduction

Testicular torsion (TT) is a common urologic emergency and is characterized by twisting of the spermatic cord, with resultant obstruction of the testicular artery, which causes testicular ischemia. TT carries an incidence of 3.8 per 100,000 boys or adolescents under the age of 18 years [1]. TT presents in a bimodal age distribution, in the neonatal period and during puberty; it may however still occur at other ages, albeit less commonly in older adults [2]. Prompt evaluation with history, physical examination, and in some cases Doppler ultrasound, and a consultation with a urologist or pediatric surgeon is warranted. Management for TT is immediate surgical exploration of the scrotum, and detorting the affected testis and spermatic cord.

This chapter will first capture TT from a clinical standpoint and will then discuss the negative ramifications on testicular tissue and its effects on spermatogenesis through several hypothesized mechanisms.

Patient Presentation

Presentation varies based upon age. Antenatal or perinatal TT often presents with painless scrotal swelling, variable acute inflammation, and in some circumstances firm or blue discoloration of the affected testis. Unfortunately, TT among neonates is associated with a very poor salvage rate as (1) antenatal torsion may be

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non-reversible by the time the neonate is delivered and TT is diagnosed, (2) the limited clinical signs for families and care providers to recognize scrotal changes, and (3) access medical care in a timely matter where salvage is possible may be limited. As such, salvage rate among 18 cumulative case series including 284 neonates was only 9% [3]. Among children, adolescents and adults, presentation typically includes acute onset of scrotal or testicular pain. It is often associated with nausea and occasionally vomiting. Pain may radiate into the inguinal or abdominal regions. The testis often assumes an elevated and horizontal lie and is exquisitely tender to touch or palpation.

Patient Evaluation

Presentation of an acute scrotum should prompt an immediate evaluation including history, physical examination, possibly color Doppler scrotal ultrasound and involvement of a urologist or pediatric surgeon [4]. Patients presenting should also be kept NPO (nothing by mouth) in anticipation for possible surgical management. The evaluation is utilized to distinguish TT from other clinical entities on the differential diagnosis (Table 1). History and physical examination are incredibly important in identifying features of TT and differentiating it from other entities on the differential diagnosis. It is important to recognize that infants, children and adolescents may have difficulty describing their symptom localization, severity and time course; thus, revisiting features on history or gaining corroborating history from family may help. Classically, TT presents with sudden onset of unilateral scrotal or testicular pain, associated nausea and/or vomiting [5–11].

On physical examination, the testicle is exquisitely tender, and often assumes an elevated ‘high-riding’ position and horizontal lie; scrotal skin may be erythematous and thickened [7, 9, 12]. The cremasteric reflex is often blunted or absent in TT [7, 8, 13, 14]. In patients that present with high likelihood of TT, surgical exploration for therapy should be performed without further delay pursuing imaging [15] in order to limit prolonged ischemia and improve testicular salvage rate [6]. However, in cases that remain unclear, scrotal imaging in the form of color Doppler scrotal ultrasound [16] or radionuclide imaging may be performed [17]. Color Doppler ultrasound is the most commonly utilized imaging modality and has a sensitivity of (88.9%) and specificity of (98.8%) with a false negative rate of 1% [15]. Color Doppler ultrasound demonstrates decreased or absent relative flow on the affected testis in cases of TT [15, 16, 18, 19]. However, If a patient receives a scrotal ultrasound with negative results (i.e. positive testicular perfusion) despite strong history and physical examination, management should still be surgical exploration [5, 6, 18–20].

Table 1 Differential diagnosis for acute scrotum and salient features on investigation. (Content adapted from Sharp et al. 2013)

Differential Diagnosis	History	Physical Exam	Investigation
Testicular torsion	Acute onset, significant pain, ± nausea and vomiting	Elevated or high-riding testis, horizontal testis orientation, acutely tender testis, ± blunted or absent cremasteric reflex, ± scrotal wall edema and erythema	U/A: Unremarkable Scrotal ultrasound: Lack of perfusion to affected testis
Epididymitis	Gradual onset, may coincide with symptoms of urinary tract infection (dysuria, hematuria, malodorous urine, fever)	Scrotal erythema, scrotal skin edema, tenderness localizes to epididymis	U/A: Positive leukocytes, ± nitrites ± blood. CBC: ± leukocytosis Scrotal ultrasound: Good perfusion to testes, ± hypervascularity to epididymis
Epididymo-orchitis	Gradual onset, may coincide with symptoms of urinary tract infection (dysuria, hematuria, malodorous urine, fever)	Scrotal erythema, scrotal skin edema, tenderness localizes to both testis and epididymis	U/A: Positive leukocytes, ± nitrites ± blood. CBC: ± leukocytosis Scrotal ultrasound: ± hypervascularity to epididymis and/or affected testis
Orchitis	Typically surrounding viral infection. Mumps orchitis often bilateral, fever	Scrotal erythema, scrotal skin edema, 1 or both testes tender	U/A: ± leukocytes, ± nitrites ± blood. CBC: ± leukocytosis Scrotal ultrasound: ± hypervascularity to affected testis(es)
Hematologic disorders	History of hematologic disorders, systemic B symptoms	Diffusely firm testes (leukemia or lymphoma), not exquisitely tender	CBC hematologic abnormalities Normal U/A
Inguinal hernia	In adults: Inguinal pain, symptoms may worsen with exertion, abdominal pain, nausea and vomiting if incarcerated and obstructed Children: Fluctuant swelling associated with patent processus vaginalis	± scrotal mass (unable to trans-illuminate in adults, but may in children), ± abdominal discomfort, ± inguinal swelling	Normal U/A Imaging may demonstrate dilated bowel or inguinal herniation of bowel

(continued)

Table 1 (continued)

Differential Diagnosis	History	Physical Exam	Investigation
Torsion of appendix testis	Acute onset pain, lack of systemic features	±blue dot sign to upper pole of testis, tenderness localizes to upper pole of testis, ± scrotal erythema or edema	U/A: Unremarkable Scrotal ultrasound: Normal testicular perfusion.
Torsion of appendix epididymis	Acute onset pain, lack of systemic features	±blue dot sign to head of epididymis, tenderness localizes to epididymis, ± scrotal erythema or edema	U/A: Unremarkable Scrotal ultrasound: Normal testicular perfusion.
Scrotal trauma	History of blunt or penetrating trauma	Eccymosis, laceration, hematoma	Scrotal ultrasound: ±testicular rupture U/A: ± gross hematuria
Renal colic	History of flank or abdominal pain. May radiate to testis, ± nausea or vomiting, ± dysuria, ± hematuria	± flank percussion tenderness or abdominal tenderness, unremarkable scrotal examination	U/A: +blood, ± leukocytes Imaging: Hydro-ureter or hydro-nephrosis ± visualized stone Bloodwork: ± elevated creatinine

Mechanisms of Testicular Torsion

TT is an occurrence that is usually not precipitated by an event, although it results from trauma in a small minority of cases [21]. The increase in testicular volume during puberty or from the presence of a tumor, testicular horizontal lie, and a history of cryptorchidism can all act as predisposing factors to TT [22].

Torsion of the testis is divided anatomically into intravaginal and extravaginal categories based on the axis of torsion. Neonatal torsion predominately occurs via extravaginal torsion where the entire contents of the spermatic cord and processus vaginalis torts, resulting in eventual obstructed arterial inflow and subsequent ischemia (Fig. 1). In children, adolescents and adults, TT typically occurs through intravaginal twisting of the spermatic cord, where the axis of rotation is within the tunica vaginalis, eventually obstructing arterial inflow similarly resulting in testicular ischemia [23, 24]. It is believed that boys with TT have abnormal fixation of the testis to the tunica vaginalis, promoting increased testicular mobility and a propensity to torsion. Here, the normal attachment of the tunica vaginalis to the posterior testicle and epididymis occurs more proximally [25] allowing increased mobility and is termed a Bell-Clapper deformity [26]. This process is initially mediated by venous obstruction and congestion, and leads to subsequent disruption of arterial inflow and resultant ischemia. The mechanism that induces twisting or rotation of the spermatic cord is not fully understood. However, strong activation of the cremasteric muscles which induces testicular retraction or elevation may have an effect [25].

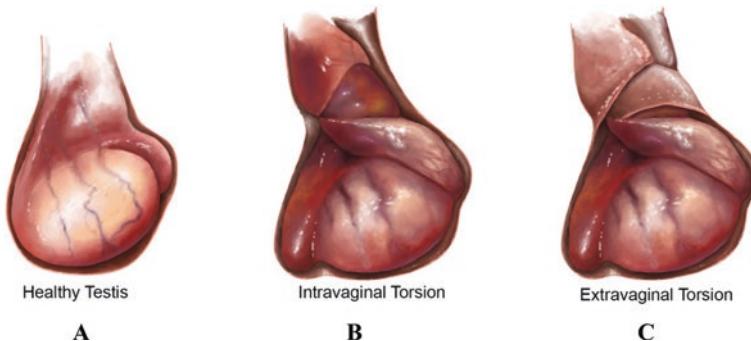


Fig. 1 Demonstrates testicular torsion where the spermatic cord twists resulting in vascular obstruction and testicular ischemia. (a). Normal anatomy of spermatic cord and testicle. (b). Intravaginal torsion common to children, adolescents and adults. (c). Extravaginal torsion common to neonates

Clinical Management

The gold standard treatment of TT is surgical scrotal exploration and detorsion of the spermatic cord. Expedient diagnosis and management is a priority with TT, since the window for intervention to prevent histopathologic changes and preserve spermatogenic and endocrine function is between 4 and 8 h [27].

A meta-analysis recently pooled all of the case series in the literature to report on testis salvage rates per time interval; their findings included:

- 97.2% between 0 and 6 h,
- 79.3% between 7 and 12 h,
- 61.3% between 13 and 18 h,
- 42.5% between 19 and 24 h,
- 24.4% between 25 and 48 h,
- 7.4% beyond 48 h [28].

Testicular salvage through scrotal exploration occurs in 90–100% of cases when performed within 6 h of symptom onset, and as low as 10% among durations of 24 h [29–31].

Due to the heterogeneity in testicular viability extending beyond 8 h, immediate scrotal exploration is indicated in cases of longer duration for potential salvage [11, 25]. Surgeons should avoid the use of epinephrine in spermatic cord blocks during the procedure to avoid risk of vasospasm and subsequent worsened ischemia [32]. At the time of de-torsion and orchiopexy, surgeons should perform an orchiopexy of the contralateral testis. This is because the anatomic abnormality predisposing an individual to torsion is presumed to be bilateral even in most events where clinical torsion is unilateral [26]. Among cadaveric studies, a Bell-Clapper deformity was identified in 12% of men and was most often bilateral [33]. In cases of non-viable and necrotic testes, an orchiectomy is performed to avoid additional discomfort, risk

of infection with necrotic tissue and additional autosensitization due to germ cell antigen exposure [34, 35]; the frequency of orchietomy is reported between 39 and 71% [20, 36, 37]. Only in cases where immediate surgical exploration is not available, should manual trans-scrotal detorsion be considered while awaiting surgical management [20, 29, 38, 39].

Effect on Fertility and Spermatogenesis

Abnormal Semen Parameters

Numerous studies have reported that men who have had TT, have a propensity towards abnormal semen analyses. Between 50 and 87% of men following torsion have abnormal semen analyses to follow [27, 40–42]. A review of 240 men with semen evaluations up to 12 years since TT, determined that 37% have oligozoospermia (defined as <20 million/ml), 56% have asthenozoospermia (<40% motility), and 53% have abnormal morphology (defined as <50% normal forms) [43]. However, the effect of TT on fertility has been heterogeneous in the literature. Reports suggest that individuals with testicular torsion prior to puberty and onset of complete spermatogenesis have normal fertility following the event, while men that develop TT beyond puberty may develop some impairment [44, 45]. Among men that have detorsion and testicular salvage, upwards of 68% have testicular atrophy [46]. The degree of atrophy is highly associated with the duration of ischemia and the degree of ischemia based upon the number of twists of the spermatic cord [27, 46–48]. Theoretically, men with one testis should have adequate spermatogenesis to maintain normal semen parameters. Thus, the rationale ensued that if impairments exist in the semen analysis, abnormalities may occur in the contralateral testis. Several hypothesis and potential mechanisms of TT induced infertility have been postulated and tested in the following sections.

Clinical Pregnancy Rates

Despite the plethora of studies investigating the mechanisms in which TT induces pathological changes, few studies have evaluated the effect on fecundity rates. Among animal studies, Madgar et al. reported no differences in pregnancy rates among rats that underwent sham surgery compared to testicular artery ligation and release after 24 or 48 h, or orchietomy following ligation at 24 or 48 h [49]. Merimsky performed another rat study that demonstrated no reduction in fertility among rats undergoing orchietomy of ligated testes. However, among those rats with ligated testes left insitu for 3 months duration, fertility rates were significantly reduced [50]. To the contrary, Nagler and colleagues report decreased fertility rates after 8 h of torsion and detorsion among rats [51]. Even fewer studies have been reported among humans assessing pregnancy rates among men with TT. Gielchinsky

et al. recently reported outcomes among 63 men that had previously had TT, of whom 41 were treated with orchiopexy, and 22 with orchiectomy. Pregnancy rates among these cohorts were 90.2% and 90.9% respectively which was comparable to pregnancy rates in the general population ranging between 82 and 92% [52]. These findings would suggest that despite validated mechanisms of TT induced damage to spermatogenesis often resulting in abnormal semen analysis, clinical pregnancy rates may not be affected at all, or to the degree that was once hypothesized. However, additional work is required to investigate the pregnancy rates among sub-groups that have endured TT to increase clinical granularity.

Mechanisms of Torsion-Induced Deficits to Spermatogenesis

Several mechanisms have been put forth to delineate the effects of TT on spermatogenesis and testicular tissue. Some studies have shown that torsion-induced damage is not only limited to the affected testis but can also extend to affect the contralateral side as well. It is noteworthy that the vast majority of studies that have looked into the mechanisms of torsion-induced testicular damage have utilized animal models.

Ischemia, Ischemia-Reperfusion (I/R) Injury and Oxidative Stress

There is a consensus in the literature that the effects of ischemia and I/R injury along with the oxidative stress that follows are the most destructive to testicular tissue and spermatogenesis compared to the other proposed mechanisms which will be discussed in this chapter.

Torsion induced-testicular damage consists of two injurious components that are complementary to one another; a hypoxic injury and a reperfusion injury [53]. The exact mechanism by which these injuries inflict cellular damage is not completely understood [54]. The initial interruption of blood supply to the testis damages metabolically active tissues and causes a rise in Ca^{2+} intracellularly, with the resulting overproduction of reactive oxygen species (ROS) following restoration of blood flow accompanied by lipid peroxidation, leading to further tissue damage [55, 56]. This I/R injury was in fact found to be more severe than the tissue damage induced by ischemia [57].

Ischemia is the first and most pivotal event in TT. The disruption of blood flow to the testis as a result of torsion is first initiated by obstruction to venous return. This obstruction is followed by the equalization of venous and arterial pressures which ultimately leads to testicular ischemia [30]. The testicular tissue then becomes hypoxic, which leads to a decrease in adenosine triphosphate (ATP) production due to limited oxygen availability. The drop in tissue ATP levels is attributed to breakdown into its degradation product hypoxanthine, along with proteolytic conversion of the enzyme xanthine dehydrogenase into the super oxide generator enzyme xanthine oxidase. The enzyme conversion is facilitated by an influx of Ca^{2+} and a rise in

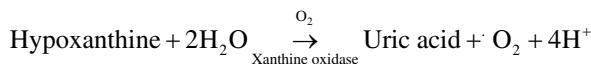
its intracellular concentration [58, 59]. The alteration in the intracellular concentration of Ca^{2+} , specifically in the mitochondria, has an additional consequence of irreversibly initiating events leading to cellular apoptosis [55].

Ischemia also has negative effects on germ cell viability with subsequent decreases in testis weight, number of type B spermatogonia, preleptotene and leptotene spermatocytes and daily sperm production [11, 60]; this may occur as early as 100 min of ischemic time in animal models [61]. In a study by Cosentino et al. which examined the histopathology of the testes in prepubertal rats that were subjected to various durations of ischemia, the first changes that were observed within the seminiferous tubules included the degeneration of germ cells along with spermatozoa and spermatids. The disarray of germ cell layers and complete damage or rupture of the tubules proved more resistant as they required longer periods of ischemia [62]. Young and Goldstein also reported that testicular ischemia or torsion induced severe testicular damage negatively effecting spermatogenesis leading to Sertoli-Cell Only (SCO) syndrome in animals [63].

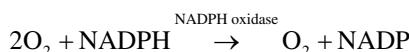
With the restoration of blood flow after detorsion, testicular tissue damage is aggravated. This cellular damage involves anoxia, altered microvascular blood flow, production of ROS, oxidation of membrane lipids, proteins and DNA, apoptosis, recruitment of neutrophils, pro-inflammatory cytokines and adhesion molecules, along with the stimulation of tumor necrosis factor alpha and interleukin 1 beta and the activation of nitric oxide synthase (NOS) [64–67]. A large delivery of ROS ensues damaging the seminiferous epithelium [61, 68]. Unfortunately, this reperfusion injury is not limited to the side of TT; the contralateral testis also experiences a time dependent injury [69].

Among the generated ROS in testicular reperfusion, they most commonly include hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^-), superoxide anions (O_2^-), and nitric oxide (NO) [70]. The mechanisms by which ROS are mostly generated include:

1. Xanthine oxidase catalyzes the conversion of hypoxanthine into uric acid which causes the release of superoxide radicals [59].



2. The migration of polymorphonuclear leukocytes to the ischemic region as a result of the ischemia-induced stimulation of chemotactic factors is followed by the release of ROS on reperfusion [71].
3. Tissue-invading neutrophils, macrophages and resident parenchymal cells cause a toxic burst of oxygen radicals 60–90 min after initial reperfusion. Neutrophils are perhaps the most implicated through their respiratory burst facilitated by the cell membrane-bound enzyme NADPH [53].



Testicular tissue relies primarily on two protective endogenous mechanisms to protect it against oxidative stress. The first is the low oxygen tension contained within the testicular microenvironment, and the second is a collection of enzymes which form an antioxidative defense system. Those enzymes include superoxide dismutase (SOD), catalase, and glutathione peroxidase (GSH-Px). Although these mechanisms can prove sufficient in protecting the testicular tissue and spermatogenesis under normal conditions, they however become overwhelmed by the abundant overproduction of ROS during reperfusion; hence, spermatogenesis is considered vulnerable to oxidative stress [72, 73].

The imbalance created between ROS and the antioxidative defense mechanisms lead to lipid peroxidation which results in the damage of spermatozoa and in the loss of sperm membrane fluidity which decreases sperm motility. The effects of lipid peroxidation can be quantified by measuring malondialdehyde (MDA) levels. Additionally, sperm motility is affected by an axonomal structural damage, perhaps induced by the peroxidative damage to axonomal proteins, which seems to cause permanent impairment in motility [74–76].

Some studies have discussed the role of ROS in the apoptosis of cells in the male reproductive system; however the exact mechanism remains obscure. Oxidative stress seems to induce damage to the integrity of the DNA in the sperm nucleus which accelerates the process of apoptosis in germ cells and ultimately decreases sperm count [77]. In their literature review, Agarwal et al. denoted the effect of oxidative stress on the mitochondrial membranes. The disruption of the membranes by increasing levels of ROS induces a release of cytochrome c, which activates caspases, specifically 9 and 3 [78]. Normally, caspases have a regulatory role in cell apoptosis in the seminiferous epithelium, sperm differentiation, and testicular maturity [74]; however, in testicular torsion, caspases can lead to impaired spermatogenesis and DNA fragmentation in sperm along with apoptosis [79].

Mitogen activated protein kinases (MAPK) have also been implicated in the pathogenesis of I/R injury of the testis [80, 81]. The activation of this signal transducing group of enzymes is triggered by lipid peroxidation via oxidative stress [82]. Once activated, MAPKs phosphorylate transcription factors and also lead to the production of proinflammatory cytokines that have various cellular effects, among which is apoptosis [74]. Minutoli et al. demonstrated that reperfusion after detorsion causes an early activation of MAPKs, this activation was however not induced upon ischemia only. The same group also found that this activation is blunted by the flavonoid inhibitor PD98059 which improved testicular I/R damage bilaterally, thus presenting a potential therapeutic role [80].

Treating Ischemia-Reperfusion Injury & Oxidative Stress

Pharmacologic agents to limit damage to the testis surrounding TT has been extensively reviewed by Karaguzel et al. in [83]; below we provide a brief summary of the agents that have been investigated.

Pharmacologic Agents

Treating I/R injury and oxidative stress has been addressed by many groups and generally includes agents that have antioxidant, ROS scavenging and anti-inflammatory properties [83]. Phosphodiesterase inhibitors have been used with varying success. Erol et al. found reduced cellular damage and lipid peroxidation with intraperitoneal administration of vardenafil in a rat model of TT [84]; while Yildiz et al. 2011 also demonstrated attenuated levels of antioxidant level reductions including glutathione, GSH-Px leading to less significant histologic changes following low dose sildenafil [85]. However, Istanbuloglu et al. found results to the contrary using a pig-based model demonstrating increased apoptosis of cells and inflated levels of the isoforms of NOS, iNOS and eNOS levels [86]. A more specific phosphodiesterase 5A inhibitor dipyridamole administration in a rat TT model also attenuated the histopathological changes [87].

Other antioxidant mediated therapies tested include intraperitoneal administration of selenium, prior to testis detorsion in a rat model which also attenuated histopathologic changes to both testes [70, 88]. α -lipoic acid, an agent that promotes endogenous antioxidant capacity, administration prior to TT resulted in favorable histopathology, and oxidative stress markers [89]. Similarly, administration of dexpanthenol prior to detorsion in an animal model prevented testicular atrophy at 60 days from the event [90]. Raxofelast administration to rats results in metabolism to a ROS scavenger and demonstrated favorable changes following I/R injury in a rat model of TT [81, 91]. Vitamin C and dopamine were studied by Azizollahi et al. and identified to reduce markers of oxidative stress, i.e. MDA levels, following TT in rats [92]. Zofenopril is an angiotensin converting enzyme inhibitor that protects endothelial cells from free radical damage [93]. Verapamil, a calcium channel blocker has demonstrated improved outcomes following animal model induced TT [53, 94–96]. Administration to rats with TT, demonstrated improved antioxidant parameters and reduced testis cellular damage [97]. Polydeoxyribonucleotide stimulates vascular endothelial growth factor (VEGF) and when used in an animal model of TT, results in reduced histopathologic damage [98]. Gabexate mesilate, improves antioxidant levels and testicular microcirculation among rats undergoing TT [99]. Anesthetic agents such as propofol and thiopental have been demonstrated to elicit antioxidant properties; when compared in a rat model of TT, rats treated with propofol demonstrated less histopathologic damage compared with thiopental [100]. Dexmedetomidine has also been demonstrated to inhibit lipid peroxidation and reduce germ cell apoptosis in a TT model [101]. Non-steroidal anti-inflammatory agent ibuprofen has been shown to decrease NO and cytokine production while diclofenac has been shown to reduce germ cell apoptosis following TT [102, 103]. Dimethylsulfoxide (DMSO) has been shown to induce favorable antioxidants levels, i.e. decreased malondialdehyde, following TT in rats [104]. Finally, N-acetylcysteine (NAC), is a common agent used clinically to reduce cellular injury. Several studies have demonstrated improved histopathological outcomes compared to rats not receiving NAC [58, 105, 106].

Natural Extracts

Numerous plant extracts have demonstrated favorable antioxidant effects in the testes. Ternatin [107], quercetin [108], lycopene [109], *P. ginseng* ie. Korean red ginseng [110], thymoquinone [111], kastamonu [57], L-carnitine [56], and ginkgo biloba [112] have all demonstrated reduced levels of oxidative stress and some preservation of testis histopathology in animal models of TT.

Endogenous Hormones & Analogues

Endogenously produced substances such as melatonin have also been demonstrated to have antioxidant properties and temper the histopathologic changes following TT in a rat model [113]. Melanocortin analogues have been used in their anti-inflammatory capacity and demonstrate favorable changes in spermatogenesis compared to controls, as well as improved histopathology and reduced inflammatory markers [114]. Erythropoietin has also demonstrated attenuated histopathological changes following TT in rats as well as decreased inflammatory markers [115]. Dehydroepiandrosterone (DHEA) has also demonstrated favorable results with reduced injury and apoptosis in rats. Peroxisome proliferator-activated receptor (PPAR) activation via L-165,041 reduced the histologic damage and IL6 and TNF α expression via western blot detection [116].

Physical Therapies

Numerous methods are being experimentally investigated to reduce the effects of both ischemia and I/R injury. Surgical strategies beyond gold standard therapy of exploration and detorsion include fasciotomy incisions to the tunica albuginea. Results have been limited to animal experiments that demonstrate decreased cellular apoptosis [117] as well as limited case series in humans [118]. The rationale is to decrease edematous changes and increased parenchymal pressures associated with ischemia-reperfusion injury. Hyperbaric oxygen therapy has also been tested in animal models with favorable results following reperfusion [119]. This has been thought to occur due to reduction of monocyte and macrophage mediated inflammatory cytokine release and preventing a degree of oxidative stress [120–123].

Other Mechanisms

Auto-Antibodies

One hypothesis is during testicular ischemia and necrosis, the blood testis barrier is disrupted and the immunologically privileged germ cells are exposed to the body's immune system producing anti-testis antibodies capable of harming the contralateral side [43]. This process was termed sympathetic orchopathia [124]. This hypothesis is applicable to those who develop testicular torsion beyond puberty, since spermatogenesis must be present for antigen exposure in order for the immune system to generate auto-antibodies [44]. Animal experiments have evaluated the contralateral testis following models of induced torsion. Numerous animals have been used ranging from mouse, rat, hamster, guinea pig, canine and rats, and each demonstrate different susceptibilities to testicular alterations [125]. The degree of rotation (180–1440°) and duration of torsion seem to play an effect on testicular perfusion as tested in magnetic resonance imaging studies, and contribute to the discordant results [60, 126–128]. Among experimentally induced TT in pre-pubertal rats, several groups have demonstrated significant reduction in contralateral seminiferous tubule diameters and detection of haploid cells if TT was left for 9–12 h [129–131], while other groups failed to identify abnormalities after only 4 h of untreated TT [132]. Animal experiments of testicular torsion and detorsion detected increased serum levels of anti-sperm antibodies using indirect immunofluorescence [133, 134], and tissue deposition in the contralateral testis [134]. However, among animals that had an orchiectomy following induced torsion, no serum anti-sperm antibodies were produced. However, among pre-pubertal studies of experimentally induced TT in rats, did not identify auto antibodies or contralateral testis damage [135]. Anti-sperm antibodies have been previously demonstrated to penetrate the blood-testis barrier and induce germinal cell apoptosis and impair spermatogenesis [136]. However, results on the topic of TT induced anti-sperm or anti-testis antibodies are heterogeneous with other studies reporting negative results using variations in experimental methodology [137]. Furthermore, human studies have had extremely variable results with 0 to 20% of post-TT men having detectable sperm antibodies [44, 138–142], with some studies demonstrating no detection of auto antibodies among 58 patients [139], while others reporting detection in 8 of 9 patients with TT beyond 6 h duration [143].

Congenital Abnormalities & Intermittent Torsion

Two additional hypotheses that are difficult to distinguish from one another include the presence of congenital abnormalities among these testes or abnormalities due to brief intermittent episodes of self-resolving torsion, resulting in accumulative damage to tissue bilaterally [43]. Studies evaluating biopsies at the time of scrotal exploration revealed histologic abnormalities among 53% of cases [144]. Some groups

report abnormal findings in the contralateral testis in 60% of men with observations of maturation arrest, tubular hyalinization and focal basement membrane thickening [145]. One group identified histologic abnormalities consistent with congenital dysplasia in 88% of contralateral testes examined, while an additional group found abnormalities in all 7 of 7 patients [146]. Similarly, biopsies from pre-pubertal boys at the time of scrotal exploration demonstrated reduced numbers of spermatogonia present and biopsies of post-pubertal boys demonstrated fewer spermatids per tubule [147]. Anderson and Williamson tested the hypothesis that abnormalities in spermatogenesis accompany developmental abnormalities such as Bell-clapper deformity and cryptorchid testes that demonstrate impaired spermatogenesis. This was performed via contralateral testis biopsies among boys with torsion and abnormal tunica vaginalis development; they identified late maturation arrest among 57% of tubules with 46% of patients demonstrated oligozoospermia on semen analysis [138].

Conclusion

Testicular torsion is a urologic emergency that requires expedient diagnosis and surgical exploration and detorsion. The impact of unilateral TT on fertility presents a significant degree of heterogeneity in the literature. Most studies report abnormalities among semen analyses in these men following TT. The most important fertility outcomes, live birth and clinical pregnancies have limited reports in the literature and suggest pregnancy rates may be on par with the general population. However, further details need to be addressed including duration of attempting pregnancy, techniques used, and if abnormalities exist in sub-populations of men developing TT such as those prior to or after puberty. Numerous mechanisms have been postulated and tested again with heterogeneous results. Data perhaps most consistently points to a potential underlying developmental abnormality in the testes prior to and thus, following TT leading to abnormal spermatogenesis. I/R injury appears to be the dominant pathology affecting spermatogenic function. Various techniques and pharmacologic approaches are being investigated to reduce the negative physiological implications of TT and preserve testicular function. Further research is required to clearly elucidate the suggested mechanisms which underlie the damage induced by testicular torsion.

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Testis Toxicants: Lesson from Traditional Chinese Medicine (TCM)



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Introduction

Through thousands of years of human history across the world, including Asia, Europe, Americans, Australia and Pacific Island nations, natural products have been used as the major source of therapeutic agents and drugs. According to reports from WHO, 80% of the world's population, mostly in developing countries, rely on traditional medicine to sustain their daily healthcare needs [1–4]. For the use of Traditional Chinese Medicine (TCM) for treatment of illnesses in China, studies in recent decades have demonstrated the use of TCM perturbs male fertility in many cases based on studies in vitro and in vivo (Table 1). As such, systematic studies of natural ingredients isolated from different TCMs to assess their potency and side-effects are becoming one of the matters of interest among scientists. Interestingly, besides identifying therapeutic activities from most active ingredients from TCMs, some of these TCMs often associate with antifertility as one of the side effects. A wide range of plants, in particular their phytocomponents and their derivatives were found to have disrupting effects on rodent testes or Sertoli cells as noted in selected studies summarized in Table 1. Many of the toxicants from TCMs are presently

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Table 1 Disruptive effects of toxicants from TCMs on adult rodent testes in vivo or on Sertoli (or germ/sperm) cells in vitro

Toxicant/drug	Phenotypes	Treatment doses	Species	Refs
Triptolide	Sertoli cell injury, likely due to a down-regulation of β -actin, Rho GTPase (e.g., RhoA, RhoB, Cdc42 and Rac1) expression	In vivo: 100 μ g/kg/ b.w.	Rat	[5]
	Testis injury due to failure in intracellular transport of fatty acids and a down-regulation of PPARs	In vivo: 60 μ g/kg/ i.p.	Mice	[6]
	Reduction of lactate and dysregulation of fatty acid metabolism in Sertoli cells; mitochondrial damage through the suppression of PGC-1 α ; reduction of lactate production and dysregulated fatty acid metabolism; mitochondrial dysfunction, initiated by the inhibition of SIRT1 with the regulation of AMPK.	In vivo: 50–100 μ g/kg/i.g.	Mice	[7]
	Expression of STAR, P450scc, P450c17, 3 β -HSD, and 17 β -HSD mRNAs and proteins in the testes are down-regulated.	In vivo: 4.5–18 mg/kg/ b.w.	Rat	
	Sertoli cell injury, due to the down-regulation of gap junctional intracellular communication.			
Gossypol	Mitochondrial dysfunction and calcium dysregulation of cytosol.	In vitro: 1.25–10 μ M	TM4	[8]
		In vivo: 10 mg/kg/ b.w.	Mice	
		In vivo: 15–35 mg/kg/ b.w.	Mice	[9]
Cannabidiol	Stages VII–VIII and XII spermatogenesis decrease, loss of Sertoli cells; 30% reduction in fertility rate and a 23% reduction in the number of litters. Inhibition of re-uptake and degradation of endocannabinoids, particularly the hydrolytic enzyme fatty acid amide hydrolase	In vivo: 10 mg/kg/ b.w.	Rats	[10]
		In vivo: 5–10 mg/kg/ b.w.	Rats	

(continued)

Table 1 (continued)

Toxicant/ drug	Phenotypes	Treatment doses	Species	Refs
Piperine	Reduction of testicular hormone synthesis; impairment of testicular antioxidant system by promoting the ROS production and hydroxyl radical generation; a stimulation of pubertal Leydig cell development by increasing Leydig cell number and promotion of its maturation while inhibiting spermatogenesis. ERK1/2 and AKT pathways may involve in the piperine-mediated stimulation of Leydig cell development.	In vitro: 5–20µM; in vivo: 2.5 mg/kg/ i.p.	TM3,TM4	[11, 12]
	Down-regulation of the cell cycle checkpoint genes <i>Ccnd1</i> and <i>Ccne1</i> , changes in the mitochondrial membrane potential, reduction on <i>Inha</i> and <i>Inhba</i> mRNA expression.		Mice	
α-Solanine	Inhibits mouse sperm functions by a [Ca ²⁺]i-related mechanism via CatSper channel	In vivo:1–50 mg/kg/ b.w.	Mice	[13]
Matrine	Activation of apoptosis-induced cell death, reduction on the size and weight of mouse testes, inhibition of ERK1/2 and Akt activation	In vitro: 10–1000µM; in vivo:10 mg/kg/ i.p.	TM4, mice	[14]
	Inhibition on amino acid metabolism in mouse testicular cells, disruptive changes in the mitochondria of spermatogonial cells, down regulation on the androgen receptor and hormone levels	In vivo:0.2–20 mg/kg/ b.w.	Mice	
Aristolochic acid I	Testis injury, including apoptosis via the IGF-1 receptor signaling pathway	In vivo:50–1000 mg/kg/ b.w.	Mice	[15]
Emodin	Reduction in sperm motility, progressive motility and linear velocity inhibition, reduction in sperm [Ca ²⁺]i and suppressing tyrosine phosphorylation.	In vitro: 25–400 µM	Human sperm	[16– 20]

PGC-1α proliferator-activated receptor coactivator-1 alpha, *SIRT1* sirtuin 1, *AMPK* AMP-activated protein kinase, *StAR* steroidogenic acute regulatory protein, *P450scc* P450 side chain cleavage enzyme, *P450c17* cytochrome P450 17-hydroxylase, *3β-HSD* 3β-hydroxysteroid dehydrogenase, *17β-HSD* 17β-hydroxysteroid dehydrogenase

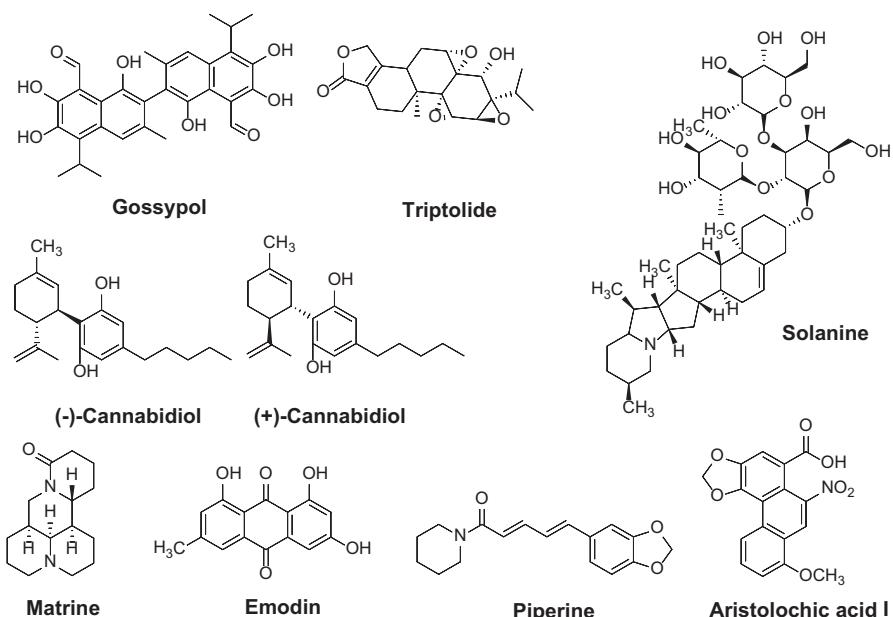


Fig. 1 A schematic drawing that illustrates the structures of TCMs with male reproductive toxicity

known to induce testis injury by disrupting Sertoli cell, Leydig cell and sperm functions, which in turn, impede spermatogenesis, leading to male reproductive dysfunction and infertility. Herein, we provide a brief summary on the effects of several active ingredients in TCMs that induce testis injury and some discussions on the underlying molecular and cellular mechanism(s). Such information should be useful to develop approaches to alleviate (or manage) their toxic side effects without reducing its therapeutic usefulness to treat specific diseases in humans.

Triptolide induces testis injury by perturbing Sertoli cell cytoskeletons and mitochondrial function.

Triptolide is a major active ingredient of medicinal plant *Tripterygium wilfordii* Hook F, which is used for the therapy of immune related diseases such as allergic purpura, rheumatoid arthritis in China for thousands of years [21, 22]. Triptolide is a diterpene triepoxide (Fig. 1) with a variety of biological and pharmacological activities, such as anti-tumor, anti-inflammatory and anti-fertility activities [23]. Thus, its clinical applications and other therapeutic uses, especially in pediatrics, are hindered by its reported disruptive effects in the testis and the reproductive tract, such as infertility, including reduced sperm count and sperm motility [5, 8, 24]. It was shown that treatment of rodents by triptolide in rats at 100 μ g/kg b.w. (oral gavage) induced weight loss in testes and epididymides, and accompanied by germ cell degeneration and exfoliation [25]. Also, the level of β -actin, an important component of Sertoli cell cytoskeleton, was considerably down-regulated after triptolide

administration at 100 μ g/kg in rats for 4 weeks. The expression of Rho GTPases including RhoA, RhoB, Cdc42 and Rac1, was also considerably reduced in the testis. A study by immunofluorescence analysis indicated that the organization of F-actin across the Sertoli cell cytosol following triptolide treatment was grossly disrupted, and accompanied by a surge in expression of testin [5], which is a marker of Sertoli cell function [26, 27].

It has been reported that treatment of mice with triptolide at 60 μ g/kg b.w./day for 2 weeks suppressed the well-known markers (SDH, ACP and LDH) and key regulators PPAR α and PPAR β/δ in fatty acid metabolism that support spermatogenesis and also reduced testosterone levels, reduced sperm counts, reduced gonad functional index and induced extensive seminiferous epithelial damage in the testis [6]. Abnormal levels of fatty acids were also noted in the testis due to a reduced expression of PPAR [6]. Also, triptolide was found to inhibit peroxisome proliferator-activated receptor coactivator-1 alpha (PGC-1 α , a member of a family of transcription coactivators that are crucial to regulate cell energy metabolism) activity and its expression in Sertoli TM4 cells, triptolide also disrupted mitochondrial fatty acid oxidation by inducing AMPK phosphorylation which, in turn, led to sirtuin1 (SIRT1, also known as NAD-dependent deacetylase sirtuin-1) inhibition, eliciting mitochondrial dysfunction in Sertoli cells [7].

Glycosides of *Tripterygium wilfordii* Hook F (GTW) are authorized native drug widely used in China for treatment of different illnesses including rheumatoid arthritis, nephritis, ankylosing spondylitis, skin disorders, and cancer which contain triptolide [28–33]. Following treatment of adult rats with GTW at 4.5–18 mg/kg b.w. for 4–12 weeks, the expression of StAR, P450scc, P450c17, 3 β -HSD, and 17 β -HSD mRNAs and their proteins were all down-regulated in the rat testis. GTW also induced male reproductive toxicity via a concentration- and time-dependent manner by inhibiting the expression of the key enzymes, but also total cholesterol level which in turn impeded testosterone synthesis [8].

Taken collectively, these findings thus have demonstrated unequivocally that the use of triptolide for therapeutic treatments of different diseases would impede male fertility, in particular its long-term use in humans, likely through its effects to perturb cytoskeletal function (and also mitochondrial and Leydig cell functions) in the testis. Perhaps it is necessary to investigate if the triptolide-induced male reproductive dysfunction in rodents is transient and reversible. Studies are also needed to examine if the use of regulatory peptides and/or signaling proteins recently shown to correct toxicant-induced testis injury would alleviate triptolide-induced toxicity to the testis. For instance, recent reports have shown that the use of a phosphomimetic mutant of p-FAK-Y407, namely p-FAK-Y407E (a constitutively active mutant), is able to block and rescue PFOS-induced Sertoli cell injury in both rats [34] and humans [35], including correcting the toxicant-induced Sertoli cell cytoskeletal disorganization.

Gossypol represses Sertoli cell gap junctional intercellular communications (GJIC) and Leydig cell steroidogenesis in mouse testes.

Gossypol is a polyphenolic compound with a terpenoid aldehyde structure (Fig. 1). Gossypol derives from cotton seeds, leaves, stems, and flower buds. It has

been used for several medical applications due to its antioxidant, anti-viral, anti-microbial, anti-parasitic, and anti-fertility activities [36]. In fact, gossypol has been actively investigated as a potential male contraceptive for decades until its toxic effects based on studies in animals and humans were unraveled which included weight loss, male infertility and kidney damage [37]. It is of interest to note that the use of a Sertoli cell line, TM4, for a study by treating Sertoli cells with gossypol at different concentrations ranging from 1.25 to 10 μ M for 6–48 h, gossypol induced considerably down-regulation on the expression of Cx43, suggesting the gap junctional intercellular communication (GJIC) was perturbed [9]. Another study showed junction proteins occludin, ZO-1, N-cadherin and specifically Cx43 could be early targets for testicular toxicants including gossypol in SerW3 Sertoli cell line [38]. Gossypol irreversibly impaired intercellular communications between homologous pairs of cultured cells, from men or rats dose-dependently. Gossypol at 5 μ M (approximately twice the peak plasma concentration measured in human patients during chronic administration), gap junctional communication was interrupted within 4–10 min [39]. Studies have shown that GJIC induced by toxicants, such as PFOS, as noted based on a functional assay (and also down-regulation of Cx43 that supports GJIC), and the associated Sertoli cell cytoskeletal disorganization can be considerably blocked and reversed by overexpression of a p-FAK-Y407E phosphomimetic (and constitutively active) mutant [40]. Earlier studies have also shown that p-FAK-Y407 (or the use of its mutant p-FAK-Y407E) promotes BTB and spermatogenic function by maintaining the cytoskeletal homeostasis in the testis through its effects on Sertoli cells to support spermatogenesis. On the other hand, this lack of GJIC function can also be restored by overexpressing Cx43 in the testis as noted in a recent report wherein overexpression of Cx43 was capable of rescuing spermatogenic dysfunction caused by treatment of rats with an acute toxic dose of adju-din [41].

Gossypol was recently shown to inhibit testicular 3 β -hydroxysteroid dehydrogenase, thereby interfering with androgen synthesis [42]. Gossypol was also found to inhibit luteinizing hormone (LH), 8-bromo-adenosine 3',5'-monophosphate-induced testosterone formation, and the conversion of pregnenolone to testosterone in interstitial cells isolated from rat testes [43]. Gossypol also causes a disruption of steroidogenesis and spermatogenesis at the level of transcription by affecting the expression of various genes to support cell cycle, testicular development, steroidogenic enzymes, and hormone receptors in mice. Gossypol suppresses mitotic proliferation of TM3 and TM4 cells via mitochondrial damage and disruption of cell calcium homeostasis. Gossypol also down-regulates the expression of steroidogenic enzymes, LH receptors, and transcriptional factors, thereby perturbing testis physiology in the mouse testis [10]. In brief, the efforts of developing gossypol as a male contraceptive were abandoned due to its ability to lower serum potassium which cannot be corrected by either potassium supplements or triamterene [44, 45].

Cannabidiol impairs sexual behavior, fertility, and the ability to re-uptake and degradation of endocannabinoids in male mice.

Cannabis sativa L. (marijuana) is a plant species that contains more than 400 compounds, and approximately 100 of them are phytocannabinoids. Cannabidiol is

the major phytocannabinoid (Fig. 1), accounting for up to 40% of marijuana extract [46]. *Cannabis sativa L.* has a long history of utilization as a fiber and seed crop in China for years [47]. However, cannabinoid consumption is linked to mammalian reproductive dysfunction in humans. In male mice, these include inhibition of spermatogenesis, reduced the levels of hypothalamic, pituitary and gonadal hormones in circulation, and changes in sperm morphology [12, 48]. The results revealed that the administration of cannabidiol at doses of 15–30 mg/kg b.w. for 34 consecutive days, followed by a 35-day recovery period caused significant changes in the testes such as an alteration on the frequency of stages of the epithelial cycle of spermatogenesis, and a considerable reduction in serum testosterone levels at the high doses. The cannabidiol treatment group at 30 mg/kg b.w. showed a 30% reduction in fertility rate and a 23% reduction in the litter size [12]. Furthermore, males in the experimental groups showed an increase in the frequency of stage I-VI tubules (mitotic stages), but a considerable decline in stage VII–VIII tubules (spermiation) and also stage XII tubules (meiotic phase) [48]. Cannabidiol also involves in the inhibition of the re-uptake and degradation of endocannabinoids, particularly the inhibition of the hydrolytic enzyme fatty acid amide hydrolase. These results should be expanded to better understand the mechanism(s) by which cannabidiol induces defects in spermatogenesis, so that the male reproductive dysfunction caused by cannabidiol consumption in humans can be therapeutically managed due to its wide-spread use among men.

Piperine inhibits spermatogenesis by reducing testis hormone synthesis, but it stimulates pubertal Leydig cell development via the ERK1/2 and AKT signaling pathways.

Piperine (PIP, 1-peperoylpiperidine) (Fig. 1) is the primary pungent alkaloid isolated from *Piper nigrum Linn.* and *Piper longum Linn.*, which belongs to the family of *Piperaceae*. *Piper nigrum* is a common food supplement all over the world. In TCMs, a formulation containing these herbs is used for skin disease such as eczema and acne. Studies in vitro have shown that piperine protects cells against oxidative damage by inhibiting or quenching free radicals and reactive oxygen species [49]. Piperine induces hormonal imbalance in male rats by altering the serum levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH), and sex hormone binding globulin (SHBG), but also serum and testicular testosterone at a dose of 10 mg/kg b.w. (for 60 days) [13]. Detailed analysis of the piperine-androgen-binding protein interactions shows that piperine interacts with Ser42 of the androgen-binding protein and could block the binding with its natural ligands dihydrotestosterone/testosterone. Moreover, piperine interacts with Thr577 of the androgen receptors in a manner similar to the antagonist cyproterone acetate [50].

In another recent report, thirty-day treatment of rats with piperine was found to significantly increase serum testosterone levels without affecting LH concentrations. However, this also accompanied by a reduced serum FSH level, an increase in Leydig cell number, cell size, and multiple steroidogenic pathway proteins, including steroidogenic acute regulatory protein, cholesterol side-chain cleavage enzyme, 3 β -hydroxysteroid dehydrogenase 1, 17 α -hydroxylase/20-lyase, and steroidogenic factor 1 expression levels. Piperine also considerably increased the ratio of

phospho-AKT1 (pAKT1)/AKT1, phosphos-AKT2 (pAKT2)/AKT2, and phospho-ERK1/2 (pERK1/2)/ERK1/2 in the testis [14]. Piperine in vitro also increased androgen production and stimulated cholesterol side-chain cleavage enzyme and 17 α -hydroxylase/20-lyase activities in immature Leydig cells [14]. These findings thus suggest the promoting effects of piperine on pubertal Leydig cell development, which should be carefully evaluated and expanded in future studies.

α -Solanine Inhibits Cell Proliferation in Mouse Testes

α -Solanine is a trisaccharide glycoalkaloid (Fig. 1) which is naturally found in leaves and unripe potatoes, tomatoes and eggplants. It was reported to have toxic effects in humans [51]. α -Solanine treatment caused the loss of MMP and cytosolic calcium ions, and accompanied by an increase in PI3K/AKT, ERK1/2, and JNK MAPK phosphorylation in both mouse Leydig (TM3) and Sertoli (TM4) cells. α -Solanine reduced cell viability in TM3 and TM4 cells but also down-regulated the expression of the cell cycle checkpoint genes Ccnd1 and Ccne1. In addition, Inha and Inhba mRNA expression also decreased in both cell lines and C57BL/6 mice with 100 μ L of α -solanine (2.5 mg/mL) every 3 days i.p. for 10 administrations [15]. In this report, mouse Sertoli cells were found to be arrested at S-phase of cell cycle by solanine, and the expression of cyclin A and CDK2 proteins, as well as vimentin and androgen binding protein were also down-regulated [15].

Matrine inhibits mouse sperm functions by a $[Ca^{2+}]_i$ -related mechanism via CatSper channel.

Matrine is a quino-lizidine alkaloid (Fig. 1) with various biological effects, such as anti-viral, anti-arrhythmic, anti-fibrotic, anti-inflammatory, anti-tumor and insecticidal activities [52, 53]. Matrine is the main bioactive component of *Sophora flavescens* (kushen) and *Sophora subprostrata* (shandougen) which are two well-known TCMs used in the treatment of cancer, psoriasis and other diseases [54]. In a study using C57BL/6 J mice, which were treated with daily doses of 0, 1, 10 and 50 mg/kg matrine by intraperitoneal injection for 30 days. Matrine did not affect testis size, testis weight, sperm count and sperm viability, but it considerably inhibited motility, progressive motility, linear velocity, capacitation and the progesterone-induced acrosome reaction of mouse sperm [16]. Furthermore, the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), a key regulator of sperm function, was reduced in sperm of matrine treated mice. The ion current and gene expression of the sperm specific Ca^{2+} channel, CatSper, which modulates Ca^{2+} influx in sperm, were considerably down-regulated in testes of matrine treated mice [16].

Aristolochic acid I induces testis injury and cell apoptosis through an inhibition of amino acid and glucose metabolism, β -oxidation of fatty acids and the TCA cycle in male mice.

Aristolochic acid (AA) is a natural herbal product derived from the genera *Aristolochia* and *Asarum* (Fig. 1). For a long period of time, TCM containing AA has been used to treat various diseases including arthritis, gout, rheumatism,

suppurative wounds and snake bites, as well as for obstetrics and anti-tumor treatment [55]. As the main toxic component of aristolochic acid, aristolochic acid I (AAI) has been proven to be carcinogenic, mutagenic and nephrotoxic. AAI inhibited amino acid metabolism in mouse testicular cells, impeded the uptake and oxidative decomposition of fatty acids, prevented normal glucose uptake by testicular cells which inhibited glycolysis and gluconeogenesis, thereby affecting the mitochondrial tricarboxylic acid (TCA) cycle and impairing the ATP energy supply [18]. In brief, AAI reduced the number of spermatogenic cells and sperm in the testes, induced changes in the mitochondrial state of spermatogonial cells, and ultimately led to physiological and pathological changes in the mouse testis. AAI also regulated the testicular physiological activity by regulating the androgen receptor and hormone levels [18]. Besides, AAI strongly inhibited Sertoli TM4 cell survival by inducing cell apoptosis and testis tissue in mice. In addition, AAI suppressed the expression of B-cell lymphoma 2 (Bcl-2), a factor related to anti-apoptosis. AAI markedly up-regulated pro-apoptotic protein expression, including Bcl-2-associated X protein, poly(ADP-ribose) polymerase, and caspase-3 and -9. Furthermore, AAI at 10 mg/kg (i.p., for 4 weeks) significantly reduced the size and weight of mouse testes. Moreover, germ cells and somatic cells in testis were markedly damaged by AAI. In addition, AAI caused severe injury and testis development by inducing apoptosis through the Akt and ERK1/2 signaling pathway [17].

Emodin inhibits human sperm functions by reducing sperm $[Ca^{2+}]_i$ and tyrosine phosphorylation.

Emodin (1,3,8-trihydroxy-6-methylanthraquinone) (Fig. 1) is a naturally occurring anthraquinone in the rhizomes of numerous plants, such as *Rheum palmatum* and *Polygonum cuspidatum* which has been widely used in anti-inflammatory and anti-cancer drugs in TCMs. Pharmacological investigations have demonstrated that emodin possesses an array of pharmacological properties including antibacterial, purgative, immunosuppressive, vasorelaxant, anti-inflammatory, hepatoprotective, anti-oxidant and anti-cancer effects [56, 57]. When emodin used at different doses (25, 50, 100, 200 and 400 μ M) to incubate with ejaculated human sperm, emodin significantly inhibited the total motility, progressive motility and linear velocity of human sperm. In addition, acrosome reaction was also adversely affected by emodin. However, emodin did not affect sperm viability. Furthermore, intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and tyrosine phosphorylation, which serve as key regulators of sperm function, were reduced by emodin dose-dependently [20]. Another report indicates that emodin caused testicular toxicity, including apoptosis through the IGF-1 receptor signaling pathway [19].

Concluding Remarks and Future Perspectives

Due to the wide variety of anti-spermatogenic effects from toxicants derived from TCMs, future investigations should probably be focused not just on how these toxicants affect the testis mechanistically, but also approaches on how to block (or

avoid) their toxic effects on the testis, so that these TCMs can be used to treat different pathological conditions and diseases without inducing male reproductive dysfunction. In fact, the efforts to develop novel male contraceptive drugs based on the use of toxicants derived from TCMs due to their inherent anti-fertility activity are well underway. Since there are numerous types of new components isolated from TCMs periodically, the list of toxicants may continue to grow. Based on emerging molecular mechanistic findings, in particular the cellular targets and signaling proteins that are involved in toxicant-induced Sertoli cell and testis injury through the use of TCMs, the disruptive effects of these toxicants can possibly be managed when used for treatment of certain diseases. It is also expected that some of these toxicants found in TCMs can be explored to serve as potential male contraceptives in future years.

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