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REGULATION AND CHARACTERIZATION OF THE ATP-BINDING CASSETTE TRANSPORTER-B1 IN THE EPIDIDYMIS AND EPIDIDYMAL SPERMATOZOA OF THE RAT

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I dedicate this thesis to my father, Thomas R. Jones, who inspired me to enter scientific research.

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List of Abbreviations

ABC	ATP-binding cassette
ABP	androgen binding protein
AP	alkylphenol
APE	alkylphenol ethoxylates
apo-A-1	apolipoproteins A-1
ATP	adenosine triphosphate
BCRP	breast cancer resistance protein
BEB	blood epididymis barrier
BPA	bisphenol-A
BTB	blood testis barrier
CA	cauda
CAR	constitutive androstane receptor
CFTR	cystic fibrosis transmembrane conductance regulator
Cl	chloride
CS	corpus
CT	caput
CYP	cytochrome P450
CYP1A1	cytochrome P450-1A1
CYP2E1	cytochrome P450-2E1
CYP450	cytochrome P450
DDT	dichlorodiphenyltrichloroethane
DHT	dihydrotestosterone
DNA	deoxyribonucleic acid
ED	endocrine disruptors
FSH	follicle stimulating hormone
GJIC	gap junctional intercellular communication
GnRH	gonadotropin releasing hormone
GPI	glycosylphosphatidylinositol
GSH	glutathione
GST	glutathion S-transferase
HDL	high-density-lipoproteins
HSF1	heat shock factor protein-1
IGF-1	insulin like growth factor-1
IS	initial segment
KO	knock out
LH	luteinizing hormone
MAPK	mitogen-activated protein kinase
MDR	multidrug resistance
MDR1	multidrug resistance-1
MDs	membrane binding domains
MRP1	multidrug resistance protein-1
MT	metallothioneins
NBDs	nucleotide binding domains
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
NP	nonylphenol

NPE	nonylphenol ethoxylates
OP	octylphenol
OPE	octylphenol ethoxylates
PAF	platelet activating factor
P-gp	p-glycoprotein
PKC	protein kinase C
PTU	propylthiouraci
PVC	polyvinyl chloride
PXR	pregnane x receptor
ROS	reactive oxygen species
SUR1	sulfonylurea receptor-1
TH	thyroid hormone
TNPP	trisonylphenol phsophite
T3	triiodothyronine
UV	ultraviolet

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Chapter 2

Summary

The epididymis and maturing spermatozoa are susceptible to the effects of xenobiotics. A functional blood-epididymis barrier (BEB) attenuates the entry and facilitates the elimination of potentially harmful substances from the epididymal epithelium. In addition to providing a sophisticated immunological barrier, the BEB barrier provides a specialized, protected environment where sperm transit, are concentrated and acquire both motility and the ability to fertilize. The BEB is composed of both tight-junctions and specialized transporters capable of regulating the types and quantities of compounds entering the epididymal lumen.

Recent evidence suggests that oral administration of alkylphenols (AP), while being able to reach the testis and epididymis, fail to accumulate in these tissues. This suggested that these tissues could either rapidly metabolize AP or expulse them from the cells. Several specialized efflux transporters that function to exclude xenobiotics from target cells have been identified. ABCB1 is a broad specificity ATP-binding cassette (ABC) transporter capable of excluding xenobiotics out of cancerous and normal tissues serving barrier or secretory function.

Little is known about the role of ABC xenobiotic efflux transporters in the epididmyis. The objectives of this study were to characterize the expression profile and functional role of ABCB1a and ABCB1b along the different regions (initial segment, caput, corpus, cauda) of the rat epididymis.

ABCB1a and ABCB1b transcripts were detected in all regions of the epididymis. Immunohistochemistry conducted with an antibody that recognizes both forms of rodent ABCB1 revealed a unique expression gradient in both the epididymal epithelium and epididymal spermatozoa along the length of the tissue. Little to no staining was present on the sperm present in the lumen or epithelium in proximal regions of the epididymis, whereas a strong immunoreaction was observed in distal caput, corpus and cauda. Expression gradients in the tissue and epididymal spermatozoa were confirmed by western blot. These results suggested an important role for ABCB1 in epididymal tissue defense

and spermatozoal protection, as well as a unique process where sperm acquire ABCB1 protein during epididymal transit.

ABCB1 functionality in epididymal principal cells and epididymal spermatozoa was confirmed using a multi drug resistance (MDR) assay. A rat principal cell line (RCE) and epididymal spermatozoa exctracted from proximal and distal regions of the epididymis demonstrated an inhibitable MDR phenotype when incubated with calcein, an ABCB1 substrate, in the presence of specific ABCB1 inhibitors.

Lastly, to assess whether or not the system was inducible by xenobiotics, RCE cells were exposed to various concentrations of nonylphenol (NP), an AP, and doxorubicin (DOX), an anti-cancer agent and known inducer of ABCB1. RCE cells exposed to NP and DOX revealed a significant induction of ABCB1a and ABCB1b mRNA, as well ABCB1 protein, suggesting the existence of a dynamic defense mechanism that can respond to chemical insult.

The unique expression profile and induction of ABCB1 in epididymis suggests a role in regulating epididymal barrier function and protection of spermatozoa. ABCB1 may be an important toxicokinetic regulator of AP and other xenobiotics in the adult rat epididymis.

Student

Director of Research

INTRODUCTION TO THESIS

Xenobiotics present in the environment can negatively impact epididymal function, sperm maturation and male fertility (Hess, 1998; Phillips and Tanphaichitr, 2008). Sperm are transcriptionaly incompetent and are therefore particularly vulnerable to chemical insult. A functional blood-epididymis barrier (BEB) exists in the form of tight junctions between adjacent cells in the epididymal epithelium and specialized transporters that actively regulate the composition of the luminal environment and the types and quantities of compounds coming into contact with maturing sperm. Although the structural components of the BEB have been well characterized (Agarwal and Hoffer, 1989; Cyr *et al.*, 1995; Cyr *et al.*, 2007), little information exists on the distribution and function of xenobiotic efflux transporters in the adult rat epididymis and their role in the maintenance of male reproductive potential. It has been suggested that members of the ATP-binding cassette (ABC) transporters family may be important toxicokinetic regulators in the epididymis; ABCG2 knock-out animals demonstrated increased epididymal accumulation of phytoestrogens (Enokizono *et al.*, 2007). Additionally, common environmental pollutants, such as industrial surfactants, were shown to be rapidly excluded from the adult rat epididymis, suggesting a role for efflux transports in epididymal tissue defence (Hamelin *et al.*, 2009). ABCB1 is a broad-specificity xenobiotic efflux transporter that had been demonstrated to be an important toxicokinetic and pharmacokinetic regulator in both cancerous and normal tissues serving a barrier or secretory function (Staud *et al.*, 2010).

The hypothesis of the current study was that ABCB1 is expressed in both the epithelium of adult rat epididymis and epididymal spermatozoa and is inducible by industrial surfactants. To address this hypothesis, the expression profile and localizaton of ABCB1 in the adult rat epididymis and epididymal spermatozoa was evaluated. The study also evaluated ABCB1 functionality in a rat epididymal cells and epididymal spermatozoa and the ability of a common environmental pollutant, NP, to induce ABCB1 expression.

The thesis is divided into four chapters; the first chapter of this thesis contains an extensive literature review of epididymal function, industrial surfactants with a particular

focus on AP (NP), and the ABC transporter family. The second chapter of this thesis contains original research entitled: *Regulation and Characterization of the ATP-binding Cassette Transporter-b1 in the Epididymis and Epididymal Spermatozoa of the Rat.* The implications of this thesis work are further discussed in the third chapter of this thesis. Lastly, a French summary of the thesis is provided in the fourth chapter.

CHAPTER 1: LITERATURE REVIEW

1.0 The Male Reproductive System

The male reproductive system contains organs required for the production of germ spermatozoa and accessory organs for supporting the reproductive process. Germ cell production occurs in the seminiferous tubules of the testis through spermatogenesis (Clermont, 1972; de Kretser and Kerr, 1988). Accessory organs are required for germ cell transit out of the body and maturation, concentration, protection and storage prior to ejaculation. Male accessory organs include the excurrent duct system (efferent ducts, epididymis and vas deferens), glands and supporting structures (Setchell and Brooks, 1988; Setchell and Breed, 2006). Accessory glands (seminal vesicles, prostate and bulbourethal) also aid the reproductive process by nourishing and maturing sperm. Supporting structures include the testes containing scrotum, penis and spermatic cords that include the vas deferens, testicular, deferential and cremasteric arteries, nerves and lymphatic vessels (Setchell and Brooks, 1988; Setchell and Breed, 2006).

1.1.0 Spermatogenesis

1.1.1 Hormonal control of Spermatogenesis

The primary function of the testes is the production of viable gametes. Secondary functions include the production of hormones and testicular factors, including androgens, estrogens, retinoids, oxytocin, peptides and growth factors (de Kretser *et al.*, 1998; Hermo *et al.*, 2010a). Spermatogenesis is an extremely complex, dynamic process that is highly regulated by endocrine signals, including luteinizing hormone (LH), follicle stimulating hormone (FSH) and thyroid hormone (TH), paracrine signals (androgens, estrogens, growth factors, cytokines) and direct cell-cell contact (Gilula *et al.*, 1976; de Kretser *et al.*, 1998; Cheng and Mruk, 2002; Holdcraft and Braun, 2004; Mruk and Cheng, 2004; Hermo *et al.*, 2010a).

The primary hormonal regulators of spermatogenesis are FSH and androgens (De Kretser *et al.*, 1971; Wahlstrom *et al.*, 1983; Ruwanpura *et al.*, 2010). Hormonal signaling to stimulate spermatogenesis begins with release of gonadotropin releasing hormone (GnRH) produced by the hypothalamus. Pulsatile release of GnRH signals the anterior pituitary to produce LH and FSH. LH binds to receptors on the surface of Leydig cells in the testis, triggering the production of androgens (De Kretser *et al.*, 1971; Steinberger *et al.*, 1973; Wahlstrom *et al.*, 1983; Komori *et al.*, 2007). Several androgens, including androstenedione, testosterone and DHT (dihydrotestosterone) are synthesized from cholesterols in Leydig cells (Dohle *et al.*, 2003; Hugues and Durnerin, 2005; Lombardo *et al.*, 2005). In rodents, androgens transported within the testis and excurrent duct system remain bound to androgen binding protein (ABP) (Turner, 1991). Testosterone is converted to DHT, a steroid possessing a greater affinity for the androgen receptor, by 5α-reductase in the epididymal epithelium and other androgen-sensitive target tissues (Viger and Robaire, 1994; Robaire and Viger, 1995). Androgens are also synthesized in peripheral adrenal glands (Liu *et al.*, 1955). Androgens serve a diverse array of biological functions both inside and outside of the male reproductive tract. They promote normal development and maintenance of secondary sexual characteristics and accessory sex organs including the prostate, seminal vesicles and epididmyis (Hughes *et al.*, 2001; Holdcraft and Braun, 2004; O'Donnell *et al.*, 2006). Androgens also regulate anabolic metabolism, fluid and electrolyte balance and feedback on the hypothalamo-pituitary gonadal axis (Gupta and Bhatia, 2008; Miller, 2009; Callewaert *et al.*, 2010). Testosterone diffuses into the seminiferous tubules and along with FSH, interacts with Sertoli cells, which express receptors for both FSH and testosterone (Wilson and Smith, 1975; Salhanick and Wiebe, 1980; Verhoeven *et al.*, 2010). Sertoli cells are the only somatic cell type in the seminiferous tubules. Their presence is essential for both testis formation and spermatogenesis; functional testes lacking Sertoli cells have never been observed and the number of male germ cells is directly proportion to the number of Sertoli cells (Griswold, 1998). Ablation of the androgen receptor in Sertoli cells completely blocks meiosis in spermatogenesis, indicating Sertoli cells are the primary mediators of androgen action in the testis (Verhoeven, 2005; Verhoeven *et al.*, 2010). Evidence suggests androgens may also utilize secondary

messengers and signaling pathways to elicit non-genomic regulation of Sertoli cell function (Walker, 2003).

Sertoli cells provide factors that fuel germ cell metabolism such as lactate, transferring and ABP (Tindall *et al.*, 1974; Jutte *et al.*, 1983). They also provide growth regulatory factors (stem cell factor, transforming growth factors), insulin-like growth factor-1 (IGF-1), fibroblast growth factor and epidermal growth factor and hormones that regulate the development of male reproductive structures or feedback to inhibit signals affecting Sertoli cells (Mullerian inhibiting substance and inhibin) (Walker and Cheng, 2005; Loss *et al.*, 2007). Dynamically regulated Sertoli-Sertoli tight junctions form a functional blood-testis barrier (BTB) that physically divides basal and apical compartments of the seminiferous epithelium (Kopera *et al.*, 2010). In addition to serving as a protective immunological barrier, the BTB also creates a unique microenvironment that allows the maturation and movement of proliferating germ cells (Kopera *et al.*, 2010). Several protein families, including claudin, occludin and tight junction proteins (ZO-1/TJP1, ZO-2/TJP2) have been identified as essential components of tight junctions of the BTB (Lui *et al.*, 2003). Gap junctions present in the cells of seminiferous epithelium and interstitial space also support spermatogenesis by allowing the transport of small molecular signals between cytoplasmic compartments of adjacent cells (Pointis et al., 2005). Androgen regulation of BTB permeability and tight junction protein expression has been demonstrated recently, suggesting an important role for androgens in germ cell movement within the seminiferous epithelium (Meng *et al.*, 2005; Wang *et al.*, 2006b; Kaitu'u-Lino *et al.*, 2007).

FSH exerts biological effects by interacting with Sertoli cell surface receptors; FSH signaling activates several signaling pathways and response elements that transcribe genes implicated in spermatogenesis (Simoni *et al.*, 1997; Heckert and Griswold, 2002; Walker and Cheng, 2005). FSH supplementation has been demonstrated to improve sperm counts in infertile men (Baccetti *et al.*, 1997). FSH, LH and testosterone have also all been demonstrated to regulate germ cell apoptosis, suggesting an important role for these hormones in germ cell survival (Erkkila *et al.*, 1997; Tesarik *et al.*, 2001). Interestingly, the importance of FSH in male fertility has also been subject to debate; testosterone alone was

sufficient to promote normal testicular maturation and fertility in GnRH receptor KO mice, suggesting a less important role for FSH (Singh *et al.*, 1995). FSH receptor mutations have also been associated with variable reductions in sperm counts while maintaining overall fertility (Tapanainen *et al.*, 1997).

In addition to gonadotrophic hormones and testosterone, estrogens are also important regulators of spermatogenesis. Targeted disruption of the estrogen receptor resulted in altered mating frequency, lower sperm counts and defective sperm (Eddy *et al.*, 1996; Joseph *et al.*, 2010). Moreover, several recent epidemiological studies have also attributed increases in number male reproductive abnormalities (infertility, cryptorchidism, hypospadias and testicular cancer) to environmental exposures of compounds possessing estrogenic or anti-androgenic properties (Main *et al.*, 2010). Aromatase present in the Sertoli (immature animals), Leydig cells (adults) and germ cells irreversibly converts androgens into estrogens (Payne *et al.*, 1976; Carreau, 1996; Lambard *et al.*, 2003; Rago *et al.*, 2003). Estrogen receptors have been reported in several germ cell types, including spermatocytes, spermatids and ejaculated sperm, suggesting an important role in spermatogenesis (Saunders *et al.*, 1998; Saunders *et al.*, 2001).

Several studies suggest that TH also play a role in testicular function and maintenance of spermatogenesis. Normal physiological functioning of the thyroid is essential for testicular development and functionality during adulthood (Wagner et al., 2008). Neonatal hypothyroidism increased testicular weight and daily sperm production in adult rats (Cooke et al., 1991; Cooke and Meisami, 1991). This effect has been related to an increased number of Sertoli cells that was attributable to delayed differentiation, and an increased number of Leydig cells (Hardy et al., 1993; Hess et al., 1993). A large body of evidence has identified triiodothyronine (T3) as a regulator of Sertoli and Leydig cell proliferation and maturation (Holsberger and Cooke, 2005; Mendis-Handagama and Siril Ariyaratne, 2005). The precise mechanism for TH action on Sertoli cells remains poorly understood. Several recent studies however, have suggested T3 may increase the expression of cyclin-dependent kinase inhibitors and proteins involved in gap junctional intracellular communication (GJIC; Cx43) (Holsberger et al., 2003; Gilleron et al., 2006). Mice exposed

to Propylthiouracil (PTU), a medication used to treat hyperthyroidism, showed increased Sertoli cell expression of Cx43 that was associated with increased GJIC (Gilleron et al., 2006). PTU also altered Cx43 localization in developing seminiferous tubules and reduced epididymal Cx43 mRNA levels in rats (St-Pierre et al., 2003).

Lastly, cytokines are also thought to be important hormonal regulators of BTB dynamics and spermatogenesis (Li *et al.*, 2009). Elevated levels of TNFalpha and TGFbeta3 have been reported in the seminiferous epithelium microenvironment at the point of spermiation (spermatozoa release) (De *et al.*, 1993; Teerds and Dorrington, 1993). These cytokines have been shown to disrupt BTB integrity and germ cell adhesion (Li *et al.*, 2009; Xia *et al.*, 2009; Wong *et al.*, 2010). It is hypothesized that cytokines released by germ and Sertoli cells regulate germ cell movement and BTB barrier dynamics during spermatogenesis (Li *et al.*, 2009).

1.1.2 *Spermatogenesis – Sequence of Events*

During spermatogenesis, testicular germs cells are transformed from round cells to cells with a unique shape, highly condensed nucleus and flagellar apparatus (Gatti *et al.*, 2004; Hermo *et al.*, 2010c). In spermatogenesis, spermatogonia go through several phases of differentiation to become mature spermatozoa (Fig. 1). Spermatozoa are continuously produced in the testis from a self renewing population of spermatogonial stem cells. The generation of spermatozoa from stem cells allows the passage of genetic material to offspring and ensures a constant supply of progenitors (Huckins, 1971a; Huckins, 1971c; Huckins, 1971b).

The process of spermatogenesis can be divided into 3 phases; proliferative (mitotic), meiotic and spermiogenic (differentiation) (Russel *et al.*, 1990; Hermo *et al.*, 2010c; Hermo *et al.*, 2010a). In the proliferative phase, spermatogonia numbers are increased dramatically. Spermatogonia undergo multiple divisions to generate a large population that will ultimately undergo meiosis in the next phase. In each mitosis, the cell population technically doubles. Three types of spermatogonia are present in the testis; Type A

(isolated - stem cell), proliferative (A paired and A aligned) and differentiated (A1, A2, A3, A4, B and intermediate), which can be characterized by the chromatin distribution in their nuclei (Huckins, 1971a; Huckins, 1971c; Huckins, 1971b; Russel *et al.*, 1990; Hermo *et al.*, 2010a). Both Type A and proliferative spermatogonia are considered undifferentiated. Interestingly, early studies noted a degeneration of proliferating (A1,A2,A3) spermatogonia during spermatogenesis (Clermont, 1962). The theoretical number of spermatocytes was also reported as being lower than expected.; approximately 75% of spermatogonia do not go on to become mature gametes, suggesting the involvement of apoptotic pathways in spermatogenesis (Huckins, 1978). It is now known that apoptosis of germ cells is a normal component of spermatogenesis; apoptotic mechanism maintain a germ cell-Sertoli cell balance by removing defective germ cells (Ruwanpura *et al.*, 2010). Several genetically programmed cell death mechanisms have been described in the testis, including caspase-dependent and -independent apoptosis and anoikis (Lockshin and Zakeri, 2004; Show *et al.*, 2004; Shaha, 2007; Ruwanpura *et al.*, 2010). Recent research suggests FSH and testosterone are the principal hormone regulators of apoptotic pathways in the testis (Woolveridge *et al.*, 1999; Chausiaux *et al.*, 2008; Ruwanpura *et al.*, 2010). These hormones may act as survival factors, regulating cell death rather than proliferation. Stem cell spermatogonia are often very resistant to perturbations and will often remain healthy when other cell populations die (Huckins, 1971a; Huckins, 1971c; Huckins, 1971b; Huckins and Oakberg, 1978; Jiang, 1998). Insult causing death of many stem cells however, such as in the case of high dose cancer chemotherapy treatments, can cause irreversible damage to spermatogenesis (Kadota *et al.*, 1989). The proliferative and differentiating spermatogonia have a much higher mitotic rate and thus, are more influenced by chemical insult (Huckins and Oakberg, 1978). Spermatogonia are found in the outer rim of the seminiferous tubule and usually have contact with supportive Sertoli cells that nurture developing sperm (Huckins, 1971c; Huckins, 1971b; Russel *et al.*, 1990). After the first mitotic division, the most mature daughter cells, each with 46 chromosomes, become the primary spermatocytes.

The second phase of spermatogenesis is the meiotic phase, in which genetic material is recombined and segregated and spermatocytes become haploid spermatids (Russel *et al.*,

1990; Hermo *et al.*, 2010a). The first step is Type B cells dividing to form preleptotene spermatocytes (Pl). The Pl are the last cells to go through the S phase of the cell cycle. Chromosomes recombine and genetic material is divided during later meiotic divisions. The end result is a quadrupling of the germ cell number (Russel *et al.*, 1990). Following the S phase is prophase, in which the Pl undergo the following changes; Preleptotene (Pl) followed by leptotene (L), Zygotene (Z), Pachytene (P) and Diplotene (Di). Leptotene (L) cells indicate the start of prophase (Russel *et al.*, 1990). The Leptotene (L) can be identified by the presence of condensed, unpaired chromosomes and cells with a more rounded appearance. In the Zygotene (Z), the homologous chromosomes pair. This can be identified with light microscopy. In the Pachytene (P), genetic recombination is allowed to take place. This enables germ cell chromosomes to create a unique arrangement that is different from the somatic cells (Russel *et al.*, 1990). The Pachytene (P) also show significant nuclear growth. The Diplotene (D) is marked by dissipation of the synaptonemal complex, a proteinous complex that connects chromosomes to form synapsed bivalents during prophase (Hermo *et al.*, 2010a). It was recently discovered that the chromatin of homologous chromosomes is attached to the synaptonemal complex (Costa and Cooke, 2007). Genetic analysis of synaptonemal complexes has helped identify some of the DNA sequences and proteins involved in chromosome attachment (Hernandez-Hernandez *et al.*, 2009); mice lacking SCP2 or SCP3, protein components of the synaptonemal complex, have been demonstrated to be sterile (Yuan *et al.*, 2000; Yang *et al.*, 2006). The Diplotene (D) are the largest primary spermatocytes and the largest of all the germ cells. Following prophase are metaphase, anaphase and telophase (Meiosis I) (Russel *et al.*, 1990). The end result of MI results in secondary spermatocytes. Meiosis II follows quickly after MI, producing spermatids. The end result of MI and MII is to form four haploid cells (23 chromosomes) from every diploid (46 chromosome) primary spermatocyte (Russel *et al.*, 1990; Hermo *et al.*, 2010a).

The third and final stage of spermatogenesis is the spermiogenic phase, which involves a series of complex morphological changes that contribute to sperm maturation. Significant alterations in sperm body and nucleus shape occur in a species specific manner (Clermont and Leblond, 1955; de Kretser *et al.*, 1998; Hecht, 1998). The excess cytoplasm

associated with a condensing of the sperm head is eliminated through the formation of a residual body and cytoplasmic droplet (Roberts *et al.*, 1976; Breucker *et al.*, 1985; Hermo *et al.*, 2010c; Hermo *et al.*, 2010b). Several organelles undergo modifications and other new organelles, including a radial body, anulate lamellae and chromatoid body are formed (de Kretser *et al.*, 1998; Hermo *et al.*, 2010b). The chromatoid body is a cloud-like structure of male germ cells that moves dynamically in haploid spermatids (Kotaja and Sassone-Corsi, 2007). The function of the chromatoid body is unknown. Recent studies however, indicate that that microRNA and RNA-decay pathways converge to the chromatoid body, suggesting possible role in RNA processing in male germ cells (Kotaja and Sassone-Corsi, 2007). The acrosome, a membrane bound organelle involved in fertilization, is formed from the Golgi apparatus (Clermont and Leblond, 1955; Hermo *et al.*, 2010b). The formation of the acrosome involves three phases; Golgi, cap and acrosome (Clermont and Leblond, 1955; Hermo *et al.*, 2010b). During the Golgi phase, vesicles from trans-Golgi networks bud and fuse with each other to form pro-acrosomic granules that attach to one pole of the sperm nucleus. A larger portion of the nucleus is covered by additional Golgi-derived vesicles in the cap phase. The acrosomal phase is characterized by an elongation of the sperm nucleus and formation of the mature acrosome.

Nuclear chromatin is heavily compacted to allow the formation of an elongated sperm head. Compaction of the chromatin allows for a more hydrodynamic nucleus that is required for fertilization (Zhao *et al.*, 2004; Pradeepa and Rao, 2007; Hermo *et al.*, 2010b). This process involves the replacement of most somatic linker histones by transition proteins (TP's), which are eventually replaced by protamines (Kistler *et al.*, 1994; Hecht, 1998; Woltje *et al.*, 1998; Ronfani and Bianchi, 2004). The primary known function of TP's is to prepare the chromatin for binding the protamines (Kimmins *et al.*, 2004; Kimmins and Sassone-Corsi, 2005). Post-translational modifications of histone variants, phosphorylation, ubiquitination, sumoylation and hyperacetylation facilitate the removal of somatic histones and chromatin compaction (Hazzouri *et al.*, 2000; Sassone-Corsi, 2005; Vigodner and Morris, 2005; Pradeepa and Rao, 2007). Meiotic cells have been shown to possess a higher level of histone acetylation (Wang *et al.*, 2006a). This is thought to facilitate the accessibility to DNA for transcription (Kimmins *et al.*, 2004; Kimmins and Sassone-Corsi,

2005). Increased transcription is necessary for chromosome congression, recombination and desynapsis. Post meiotic spermatids also show elevated transcription and acetylation (Wang *et al.*, 2006a). This chromatin architecture is thought to facilitate the transition from histones, to transition proteins and then protamines and eventual differentiation of the round spermatids into elongated spermatids (Kimmins *et al.*, 2004; Kimmins and Sassone-Corsi, 2005).

A flagellum is also established allowing the eventual acquisition of motility during epididymal transit. The flagellum is composed of a connecting piece, midpiece and principal piece with a ring shaped annulus, and endpiece (Fawcett, 1975; Hermo *et al.*, 2010c). Flagellum formation involves migration of paired centrioles at nuclear pole opposite the developing acrosome. Paired centrioles and accessory proteins form the centrosome, a microtubule organization center that coordinates the initiation and development of the flagellum (Paintrand *et al.*, 1992; Hermo *et al.*, 2010c). Mature testicular sperm are released at the point of spermiation into the lumen of the seminiferous tubules.

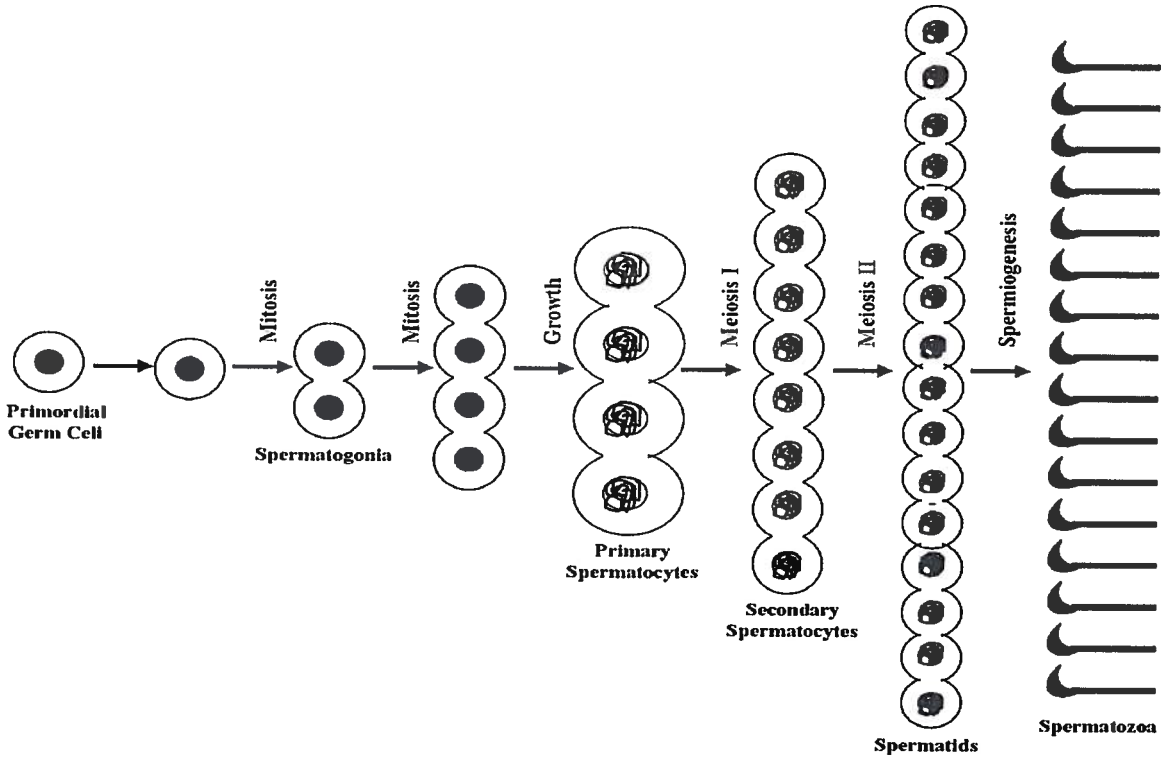

Figure 1: Schematic representation of rat spermatogenesis. Courtesy of Danton O`Day, UTM.

1.2.0 The Epididymis

Sperm are produced in the seminiferous tubules of the testes and collected by the rete testis, an anastomosing network of tubules, prior to entry into the excurrent duct system (Robaire and Hermo, 1988). Germs cells are produced in the testis, but it is during transit through the epididymis that they mature to become fully motile and fertile cells (Orgebin-Crist, 1969). The epididymis is a highly coiled and continuous duct connecting the efferent ducts to the vas deferens (Fig. 2). It contains a highly specialized epithelium with numerous cell types that differ in their localization, characterization and functionality in a region specific manner (Fig. 3) (Hermo *et al.*, 1992; Hinton and Palladino, 1995). The rodent epididymis can be divided into four segments according to connective tissue septa: initial segment (IS), caput (CT), corpus (CS) and cauda (CA)(Robaire and Hermo, 1988). These larger regions can also be subdivided into 10 or more intraregional segments (Johnston *et al.*, 2005). Each segment provides a specific luminal microenvironment necessary for sperm maturation. Branches of arteries enter the epididymis through the connective tissue and give rise to capillary networks. This system provides the tissue with a constant blood flow and permits the delivery of both endogenous and exogenous substances (Brown and Waites, 1972). Sperm transit through the epididymis is mediated by peristaltic contractions and hydrostatic pressure generated by testicular fluid (Robaire and Hermo, 1988). A thin layer of myoid cells surrounding the epididymis contribute to this process. Once fertile, sperm are stored in the CA epididymis until ejaculation. Sperm exit the CA to enter the vas deferens, ejaculatory ducts and external genitalia.

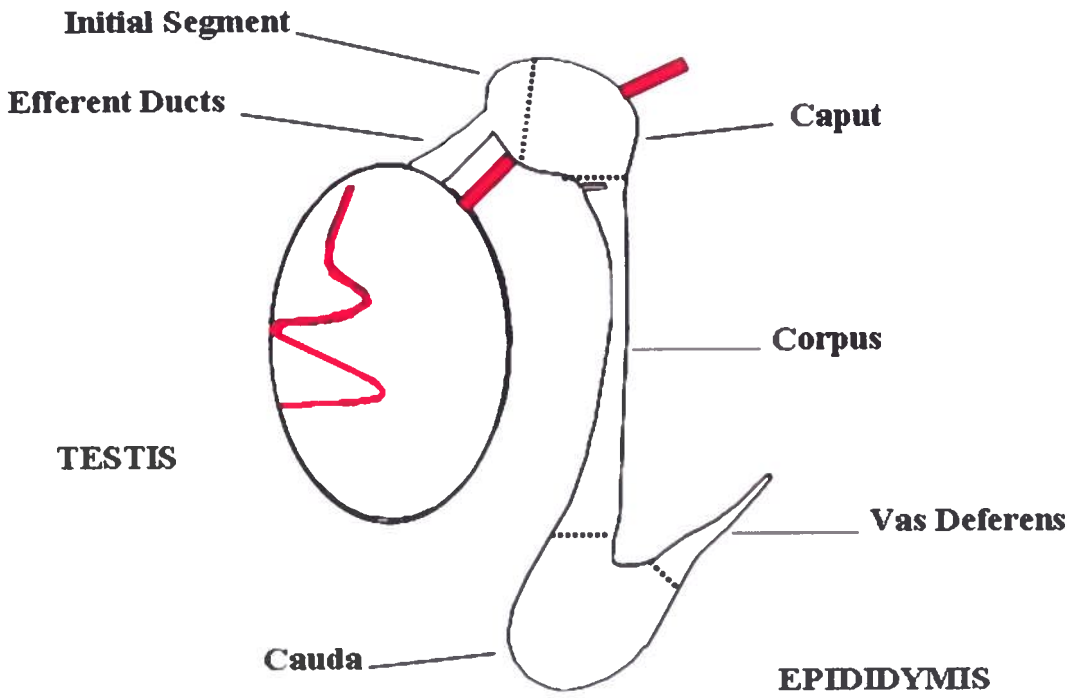

Figure 2 : Schematic representation of the adult rat testis, efferent ducts, epididymis and vas deferens. Cyr *et al.* (2001)

1.2.1 Epididymal Histology – Cell Types, Distribution and Function

The main cell type in the epididymal epithelium, representing approximated 65-80% of the total cell number, is the principal cell (Robaire and Hinton, 2001). Principal cells are important regulators of luminal composition and sperm maturation. These cells are the principle endocytic and secretory cells in the epididymal epithelium. Proteins and glycoproteins secreted from principal cells interact with maturing sperm to form functional gametes. Nearly all secreted luminal proteins identified to date have been found to be synthesized in principal cells. Principal cells from all regions of the epipidymis also display endocytic properties, including the presence of coated pits and coated vesicles, endosomes, pale and dense multivesicular bodies and lysosomes (Jones *et al.*, 1979a). Tight-junctions between adjacent principal cells and other epididymal cell types form the BEB (Cyr *et al.*, 2002; Cyr *et al.*, 2007). Principal cells are present in all regions of the epididymis, but display region specific morphology and function. From proximal to distal regions of the epididymis, the epididymal epithelium gradually decreases in height and the luminal area

increases. This trend is largely attributable to changes in principal cell morphology. Consistent with morphological changes, alterations in principal cell endocytic and secretory organelles, as well as lipid content, are observed along the length of the epididymis (Jones *et al.*, 1979a).

Apical cells represent a relatively small percentage of the cells in the epididymis and are found exclusively in the IS (Adamali and Hermo, 1996). They are located on the luminal side of the epididymal epithelium and unlike basal cells, do not cross the epididymal barrier. They are characterized by an apically localized nucleus and few apical vesicles and endosomes. Little is known about the specific functions of apical cells. The presence of GST subunits may indicate a role in spermatozoal protection from reactive oxygen species (Adamali and Hermo, 1996; Andonian and Hermo, 2003).

The localization of narrow cells is also restricted to proximal regions of the epididymis. Narrow cells are thin cells whose cytoplasm extends to the basement membrane and contain an elongated nucleus that is localized to the top half of the epithelium. Narrow cells are characterized by apically located vesicles that are implicated in endocytosis of luminal proteins and regulation of luminal pH by carbonic anhydrase and proton ATPase (Adamali and Hermo, 1996; Hermo *et al.*, 2000; Hermo *et al.*, 2005). Narrow cells, like basal and apical cells, also express GST subunits, suggesting a role in protection against reactive oxygen species (ROS)(Adamali and Hermo, 1996).

Halo cells are small cells characterized by a basally located dense nucleus, clear cytoplasm and dense core granules (Robaire *et al.*, 2006). Halo cells are found throughout the epididymis and are thought to serve an immunological function; they are comprised of monocytes, T and B lymphocytes as well as eosinophils (Nashan *et al.*, 1989; Serre and Robaire, 1999).

Basal cells represent the second largest population of cells in the epididymal epithelium. They are found throughout all regions of the epididymis, but increase in frequency in proximal regions compared to distal (Robaire and Hermo, 1988; Robaire and

Hinton, 2001). Basal cells are hemispherical in shape, with thin attenuated processes that, until recently, were not thought to be in direct contact with the epididymal lumen (Shum *et al.*, 2008). The shape of basal cells is thought to be regulated in part, by luminal volume and pressure. These cells have not been demonstrated to be under androgen control, but castration transforms them into compact dome-shaped cells with few lateral processes (Hermo and Papp, 1996). Unlike principle cells, the function of basal cells remains unclear. The presence of coated pits in the basal cell plasma membrane suggests a role in receptor-mediated endocytosis. A protective role against reactive oxygen species has been proposed; the Yb unit of glutathione S-transferase (GST) was localized to basal cells in the adult rat epididymis (Andonian and Hermo, 1999). Basal cells were also demonstrated to express metallothioneins (MT), a cytosolic protein involved in metal sequestration and regulation of oxidative stress, suggesting an additional role in epididymal protection (Cyr *et al.*, 2001). Prostaglandin biosynthesis in basal cells also suggested a potential role in the paracrine regulation of luminal water and electrolyte transport by principle cells (Leung *et al.*, 2004). Recently, basal cells were demonstrated to penetrate the BEB and express angiotensin II receptors, suggesting a role in luminal acidification through cross talk with adjacent clear cells (Shum *et al.*, 2008). Activation of angiontenin II receptors on basal cells is thought to promote proton secretion and luminal acidification by clear cells, which do not express these receptors.

Clear cells are large cuboidal endocytic cells characterized by numerous coated pits, endosomes, lysosomes, multivesicular bodies and lipid droplets. Clear cell localization is restricted to CT, CS and CA regions of the epididymis. The endocytic properties of clear cells were demonstrated in early studies using luminally injected tracers (Hermo *et al.*, 1988). Clear cells have been shown to endocytose proteins, as well as contents of the cytoplasmic droplet of sperm released during epididymal transit (Hermo *et al.*, 1988; Hermo *et al.*, 1992; Vierula *et al.*, 1995). In addition to endocytosis, the presence of a plasma membrane proton ATPase in clear cells suggests a role in luminal acidification (Brown *et al.*, 1992), which is thought to maintain sperm in a quiescent state during sperm maturation and storage in the epididymis. Clear cells may also be implicated in epididymal detoxification and protection of sperm from xenobiotics; several members of the

cytochrome P450 family, including CYP1A1 and CYP2E1 were localized to rat epididymal clear cells (Roman *et al.*, 1998; Forkert *et al.*, 2002).

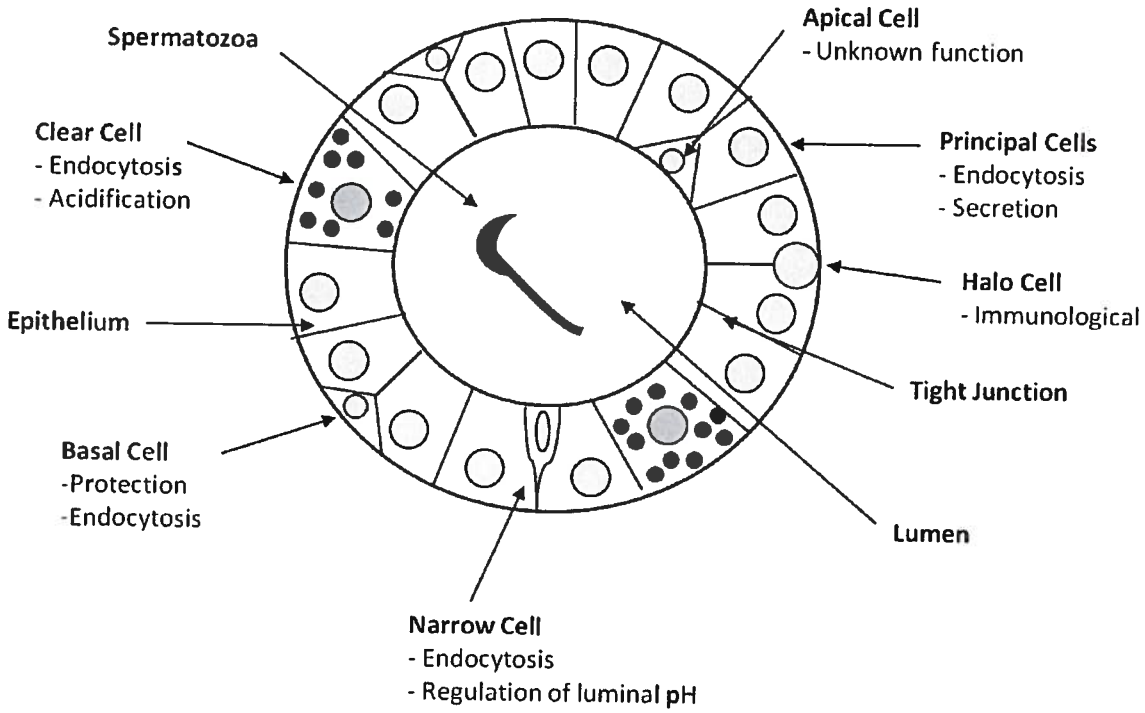

Figure 3 : Schematic representation of rat epididymal epithelium. Adapted from Robaire and Orgebin-Crist (2006)

1.2.2 Alterations in Sperm During Epididymal Transit

Sperm maturation in the epididymis is a dynamic process in which transiting sperm interact with the epididymal epithelium and respond to the luminal environment (Cornwall, 2009). Sperm alone are transcriptionally inert. The epididymal epithelium, under androgen control, is responsible for secreting proteins that interact with sperm to induce biochemical changes (Dacheux *et al.*, 2009). Major morphological changes in germs cells occur both during and after spermatogenesis. Once sperm have entered the excurrent duct system, luminal fluid reabsorption by the efferent ducts drastically increases sperm concentration (Ilio and Hess, 1994). Once inside the epididymis, the sperm undergo numerous morphological and biochemical changes that will ultimately lead to their maturation and acquisition of fertility.

Sperm extracted from proximal regions of the epididymis are immotile and infertile; motility is not initiated when proximal epididymal sperm are placed in media mimicking the composition of the female reproductive tract (Robaire *et al.*, 2006). A decrease in luminal pH along the length of the epididymis is thought to initiate the development of a mature axonemal apparatus and activate K+ channels implicated in hyperactivation (Carr *et al.*, 1985). Sperm are mature and are capable of motility in distal regions of the epididymis (Robaire *et al.*, 2006). Luminal concentrations of Na+, K+ and viscous mucoproteins that occur in distal regions of the epididymis however, maintain sperm in an inactive and immotile state prior to ejaculation (Carr *et al.*, 1985).

The main morphological change that occurs in sperm during epididymal transit is the migration of the cytoplasmic droplet, an excess of cytoplasm associated with testicular sperm (Gatti *et al.*, 2004; Hermo *et al.*, 2010c). The migration of the droplet occurs through an unknown mechanism. The movement of the droplet has been postulated to be related to epididymal peristalsis or alterations in spermatozoa volume regulation (Cooper 2010). Centrifugation has been demonstrated to shift cytoplasmic droplets in several species, demonstrating a sensitivity to physical stimuli (Kato *et al.*, 1983; Kato *et al.*, 1984). Shearing forces have also been demonstrated to completely remove cytoplasmic droplets from epididymal sperm (Jones, 1986). Migration begins when sperm transit from CT to CS. The droplet gradually slides from the head to the midpiece of sperm where it was previously thought to be released during storage in the CA or ejaculation (Hermo *et al.*, 1988). Several studies however, have reported retention of the cytoplasmic droplet until ejaculation in several species (Merton, 1939; Cooper and Yeung, 2003). Retention of the cytoplasmic droplet in ejaculated sperm has been correlated with decreased fertility, suggesting an important role in post-testicular sperm maturation (Zini *et al.*, 2000; Moore *et al.*, 2010). A recent study proposed a novel model where cytoplasmic droplet retention in infertile patients is the result of inadequate osmolyte provision in spermatozoa and regulated volume decrease (RVD) (Cooper 2010). CA sperm are stored in a unique fluid environment containing high osmolality and K+ and low Na+ and pH (Hinton and Palladino, 1995). Once ejaculated, sperm are subjected to complete reversal of fluid

composition in the female reproductive tract (Cooper 2010). A compensatory RVD prevents hypotonic shock and influx of water associated with the transition to a low osmalality and K+ and high Na+ environment (Yeung *et al.*, 2006). Cytoplasmic droplets themselves are thought to be involved in RVD; the cytoplasmic droplet contains several osmolyte channels implicated in loading and unloading of osmolytes (Yeung *et al.*, 2006). Fertile males may therefore have sufficient RVD to permit pinching off of the cytoplasmic droplet (Cooper 2010). Infertile males however, have compromised RVD, possibly resulting in droplet swelling, altered flagellar angulation and retention of the cytoplasmic droplet (Cooper 2010). The identification of a functional Golgi in the cytoplasmic droplet may also suggest a role in glycosylation of important sperm proteins during epididymal transit (Oko *et al.*, 1993).

Several biochemical alterations in sperm plasma membrane composition are also thought to contribute to the acquisition of fertility and motility during epididymal transit. Biochemical changes include changes in sperm plasma membrane phospholipid content and cholesterol-lipid ratios, relocation of surface antigens, addition, modification or removal of membrane proteins and increases in disulfide bonds or net surface negative charge (Sullivan *et al.*, 2007). Alterations in cholesterol and lipid ratios are critical events in the regulation of sperm maturation and capacitation (de Lamirande *et al.*, 1997); cholesterol loss from the sperm plasma membrane destabilizes membrane lipid rafts and initiates phosphorylation-based signaling cascades (Visconti *et al.*, 1999; Morales *et al.*, 2008). Several cholesterol acceptors, including high-density-lipoproteins (HDL) and apolipoproteins have been identified in the both the epithelium and interstitial stroma of the epididymis (Olson *et al.*, 1995; Law *et al.*, 1997; Morales *et al.*, 2008). HDL is a major lipoprotein and carrier of cholesterol and triglycerides. Apolipoproteins are plasma membrane constituents and components of HDL that are involved in the lipid transport and distribution between cells and tissues. Interestingly, several candidate transporters previously implicated in sterol and phospholipid transfer to apolipoproteins and HDL, including members of the ABC family of transporters, have been localized to sperm and the epididymal epithelium (Morales *et al.*, 2008). ABCBA1, ABCA7 and ABCG2 were localized to epididymal sperm of adult mice. Additionally, antibodies raised against

ABCBA1, ABCA7 and ABCG2 were demonstrated to decrease cholesterol efflux from sperm and inhibit in vitro fertilization, suggesting an important role in sperm maturation and the acquisition of fertility (Morales *et al.*, 2008).

Comparisons of proteomic profiles of immature proximal epididymal sperm and mature CA sperm identified several proteins whose expression increases during epididymal transit and maturation (Aitken *et al.*, 2007; Cornwall, 2009). Several proteins capable being phosphorylated, including glucose-regulating protein, heat-shock protein 70, actin, β-tubulin, lactic acid dehydrogenase and the mitochondrial proteins aconitase and β subunit F1 ATPase, were shown to increase in CA sperm, suggesting the development of phosphorylaton signaling complexes are important in epididymal sperm maturation (Aitken *et al.*, 2007; Cornwall, 2009). The topography of sperm proteins has also been demonstrated to change in a region specific manner during epididymal transit (Takano and Abe, 2004; Jones *et al.*, 2007). Both sperm proteins synthesized during spermatogenesis, such as ADAM family members fertilin and cyritestin, CE9 and α-mannosidase, SPAM1 and β-galactosyltransferase, and proteins from the epididymal epithelium that interact with sperm, such as CRISP family proteins, P26h, P34h, SPAG11, eppin and SED1, have been demonstrated to undergo proteolytic processing, modification (glycosylation) or re-localization during epididymal transit (Phelps *et al.*, 1990; Takano and Abe, 2004; Jones *et al.*, 2007; Cornwall, 2009).

1.2.3 Unique Mechanism for Transfer of Proteins from the Epididymal Epithelium to Sperm

Several secreted epididymal proteins have been demonstrated to be transferred from the epithelium to maturing spermatozoa and are thought to contribute to sperm maturation. The majority of identified proteins contain typical signal sequences, indicating trafficking through the Golgi, subsequent packaging and release by merocrine secretion (Gatti *et al.*, 2004; Cornwall and von Horsten, 2007; Sullivan *et al.*, 2007). Proteins transferred in this manner are referred to as coating proteins, binding to the sperm through electrostatic interactions. Interestingly, other secreted epididymal proteins which lack signalling

sequences, have been demonstrated to behave like integral membrane proteins when biochemically separated from sperm plasma membranes (Kirchhoff and Hale, 1996; Cooper, 1998). Several proteins that have been identified, including HE5 (CDC52) and SPAM1 in humans and P26h and P25b in hamster and bulls, possess glycosylphosphatidylinositol (GPI) anchors that are not conventionally associated with proteins originating in the extracellular compartment (Legare *et al.*, 1999; Frenette and Sullivan, 2001; Yeung *et al.*, 2001). A protein that is GPI-anchored to the plasma membrane must pass through the endoplasmic reticulum-Golgi apparatus-secretory vesicle pathway, or merocrine secretion. This suggested the existence of a unique mechanism for the transfer of epididymal proteins to sperm.

Several secreted proteins have been associated with membrane bound vesicles called epididymosomes that are characterized by a high cholesterol/phospholipid ratio (Rejraji *et al.*, 2006). These vesicles have been reported in several mammalian species including mouse, rat, hamster and bull (Legare *et al.*, 1999; Eickhoff *et al.*, 2001; Frenette and Sullivan, 2001; Rejraji *et al.*, 2006; Sullivan *et al.*, 2007). Epididymosomes are formed through apocrine secretion of apical blebs from the epididymal epithelium. Similar to prostasomes previously detected in semen, epididymosomes bud and detach from the epididymal epithelium in various sizes and fuse with sperm to transfer their contents (Saez *et al.*, 2003). Proteomic analysis of epididymosomes revealed the presence of several proteins that have been demonstrated to be transferred to sperm, including P26h, P25b, macrophage inhibitory factor, ubiquitin and glutathione peroxidise (Frenette and Sullivan, 2001; Frenette *et al.*, 2003; Sullivan *et al.*, 2007; Thimon *et al.*, 2008). Interestingly, epididymosomes isolated from different regions of the epididymis vary in their protein composition (Thimon *et al.*, 2008). Additionally, selective transfer of epididymosomal proteins has been demonstrated in vitro by incubating isolated bovine caudal epididymosomes with caput spermatozoa (Frenette and Sullivan, 2001).

1.2.4 Epididymal Defence Mechanisms

The epididymis provides a specialized, protected microenvironment in which spermatozoa transit and transform into fully mature and motile cells. Several epididymal defence mechanisms, including selective transport proteins, metabolic enzymes and the presence of a functional BEB, regulate the luminal environment.

Like the testis, the epididymal epithelium contains several families of specialized junctional proteins that are essential for the maintenance of male fertility (Dufresne et al., 2003; Cyr et al., 2007). Tight junctions between adjacent principal cells form a functional BEB that is able to restrict the type and quantity of compounds that enter the lumen (Agarwal and Hoffer, 1989). Along with other secretory and endocytic processes, claudins, occludins and other tight junction proteins regulate the composition of the luminal environment to ensure the normal, protected maturation of spermatozoa (Cyr et al., 2007). The epididymis has been demonstrated to concentrate organic molecules such as carnitine and inositol 10- to 100-fold while allowing a relatively low penetration and accumulation of other compounds such as inulin, glucose and serum albumin, demonstrating selective permeability (Hinton and Howards, 1988). Adherens junctions provide the initial cellular adhesion required for the formation of tight junctions and initiate signal transduction pathways with other junctional proteins (Pincon-Raymond, 2004; Yan and Cheng, 2005; Meng and Takeichi, 2009). The physical linkages formed by adherens junctions are essential for tissue remodeling and morphogenesis and maintenance of cell polarity. Several members of the largest family of adhesion proteins, cadherins and their associated proteins, catenins, have been identified in tight and gap junctions of the testis and epididymis (Mruk and Cheng, 2004; Cyr et al., 2007).

As mentioned previously, certain epididymal cell types express GST and MT, suggesting a role in protection against cellular stresses in the epididymal epithelium (Adamali and Hermo, 1996; Cyr *et al.*, 2001; Andonian and Hermo, 2003). GST is a biosynthesized antioxidant that serves to protect cells against ROS. Reduced GST acts as an electron donor to unstable free radicals and peroxides. The ratio of reduced to oxidized

GST is commonly used as a marker of cellular stress. MT are cytoplasmic proteins that bind and sequester divalent cations such as Cd, Cu, Hg and Zn. MT are found in several cell types throughout the male reproductive tract, including epididymal basal cells, where they serve as a primary defence against metal toxicity (Moffatt and Seguin, 1998; Cyr *et al.*, 2001; Ren *et al.*, 2003a; Ren *et al.*, 2003b). Clear cells regulate luminal composition primarily through endocytic activities (Hermo *et al.*, 1988). Clear cells also however, express several drug metabolizing enzymes, such as CYP450s, suggesting a role in epididymal detoxification (Roman *et al.*, 1998; Forkert *et al.*, 2002). Clear cells may therefore utilize both their endocytic and metabolic capabilities to detoxify potentially harmful substances present in the epididymal lumen.

Depending on the species, sperm spend between 1-14 days transiting through the epididymis (Robaire *et al.*, 2006). During this time, they are not only susceptible to xenobiotics, but are also capable of themselves generating potentially harmful ROS and metabolites (Jones *et al.*, 1979b). ROS generation by spermatozoa is suggested as being involved in virtually all aspects of sperm function, including acquisition of motility, capacitation, the acrosome reaction and hyperactivated motility (de Lamirande and Gagnon, 1993; Aitken *et al.*, 1995; Griveau *et al.*, 1995; Aitken, 2000). Inappropriate levels of ROS however, may have significant detrimental effects on sperm function, maturation and viability (Agarwal and Saleh, 2002). To counteract excessive oxidative stress, several protective proteins are secreted into the luminal environment to regulate lumen fluid composition and protect sperm. Glutathione peroxidase (GPX) secreted in the lumen of the initial segment of the epididymis protects the spermatozoa lipid membrane from peroxides (Drevet, 2006). Other enzymes implicated in oxidative protection, including indole amine dioxygenase, superoxide dismutase (SOD), catalase and gamma glutamyl transpeptidase (GGT) are also secreted into the epididymal lumen (Drevet, 2006). Other small molecular mass scavengers present in the epididymal fluid may provide additional protection against oxidative stress. Taurine, an organic acid with antioxidant properties, has been previously detected in epididymal plasma (Hinton, 1990). Metals that impair lipid peroxidation, such as zinc, are secreted by the epididymis and are internalized by sperm during epididymal transit (Stoltenberg *et al.*, 1997a; Stoltenberg *et al.*, 1997b).

In addition to the physical divisions of the BEB, specialized transport proteins actively regulate the type and quantity of compounds entering the luminal environment (Enokizono *et al.*, 2007; Kujala *et al.*, 2007; Cotton *et al.*, 2010). The presence of this barrier contributes to the formation of a distinct luminal micro-environment that differs from the circulating systemic blood. These will be discussed in greater detail later in this review.

2.0 Male Fertility and Environmental Pollutants

There is increasing concern that exposure to environmental pollutants may be contributing to reported declines in male reproductive potential. Approximately one male in twenty is affected by infertility (McLachlan and de Kretser, 2001). A male cause also contributes to fifty percent of all infertility observed in couples (McLachlan and de Kretser, 2001). As early as the 1940s, occupational exposure to DDT was reported to lower sperm counts. A meta analysis examining sperm counts and semen volume of 15,000 men between 1938 and 1990 also noted a decrease that was thought to be attributed to environmental exposures (Carlsen *et al.*, 1992). An increasing body of evidence suggests that common environmental pollutants are capable of altering male reproductive function through diverse array of toxicological mechanisms (Phillips and Tanphaichitr, 2008). The complex nature of the male reproductive system allows xenobiotic disturbances from a multitude of potential pathways. Endocrine disrupters (EDs) are compounds capable of mimicking, antagonizing or altering the actions of endogenous hormones. This can be achieved though interference with hormone synthesis, storage, release, metabolism, transport or receptor binding. The hormone dependence of the male reproductive tract creates a vulnerability to EDs possessing anti-androgenic or estrogenic properties (Phillips and Tanphaichitr, 2008). Xenoestrogens, which are compounds possessing chemical and structural similarities to endogenous estrogens, are thought to produce the greatest risk to male reproductive function. Several common environmental pollutants, including chemicals utilized in the production, or as components of paints and plastics (AP, bisphenol-A (BPA), pthalates) pesticides (organochlorines, organometalics,

organophosphates), drugs (diethylstilbestrol) and other industrial compounds (polychlorinated biphenyls, polychlorinated dibenzodioxins) have a core ring structure that resembles endogenous steroid hormones and have also been demonstrated to bind the estrogen receptor (Kuiper *et al.*, 1997; Kuiper *et al.*, 1998; McKinney and Waller, 1998).

2.1.0 Alkyphenols (AP) and Alkylphenol Ethoxylates (APE)

AP are high volume chemicals commonly used in a variety of industrial sectors, including textiles, pulp and paper, steel manufacturing, and production of pest control products, paints and resins (Soares *et al.*, 2008). They are used as surfactants, wetting agents and plasticizers in industrial, as well as commercial products. APE enter the environment primarily through wastewater treatment plants, where they can biodegrade to form higher solubility and hydrophobicity AP that are more stable in the environment. Many of these compounds, including nonylphenol ethoxylates (NPE) and octylphenol ethoxylates (OPE), as well as their major degradation products, 4-nonlyphenol (NP) and 4-tert-octylphenol (OP), and BPA can be found at low concentrations in sewage sludge, water, sediments and ambient air (Ying *et al.*, 2002). AP have also been shown to bioaccumulate in fish and mammals (Ying *et al.*, 2002).

1.1.1 Reproductive Toxicity of Alkylphenols and Alkylphenolethoxylates

The reproductive toxicity of AP and APE remains controversial. Certain in vivo animal models for AP exposure have demonstrated a wide range of reproductive abnormalities. Several studies have reported that high dose oral administration of OP, OPE, NP, NPE and BPA to rats induced alterations in testis, epididymis, and prostate weight, histopathological abnormalities including damage to spermatogenic and somatic cells of the testis, altered sperm motility parameters and daily sperm production (DSP) (Boockfor and Blake, 1997; Han *et al.*, 2004; Hamelin *et al.*, 2009). There is evidence to suggest AP mediated reproductive abnormalities are related to their estrogenicity and ability to suppress gonadotropins (Han *et al.*, 2004). AP and APEs possess chemical similarities with estradiol and have been demonstrated to produce estrogenic responses in various biological

organisms (Nimrod and Benson, 1996). Other direct forms of cellular stress have also been reported in the male reproductive systems of rats chronically exposed to NP; antioxidant enzymes (catalase, glutathione reductase and GPX) decreased while levels of peroxide generation and lipid peroxidation increased in epididymal sperm of NP treated animals (Chitra *et al.*, 2002). Similarly, testis of rats chronically co-administered BPA, NP and OP displayed elevated levels of malondialdehyde, a product of ROS degradation of polyunsaturated lipid and marker of oxidative stress, and decreased levels of glutathione (GSH) (Aydogan *et al.*, 2010).

Other research indicates that AP mediated disruption of cellular junctions may also be involved in mediating downstream endocrine effects, or directly contributing to male reproductive toxicity in an endocrine - independent manner. GJIC and immunostaining of gap junction protein (Cx43) were altered via an estrogen-independent pathway in NP exposed Sertoli cell line (Aravindakshan and Cyr, 2005). Cx43 protein expression was reduced in neonatal rats exposed to BPA (Salian *et al.*, 2009). Alteration in expression and localization of several junctional proteins has also been reported in a Sertoli cell line exposed to OP and BPA (Fiorini *et al.*, 2004). Ultrastructural analyses of NP treated rats revealed incompact inter-Sertoli junctions (Han et al., 2004). Similar aberrations were found in junctional proteins investigated at the cellular and molecular level. Western blot data revealed a decrease in expression of two junction proteins, occludin and N-cadherin. Aberrant localization of these proteins was also reported; tight and adherens junction proteins were delocalized from the cellular membrane to the cytoplasm (Fiorini et al., 2004). Interestingly, the viability of the Sertoli cell line was unaffected at the doses used, possibly indicating selective targeting of cellular junction proteins.

Although industrial surfactants have been proposed as male reproductive toxicants in vivo (Boockfor and Blake, 1997; Han *et al.*, 2004), recent in vivo studies suggest that weak doses of AP may be less toxic than originally reported (Gregory *et al.*, 2009). Toxicokinetic analysis of adult male rats demonstrated a rapid clearance of OP, from the rat epididymis (Hamelin *et al.*, 2009). Contrary to earlier studies using elevated doses (250 mg/kg/day for 50 days; (Han *et al.*, 2004)), a general lack of reproductive toxicity was

observed at environmentally relevant doses (25 mg/kg/day for 60 days) of OP; testicular and epididymal weights and histology were unaltered and no significant differences in sperm counts or motility were observed (Gregory *et al.*, 2009). Interestingly, OP was detected in reproductive tissues 24 hours following exposure, but was rapidly excluded, suggesting the involvement of metabolizing enzymes or efflux proteins in tissue defence (Gregory *et al.*, 2009; Hamelin *et al.*, 2009).

2.1.2 Nonylphenol

NP is produced in a catalyzed reaction involving phenol and mixed nonenes. NPE ethoxylates are produced by the ethoxylation of NP. NPE are transformed in the environment by microorganisms to re-form NP and short chain NPE (Zhang *et al.*, 2008). Transformed compounds are more stable in the environment and have been associated with increased toxicity. NPE are extremely cost effective as industrial surfactants and have become the most widely used compounds in their class. The demand for NP in the US is estimated to be increasing by 2% annually, with current market demands being 120,000 tons per year. The synthesis of industrial and household detergents contributes approximately 80% of the current demand and is thought to be responsible for the continually increasing usage (Vasquez-Duhalt *et al.*, 2005). Other applications for NP include use in the production of TNPP (trisnonylphenol phosphite), an antioxidant used in polymer synthesis, phenolic resins, synthetic rubber, PVC, pharmaceuticals, pesticides, corrosion inhibitors, chemical stabilizers and leather (Vasquez-Duhalt *et al.*, 2005).

2.1.3 Nonylphenol in the Environment

As mentioned previously, the most common entry of NP into the environment is through wastewater. NPE present in industrial and domestic detergents, soaps and cleaners, cosmetics, paints, pesticides and dispersing agents are discharged into the sewer system and ultimately travel to wastewater treatment plants. For this reason, high levels of NP (8811 μg/L (39.97 μM)) have been reported in untreated or partially treated industrial sewage effluent (Vasquez-Duhalt *et al.*, 2005). Few studies have examined the atmospheric

concentrations of NP and NPE. The presence of NP in the atmosphere however, has been reported; NP concentrations ranged from 2.2 to 70 ng/m^3 in the coastal atmosphere of New York and New Jersey (Vasquez-Duhalt *et al.*, 2005). One other study conducted in New Brunswick reported atmospheric concentrations of NP to be between 0.3 and 81 ng/m^3. The occurrence of NP in rivers, lakes and coastal waters has been investigated in several countries; high levels of NP, (654 μg/L (2.97 μM), 53 μg/L (0.24 μM) and 95 μg/L(0.43 μM)) were detected in Spain, United-Kingdom and U.S.A, respectively (Vasquez-Duhalt *et al.*, 2005). NP concentrations in Canada were reported as being up to 10 μg/L (45.4 nM) in rivers, 0.06 μg/L (0.27 nM) in lakes and 0.98 μg/L (4.46 nM) in harbors (Sabik *et al.*, 2003; Soares *et al.*, 2008). NPE were considerably higher, with certain rivers reported as containing up to 17.6 μg/L (0.079 μM). No data exists on the levels of NP and NPE in soil.

2.1.4 Nonylphenol – Human Exposure

Human exposure occurs primarily through environmental media and use of NP and NPE containing consumer products. Environmental exposure can occur through ingestion of contaminated substances, by inhalation or absorption through the skin. Clark *et al.* (1992) reported NPE concentrations as being up to 0.129 μg/L (0.5 nM) in drinking water (U.S). Little data exists on the concentrations of NP and NPE in consumed foods. One study however, reported NP in 39 typical grocery store food items and 21 baby food items; concentrations ranged from 0.1-19.4 μg/Kg (Guenther *et al.*, 2002). Infant exposure can also occur through the consumption of contaminated breast milk or prenatally through maternal transfer.

Several studies have investigated levels of NP and NPE in human tissues and fluids. A Swiss study conducted using 25 human cadavers detected NP between 19.8 ng/g and 84.4 ng/g in lipids. The NPE investigated in this study were below the detection level limit (5 ng/g) (Muller 1997). Levels of NP were found to be two orders of magnitude higher than any other AP measured at a mean concentration of 32 ng/ml (~0.145 μM) in the breast milk of Italian women (Ademollo *et al.*, 2008). A study examining maternal and umbilical concentrations of endocrine disruptors in low-birth-weight infants also found NP at 1.51

mg/L (6.85 μM) and 1.12 mg/L (5.08 μM) in maternal and chord blood, respectively (Lin *et al.*, 2008).

3.0 Transporters and Toxicology/Pharmacology

There are many factors that can affect an individual's response to a potentially toxic or therapeutic substance. Toxicokinetics/pharmacokinetics is the study of the time dependent processes regulating how substances interact with living organisms. Toxicokinetics encompass four fundamental principles in toxicology; absorption, distribution, biotransformation and excretion (Klaasen, 2001). Dose and chemical characteristics of the substance, route of exposure and inter-individual variability are important factors capable of regulating toxicokinetic profiles. Any compound is capable of eliciting a toxic response if given at significantly high doses. Chemical lipophilicity and routes of exposure will also greatly affect how a substance is both absorbed and distributed throughout the body. As a general rule, lipid soluble compounds are more readily absorbed through the skin, the gut and lungs, and are more easily distributed between internal compartments. Other substances, while innocuous in their original form, can be bio-activated by xenobiotic metabolizing enzymes in the liver to become more toxic (Klaasen, 2001).

Fortunately, mammals contain several lines of defence that attenuate the entry and distribution, and facilitate the excretion of undesired substances (Klaasen, 2001). A substance must circumvent several structural, cellular and metabolic barriers to reach internal compartments in organs and tissues. Tight junctions between adjacent enterocytes in the small intestine and colon, endothelial cells of blood vessels and epithelial cells of various blood tissue barriers serve a "fence" function regulating the entry of toxicants. Toxicants that enter via the digestive tract must enter the hepatic portal circulation and are subject to a first-pass-effect prior to entry in the systemic blood circulation. Xenobiotic metabolizing enzymes in the liver greatly reduce the bioavailability of toxicants by transforming and conjugating substances to increase hydrophilicity. Intravenous, intraperitoneal, intramuscular, suppository and inhalation exposure surpass this effect by

allowing for direct entry into the systemic blood circulation. Transport proteins provide an additional line of defence against toxic substances (Leslie *et al.*, 2001; Seeger and van Veen, 2009; Klaassen and Aleksunes, 2010). They act in concert with physical barriers to minimize toxicity. Their expression in the gut lumen provides a first line of defence that can attenuate the entry of potentially harmful substances. Similar to tight junctions, their presence in vasculature and epithelium of various blood-tissue barriers can attenuate the transfer of substances from the systemic blood circulation to sensitive organs and tissues. Once inside organs and tissues, transport proteins also facilitate the elimination of xenobiotics and toxic metabolites through various secretory and excretory routes.

3.1.0 ABC Transporter Superfamily

ABC efflux transporters are membrane-embedded proteins capable of regulating intracellular concentrations of numerous substrates (Leslie *et al.*, 2001; Seeger and van Veen, 2009; Klaassen and Aleksunes, 2010). Through an active, energy dependent process, ABC transporters regulate the cellular disposition of a wide array of chemically diverse substrates, including drugs and toxicants, proteins, peptide, lipids and other endogenous molecules, often against steep concentration gradients. ABC transporters contain two membrane binding domains (MDs) and two nucleotide binding domains (NBDs), or ATP binding cassette domains, that allow for the binding of substrates and ATP, respectively. ATP binding and subsequent hydrolysis provides the energy necessary to translocate substrates across biological membranes. Highly conserved sequences in the NBD's of ABC transporters are the hallmark of the superfamily. MDs however, have sequence variation that allows for substrate diversity. The ABC transporter family contains 50 members (51 in rodents), that can be subdivided according to sequence homology and topology (Table 1) (Dean *et al.*, 2001). Seven subfamilies, ABCA-ABCG, each containing several members, have been characterized. The broad substrate specificity of ABC transporter family implicates them in a diverse array of physiological functions, including a fence function at various blood-tissue barriers, regulating the circulation of physiological molecules, and detoxification of drugs, toxicants and endogenous metabolites by excretion.

Table 1. ABC transporter superfamily

Symbol	Alias	Function	Symbol	Alias	Function
ABCA1	ABC1	cholesterol efflux	ABCC1	MRP1	drug resistance
ABCA2	ABC2		ABCC2	MRP2	organic anion efflux
ABCA3	ABC3		ABCC3	MRP3	drug resistance, toxicant efflux
ABCA4	ABCR	N-Retinylidiene-PE-efflux	ABCC4	MRP4	nucleoside transport
ABCA5			ABCC5	MRP5	nucleoside transport
ABCA6			ABCC6	MRP6	
ABCA7			ABCC7	CFTR	chloride ion channel
ABCA8			ABCC8	SUR	sulfonylurea receptor
ABCA9			ABCC9	SUR2	k(ATP) channel regualtion
ABCA10			ABCC10	MRP7	
ABCA11			ABCC11		
ABCA12			ABCC12		
ABCA13			ABCD1	ALD	VLCFA transport regulation
ABCB1	P-glycoprotein, PGY1, MDR1	multidrug resistance, toxicant efflux	ABCD2	ALD1, ALDR	
ABCB2	TAP1	peptide transport	ABCD3	PXMP1, PMP70	
ABCB3	TAP2	peptide transport	ABCD4	PMP69, P70R	
ABCB4	PGY3	PC transport	ABCE1	OABP, RNS4I	olygoadenylate binding protein
ABCB5			ABCF1	ABC50	
ABCB6	MTABC3	iron transport	ABCF2		
ABCB7	ABC7	Fe/S cluster transport	ABCF3		
ABCB8	MABC1		ABCG1	ABC8, white	cholesterol efflux
ABCB9			ABCG2	ABCP, BCRP, MXR	drug resistance, toxicant efflux
ABCB10	MTABC2		ABCG4	white 2	
ABCB11	SPGP	bile salt transport	ABCG5	white 3	sterol transport
			ABCG8		sterol transport

Interestingly, a subset of ABC transporters also exhibit alternative functions (channel, receptor) in addition to transport. The Cystic fibrosis transmembrane conductance regulator (CFTR/ABCC7) acts as a voltage-independent Cl- channel. Mutations in CFTR cause cystic fibrosis, a prevalent debilitating disease. ABCC8 (SUR1) acts as sulfonylurea receptors, which are commonly used to promote insulin release from pancreatic beta cells (Darendeliler *et al.*, 2002). ABC transporters are constitutively expressed in numerous tissues and organ systems, but certain members, including the xenobiotic efflux transporters, ABCB1 (P-gp, MDR1), ABCC1 (MRP1) and ABCG2 (BCRP, MXR), can also be induced by chemical insult or cellular stress. The generation of specific ABC knockout (KO) mice has provided considerable insight into their specific roles. CFTR KO mice have served as a model for CFTR gene defect related lung disease and associated male infertility (Reynaert *et al.*, 2000). ABCB1, ABCC1 and ABCG2 KO animals, although viable and fertile, have altered pharmacokinetic and toxicokinetic profiles when

challenged with xenobiotics (Leslie *et al.*, 2001). The ABC transporter family is the largest and most researched family of transport proteins. Understanding the distribution and role of these transporters is relevant not only to the understanding of fundamental cellular and molecular processes, but also disease and pathology, toxicology and pharmacology.

3.1.1 ABCB1

ABCB1 is the product of the ABCB1 gene and member of the ABC transporter superfamily. ABCB1 is an integral plasma membrane protein (170 kDa) whose apical localization allows for preferential basal-to-apical transport of various substrates. ABCB1 was originally identified as a phosphoglycoprotein expressed in drug-resistant ovarian cells (Juliano and Ling, 1976). Over-expression confers the Multidrug-Resistant (MDR) phenomenon in numerous blood-born and solid tissue cancers. Early promise as a target for circumventing chemotherapeutic resistance has made ABCB1 the most researched and best characterized ABC transporter. ABCB1 was later found to be constitutively expressed in numerous normal tissues serving a barrier or secretory function, suggesting a physiological function in host defence (Staud *et al.*, 2010). While humans express one ABCB1 efflux gene, rodents have two ABCB1 efflux genes, ABCB1a and ABCB1b. ABCB1a and ABCB1b share approximately 85% amino acid identity with each other and greater than 80% with human ABCB1 (Leslie *et al.*, 2001). ABCB1 is an important toxicokinetic regulator of a diverse array of xenobiotics. Its anatomical localization limits the absorption and distribution, and facilitates the excretion of potentially harmful substances.

3.1.2 Substrates, Modulators and Inducers

ABCB1 is capable of transporting a diverse array of primarily lipophilic and amphipathic substrates in un-modified forms (Table 2). ABCB1 is an important pharmacokinetic and toxicokinetic regulator of both clinically relevant drugs and environmental toxicants (Padowski and Pollack, 2010). Interestingly, there is considerable overlapping substrate specificity between ABCB1 and CYP3A xenobiotic metabolizing enzymes, suggesting a coordinated synergistic role in the distribution and detoxification of

xenobiotics (Fromm, 2004). Constitutive ATPase activity of ABCB1 and the ability to transport numerous endogenous compounds, including sterols, lipids and proteins, also suggests an important role in normal physiological functioning. The ability of ABCB1 to transport several diagnostic dyes, including calcein, rhodamine-123 and Hoescht 33342, and radiolabelled drugs, has proved to be an extremely useful tool in evaluating transporter function (Schwab *et al.*, 2003; Muller *et al.*, 2007; Colabufo *et al.*, 2010). Several commercially available MDR assays utilize calcein and rhodamine-123 efflux to evaluate ABCB1 functionality (Fazlina *et al.*, 2008).

The clinical relevance of ABCB1 has promoted the development of numerous ABCB1 modulators capable of inhibiting ABCB1 functionality (Baumert and Hilgeroth, 2009). Several specific ABCB1 modulators have been designed to circumvent cancer cell chemotherapeutic resistance or modulate the pharmacokinetic and toxicokinetic profile of various substances. ABCB1 inhibitors can be used to circumvent blood-tissue barriers and increase the bioavailability of compounds (Table 2). These inhibitors can be classified into two broad groups, competitive and non-competitive inhibitors (Baumert and Hilgeroth, 2009). Competitive inhibitors are ABCB1 substrates that compete for active binding sites in the ABCB1 pocket. Non-competitive inhibitors do not compete for active binding sites. Non-competitive inhibitors usually function by binding a different region of the transport protein, initiating a conformation change that alters substrate recognition (Baumert and Hilgeroth, 2009). The majority of first and second generation synthetic ABCB1 inhibitors, including cyclosporine, verapamil and quinidine were utilized for their ABCB1 binding affinity. Although first and second generation inhibitors demonstrated great promise in-vitro, several limitations were detected in vivo, including low efficacy, unintended inhibition of other ABC transporter and toxicity (Baumert and Hilgeroth, 2009). ABC transporters are also important toxicokinetic and pharmacokinetic regulator in a number of normal tissues (Fromm, 2004; Staud *et al.*, 2010). Non-specific inhibition of ABC transporters may therefore increase the potential for toxicity of co-administered compounds or the inhibitor itself. Pharmacokinetic and toxicokinetic alterations may be observed at the systemic (blood), regional (organ) or local (intracellular) level, depending on the type and quantity of inhibitor and route of administration (Padowski and Pollack, 2010).

Advancements in computational chemistry, chemoinformatics and quantititative analysis structure activity studies allowed for the development of high-specificity non-competitive third generation inhibitors, such as biricodar, tariquidar and zosuquidar that are demonstrating great clinical promise (Baumert and Hilgeroth, 2009). These new inhibitors are demonstrating minimal effects on other members of the ABC transporter family and minimal alterations in the pharmacokinetics of co-administered therapeutics.

Several compounds, including numerous chemotherapeutic agents, environmental pollutants and endogenous molecules have been demonstrated to induce ABCB1 expression and increase functionality (Staud *et al.*, 2010). Exposure to these compounds can induce an MDR phenotype in both cancerous and non-cancerous tissues (Klaassen and Aleksunes, 2010; Staud *et al.*, 2010). Interestingly, not all ABCB1 inducers are known ABCB1 substrates; Alfatoxin, a naturally occurring carcinogen and known inducer of ABCB1, did not modulate rhodamine-123 efflux in a competitive transport assay (Santoni-Rugiu and Silverman, 1997). Similarly, not all compounds capable of modulating ABCB1 functionality expression are known substrates or inducers of ABCB1 expression. This lack of consistency in the categorization of ABCB1 substrates, modulators and inducers is a great challenge in the development of efficient and specific ABCB1 modulators and the prediction of xenobiotic impacts on ABCB1 expression and function

Table 2. ABCB1 substrates, modulators and inducers

Substrates	Modulators	Inducers
Drugs	**Drugs**	**Drugs**
Analgesics: morphine, methadone	Amiodarone	clotrimazole
Anthelmintics: ivermectin, abamectin	Biricolar	doxorubicin
Antibiotics: erythomycin, tetracyclines, fluoroquinolines	Buprenorphone	ethynyl estradiol
Anticancer drugs: vinblastine, vincristine, doxorubicin,	Clacridar	midazolam
daunorubicin, mitoxantrone, etopside, methotrexate,	cramophore EL	phenobarbitol
Topotecan	Cyclosporine	rapamycin
Antidiarrheal agents: loperamide	Dexverapamil	reserpine
Antiemetics: domperidone, ondansteron	ethynyl estradiol	rifampicin
Antiepileptic drugs: phenytoin, carbamazepine,	Fumagilin	St John's Wort
lamotrigine, phenobarbital, felbamate, gabapentin,	Laniquidar	verapamil
Topiramate	Methadone	**Environmental Pollutants**
Anti-gout agents: colchicine	Morphine	aflatoxin
Calcium channel blockers: verapamil	Quinidine	arsenite
Cardiac glycosides: digoxin	Quinine	cadmium
Corticoids: dexamethasone, triamcinolone	Tariquidar	DDT
Diagnostic dyes: calcein, rhodamine, Hoescht 33342	Valspodar	diazinon
HIV protease inhibitors: saquinavir, rintonavir,	Verapamil	ivermectine
Indinavir	Zosuquidar	phthalates
Immunosuppressive agents: cyclosporine, sirolimus,	**Environmental Pollutants**	**Endogenous Sterols**
Tacrolimus	DDT	estriol
cypermethrin and fenvalerate	Diazinon	estrone
Opioids: methadone, morphine, paclitaxel	nonylphenol ethoxylates	
Psychotropic drugs: chlorpromazine, clozapine,	Procloraz	
Desipramine, domperidone, flupentixol, imipramine,	Selamectin	
Nortyrptiline, sertaline, amitryptiline, doxepin,	**Endogenous Sterols**	
Venlafaxine, paroxetine	Cholesterol	
Diagnostic dyes	Progesterone	
calcein, rhodamine, Hoescht 33342	**References**	
Endogenous Substrates	(Chin *et al.*, 1990)	(Letrent *et al.*, 1999)
Aldosterone, cholesterol, cytokines, corticosterone,	(Sturm *et al.*, 2001)	(Garrigues *et al.*, 2002)
cortisol, estriol, estrone, platelet activating factor (PAF),	(Fromm, 2004)	(Kim and Benet, 2004)
Sphingomyelin	(Doo *et al.*, 2005)	(Griffin *et al.*, 2005)
Environmental Pollutants	(Jin and Audus, 2005)	(Shabbir *et al.*, 2005)
Industrial surfactants and plasticizers: nonylphenol	(Dupuy *et al.*, 2006)	(Lecoeur *et al.*, 2006)
Ethoxylates, bisphenol A	(Kimura *et al.*, 2007)	(Sreeramulu *et al.*, 2007)
Pesticides: endosulfran, eprinomectin, ivermectin,	(Mizutani *et al.*, 2008) (Staud *et al.*, 2010)	(Kiki-Mvouaka *et al.*, 2010)

3.1.3 Regulation

ABCB1 induction in both cancerous and normal tissues is an extremely complex and multifactorial event. ABCB1 induction occurs not only through the activation of numerous transcription factors, but also through epigenetic modifications, signal transduction pathways and signal cascades (Callaghan *et al.*, 2008). The nuclear receptors, CAR (constitutive androstane receptor) and PXR (Pregnane X receptor), form a xenobiotic response module capable of up-regulating ABCB1 expression to promote the elimination of potentially harmful substances. Cellular stresses generated by the harsh intracellular environments of cancer cells or elevated intracellular levels of toxicants are also capable of stimulating ABCB1 expression through numerous pathways. Heat shock, inflammation, UV cytostatics and DNA damage have all been demonstrated to regulate ABCB1 transcription via HSF1, NF-kB and C/EBPb, and P53 transcription factors, respectively. Cellular stress inflicted by chemotherapeutic agents or environmental pollutants is thought to increase endogenous levels of ROS. This results in compensatory changes in antioxidant systems (Callaghan *et al.*, 2008). Oxidative stress can lead to the activation of several signalling pathways, including NF-kB, MAPK and PKC that ultimately lead to cell death. Transient activation of cellular stress pathways however, is thought to contribute ABCB1 up-regulation. Numerous classes of environmental pollutants, including pesticides, industrial surfactants, metals and plasticizers have been associated with generation of ROS in in-vitro and in-vivo mammalian systems (Mena *et al.*, 2009). Interestingly, alternations in oxidative stress is also thought to be a key contributor to recent declines in male reproductive potential (Sheweita *et al.*, 2005).

3.1.4 Expression and Function in Normal Tissues

Although originally discovered in cancer cells, ABCB1 was later found to be constitutively expressed in normal tissues, suggesting a role in tissue defence. ABCB1 is localized almost exclusively to the apical side of epithelial cells, allowing for basolateral to apical translocation of substrates. ABCB1 is present in the epithelia of numerous blood-tissue barriers and organs serving a barrier or secretory function (Thiebaut *et al.*, 1987).

The anatomical localization and broad substrate specificity of ABCB1 has a profound impact on the toxicokinetic and pharmacokinetic profiles of toxicants and therapeutic agents. ABCB1 acts in concert with structural barriers, such as those formed by tight-junctions between adjacent epithelial cells, and drug metabolizing enzymes to regulate the entry, disposition and bio-availability of toxicants and therapeutic substances in normal tissues. The generation of ABCB1 KO animals provided valuable insight into its physiological function; ABCB1 KO animals, although viable and fertile, had increased bio-availability of administered compounds and were more susceptible to chemical injury (Schinkel *et al.*, 1994).

Epithelial cells within epithelia throughout the body are linked by tight junctions. As mentioned previously, tight junctions act not only to physically link cells together and maintain cellular polarity, but also to restrict the types and quantities of substances passing through intracellular space. Substances that are not sufficiently small enough to diffuse between adjacent cells of biological barriers must cross directly through the epithelial cells. ABCB1 substances are generally hydrophobic in nature, and therefore can diffuse more easily across cellular membranes (Klaassen and Aleksunes, 2010; Staud *et al.*, 2010). Elevated levels of ABCB1 act to limit the passive diffusion of drugs and toxicants into the blood from the intestinal lumen and from circulating blood to internal compartments.

As a first line of defence, ABCB1 limits the bioavailability of orally administered drugs and toxins. ABCB1 expression in the brush border of the intestinal epithelium limits the rate at which substances present in the intestinal lumen enter the blood (Thiebaut *et al.*, 1987). ABCB1 actively translocates substrates back into the intestinal lumen from the epithelium. Interestingly, ABCB1a KO mice spontaneously developed inflammatory symptoms similar to those found in patients with inflammatory bowel disease, suggesting an additional role in intestinal homeostasis (Panwala *et al.*, 1998). Inflammation was suggested as being the result of accumulated endogenous bacterial toxins which are normally excluded from the intestinal wall by ABCB1a. Expression of ABCB1 on the apical surface of bronchial and bronchiolal epithelium and at the plasma membrane of alveolar macrophages is also thought to protect lungs from inhaled environmental toxicants

(Scheffer *et al.*, 2002). To date however, a physiological role for ABCB1 in the lung has not been confirmed in an in-vivo model.

ABCB1 is also expressed in blood capillary endothelial cells of several blood tissue barriers. The presence of ABCB1 in blood capillaries limits the diffusion of both therapeutic and toxic substances from circulating blood to sensitive organs and tissues. ABCB1 is expressed at significant levels in blood-brain, blood-cerebrospinal fluid, blood-testis and fetal-maternal barriers. Absence of ABCB1 in the blood-brain barrier resulted in 10- to 100- fold increases in brain penetration of administered compounds (an antiparasitic, ivermectin and carcinostatic drug, vinblastine), increased bio-availability and toxicity (Schinkel *et al.*, 1994). Administration of avermectin, a naturally occurring pesticide, to pregnant ABCB1 KO mice resulted in increased fetal accumulation and birth defects (Lankas *et al.*, 1998).

ABCB1 promotes the elimination of undesired substances through several excretory and secretory routes. Substances capable of circumventing xenobiotic defence mechanisms in the intestinal wall enter the hepatic portal system. The liver is an important site of xenobiotic metabolism and detoxification. In addition to the presence of numerous xenobiotic metabolizing enzymes, ABCB1 expression at the canalicular surface of hepatocytes allows for excretion of toxic substances and endogenous metabolites into the bile (Thiebaut *et al.*, 1987). In addition to the regulation of fluid volume and electrolyte composition, the kidney also plays an important role in the excretion of endogenous and xenobiotic metabolites. ABCB1 is expressed primarily in the proximal tubule of the kidney, where it aids in the transfer of substances from the blood to the urine (Thiebaut *et al.*, 1987). Several ABCB1 substrates, including APE, have been detected in human urine (Charuk *et al.*, 1998). Other unconventional excretory routes, including the ducts of mammary gland and epithelium of the testis are also sites of ABCB1 expression. Toxicants capable of bypassing earlier biological defense mechanisms and entering sensitive tissues are actively excluded by ABCB1. Toxic metabolites, including pesticides and industrial compounds have been previously detected in breast milk and semen (Stachel *et al.*, 1989; Stefanidou *et al.*, 2009).

Differences in the tissue distribution of ABCB1 orthologs have been reported; ABCB1a is expressed at high levels in the blood-brain barrier, intestine, placenta, liver and kidney, and ABCB1b is expressed adrenal glands, uteral endometrium, ovary, placenta and kidney ABCB1 (Schinkel, 1997; Cui *et al.*, 2009). The two rodent genes however, are thought to collectively serve a similar physiological function as human ABCB1.

3.1.5 Expression in the Male Reproductive Tract

In addition to the physical divisions of the BTB and BEB created by tight junctions, specialized xenobiotic efflux transporters actively regulate the type and quantity of compounds entering the male reproductive tract. Elevated levels of ABCB1 mRNA were originally demonstrated in Chinese hamster testes, suggesting a role in male reproductive function (Baas and Borst, 1988). Later, ABCB1 was localized to the vasculature of human and rodent testes. ABCB1 has also been reported in interstitial steroidogenic Leydig cells, and was recently identified as an integrated component of the blood-testis barrier (Melaine *et al.*, 2002; Bart *et al.*, 2004; Su *et al.*, 2009). The specific localization of ABCB1 throughout spermatogenesis remains controversial. Differences in antibody selection and immuno-detection protocols are thought to contribute to the variability of published results. The most widely used ABCB1 antibody, C219, has been reported as being sensitive to fixation (Volk *et al.*, 2005). Melaine *et al.*, (2002) reported the presence of ABCB1 in the cytoplasmic lobes of late spermatids in rat. In this study however, ABCB1 was not detected in mitotic and meiotic germ cells or BTB of several species. Contrarily, Su *et al.*, (2009) reported ABCB1 at the Sertoli-elongated spermatid interface, prior to spermiation, and BTB throughout all stages of spermatogensis in adult rat testis. ABCB1 was also found to be associated with several tight junction proteins (occluding, claudin-11 and JAM-A), suggesting a more integrated role in BTB dynamics. The protective role of ABCB1 in the male reproductive tract was confirmed upon the generation of specific ABCB1 knockout (KO) mice. In ABCB1-knockout mice, digoxin entry into the testis was significantly increased compared to wild-type mice (Schinkel *et al.*, 1995b). To date, little information

exists on the expression profile or functional role of ABCB1 in the adult epididymis of any mammalian species.

3.1.6 ABCB1 and Male Fertility

Sperm development and maturation requires a specialized, protected microenvironment. Maturing sperm are transcriptionally incompetent and therefore are thought to be more susceptible to xenobiotic insult. ABCB1 aids in the maintenance of male fertility by actively restricting the types and quantities of compounds coming into contact with maturing sperm. ABCB1 in the blood-testis and blood epididymis barriers restricts the transfer of blood toxins to epididymal and testicular lumens. As mentioned previously, ABCB1 KO animals have increased testicular levels of administered compounds and are therefore more susceptible to toxicity (Schinkel *et al.*, 1995b). Spermatogenesis is also dependent on the normal physiological functioning of steroidogenic Leydig cells and supportive Sertoli cells. Immunohistochemical data suggests ABCB1 may support spermatogenesis by protecting the viability of somatic cells in the reproductive tract (Melaine *et al.*, 2002; Bart *et al.*, 2004; Su *et al.*, 2009).

An association between male infertility and an ABCB1 polymorphism was demonstrated recently, suggesting that ABCB1 may be involved in mediating the detrimental effects of environmental toxicants on male reproductive function (Drozdzik *et al.*, 2009). ABCB1 is thought to attenuate the entry and accumulation of environmental toxins, carcinogens and metabolites in testis. Individuals possessing ABCB1 polymorphisms may therefore be more susceptible to environmental pollutants, resulting in infertility. ABCB1 may play a more physiological role in male fertility; endogenous sterols are among the wide range of ABCB1 substrates (Garrigues *et al.*, 2002). ABCB1 may be involved in mediating male reproductive function by regulating testicular transport, intracellular and extracellular concentrations of important sex steroids (Garrigues *et al.*, 2002; Enokizono *et al.*, 2007).

3.1.7 Other ABC Transporters in the Male Reproductive Tract

Several other members of the ABC family of efflux proteins have been implicated in both the phenomenon of chemotherapeutic resistance and the disposition of xenobiotics. ABCC1 is a rodent ortholog of human ABCB1 that shares 88% amino acid identity (Leslie *et al.*, 2001). Like ABCB1, ABCC1 is expressed in numerous normal tissues, including the lung, gut, liver, kidney and placenta (Cole *et al.*, 1992; Flens *et al.*, 1996; Borst *et al.*, 1999; Peng *et al.*, 1999; Borst *et al.*, 2000; Leslie *et al.*, 2001). The localization of ABCC1 is limited almost exclusively to the basolateral side of the cellular membrane, and therefore transports substrates from the epithelium towards the blood. Contrary to ABCB1, ABCC1 transports primarily GSH, glucuronate and sulphate conjugated substances of toxicological relevance (Leier *et al.*, 1994; Jedlitschky *et al.*, 1996; Leier *et al.*, 1996; Leslie *et al.*, 2001). Substrates include lipid peroxidation products, pesticides, tobacco smoke derivatives, heavy metals and pharmaceutical drugs. Despite a significant amino acid identity between human and rodent ABCC1, differences in substrate specificity have been reported (Leslie *et al.*, 2001)

High levels of ABCC1 have been reported in rodent and human testis, suggesting a role in male reproductive function. ABCC1 was detected in both interstitial Leydig cells and Sertoli cells, but was absent in meiotic and meitotic germ cells (Bart *et al.*, 2004). Mice lacking ABCC1 had increased etoposide-induced damage to the oropharyngeal cavity and seminiferous tubules of the testis (Wijnholds *et al.*, 1998). ABCC1 was also co-localized with estrogen-sulfotransferase in Leydig cells, indicating that it may be involved in maintaining low estrogen levels in the testis (Leslie *et al.*, 2001). There are currently no reports of ABCC1 expression or function in the epididymis.

A third ABC transporter, ABCG2 was originally isolated from a multi-drug resistant breast cancer cell line. Over-expression of ABCG2 confers drugs resistance to several classes of chemotherapeutic agents, including anthracyclines, mitoxantrone and topoisomerase inhibitors (Mao and Unadkat, 2005). ABCG2 is also expressed in several non-malignant tissues where it plays a role in xenobiotic defence and drug disposition.

ABCG2 is highly expressed in the endothelial cells of the blood-brain-, testis-, epididymis- and placenta-barriers, apical surface of epitheliums of the small intestine and colon, liver canalicular membrane, proximal kidney and lactating breasts (Maliepaard *et al.*, 2001; Cooray *et al.*, 2002; Mao and Unadkat, 2005). Unlike other ABC transporters, ABCG2 is a half transporter consisting of one nucleotide binding domain and one membrane spanning-domain (Mao and Unadkat, 2005). Biochemical evidence suggests ABCG2 forms a homodimer or heterodimer to efflux substrates. ABCG2 substrates include not only chemotherapeutic agents, but also numerous conjugated and un-conjugated organic anion molecules of toxicological relevance (Mao and Unadkat, 2005).

In the male reproductive tract, ABCG2 has been localized to luminal surface of testis and epididymal blood capillaries, myoid cells surrounding seminiferous tubules and luminal surface of the epithelium of the proximal epdidiymis (Fetsch *et al.*, 2006; Enokizono *et al.*, 2007). ABCG2 was also recently detected on the acrosome of mature murine sperm, suggesting a role in sperm protection or maturation (Scharenberg *et al.*, 2009). The administration of phytoestrogens to the xenobiotic efflux transporter ABCG2 knockout mice resulted in elevated epididymal concentrations of phytoestrogens as compared to wild-type mice, reinforcing a role in protecting the epididymis and maturing sperm from chemical insult (Enokizono *et al.*, 2007).

Given this information, we hypothesize that ABCB1 is expressed and functional in the epididymal epithelium and epididymal spermatozoa and is an important toxicokinetic regulator of AP and other xenobiotics in the male reproductive tract.

4.0 Objectives

Little is known about the role of ABC xenobiotic efflux transporters in the epididymis. The objectives of this thesis are to characterize the expression profile and functional role of ABCB1a and ABCB1b in the adult rat epididymis and epididymal spermatozoa. Given the lack of expression data on ABC transporters in the epididymis, the first objective is to characterize the expression profile and localization of ABCB1 in the four regions (IS, CT, CS and CA) of the adult rat epididymis. The second objective of this thesis is to determine if ABCB1 is functional in epididymal epithelial cells and epididymal spermatozoa. Lastly, to determine if ABCB1 plays a role in defending the epididymis against industrial surfactants and other xenobiotics, the third objective is to determine if ABCB1 expression is inducible by NP and DOX in rat epididymal cells.

CHAPTER 2: ARTICLE

FRENCH ABSTRACT

Lorsqu'ils sont administrés, les alkyphénols, tel que l'octylphénol, se rendent jusqu'au testicule et jusqu'à l'épididyme sans toutefois s'accumuler dans ces tissus, ce qui suggère qu'ils sont rapidement éliminés. Il existe des transporteurs spécialisés dont le rôle est de limiter l'entrée de certains composés dans les cellules-cibles. ABCB1 fait partie de la famille des transporteurs ABC (ATP-binding cassette) qui sont capables de transporter une variété de médicaments et de xénobiotiques à l'extérieur des tissus. L'objectif de cette étude était de caractériser l'expression et le rôle des protéines ABCB1a et ABCB1b dans les différentes régions de l'épididyme (segment initial, tête, corps, queue) du rat adulte. Les transcrits d'ABCB1a et d'ABCB1b sont présents dans les quatre régions de l'épididyme. Des études d'immunolocalisation ont révélé qu'ABCB1 était faiblement exprimé par les cellules épithéliales et par les spermatozoïdes dans les régions épididymaires proximales. Par la suite, son expression augmente progressivement dans le corps et dans la queue de l'épididyme. Ce gradient d'expression a été confirmé par immunobuvardage de type western suggérant qu'ABCB1 est acquis par les spermatozoïdes au cours de leur transit épididymaire. Des essais de chimiorésistance (MDR) ont démontré que les cellules de rat épididymaires ainsi que les spermatozoïdes présentent un phénotype MDR, qui peut être inhibé dans des conditions contrôles. De plus, il est possible d'induire l'expression des ARNm d'ABCB1a et d'ABCB1b et de la protéine ABCB1 dans une lignée cellulaire d'épididyme de rat (RCE) en les exposant à différentes concentrations d'un alkylphénol, le nonylphénol. L'ensemble de ces résultats démontre que, dans l'épididyme, ABCB1 joue un rôle important dans la protection des cellules épithéliales et des spermatozoïdes contre les xénobiotiques.

REGULATION AND CHARACTERIZATION OF THE ATP-BINDING CASSETTE TRANSPORTER-B1 IN THE EPIDIDYMIS AND EPIDIDYMAL SPERMATOZOA OF THE RAT.

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Running Title: ABCB1 in the epididymis

Key words: ABC transporter, ABCB1, alkylphenol, epididymis, nonylphenol, spermatozoa

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Toxicological Sciences

ABSTRACT

It has been reported that following administration, alkylphenols, such as octylphenol, reach the testis and epididymis, but fail to accumulate in these tissues, suggesting the rapid expulsion of these chemicals by transporters. Specialized transporters that function to restrict compounds that enter target cells have been identified. ABCB1 is a member of the ATP-binding cassette family of proteins capable of transporting a broad range of drugs and xenobiotics out of tissues. The objective of this study was to characterize the expression profile and functional role of ABCB1a and ABCB1b along the different regions (initial segment, caput, corpus and cauda) of the adult rat epididymis. ABCB1a and ABCB1b transcripts were detected in all four regions of the epididymis. Immunolocalization revealed minimal ABCB1 staining in epithelial cells or spermatozoa of proximal regions of the epididymis; however, this progressively increased in the corpus and cauda epididymis. This expression gradient was confirmed by western blot suggesting that spermatozoa acquire ABCB1 during epididymal maturation. Multidrug resistance (MDR) assays revealed that rat epididymal cells and epididymal spermatozoa display a MDR phenotype which can be inhibited under control conditions. To assess whether or not the system was inducible by alkylphenols, cells from an immortalized epididymal cell line (RCE) were exposed to different concentrations of nonylphenol. Results revealed a significant induction of both ABCB1a and ABCB1b mRNA, and ABCB1 protein in RCE cells. Our findings demonstrate a role for ABCB1 in protecting both epididymal principal cells and spermatozoa from xenobiotics.

INTRODUCTION

Spermatozoa are produced in the testis via spermatogenesis, but it is during epididymal transit that they acquire the ability to swim and fertilize. The epididymal epithelium consists of several specialized cell types which exhibit region-specific localization and function that contribute to sperm maturation (Hermo *et al.*, 1992; Hinton and Palladino, 1995). Tight junctions between epithelial principal cells which line the lumen, as well as receptor-mediated transport of molecules to and from the lumen, create a specific luminal environment that is different from blood and which is necessary for sperm maturation (Cyr, 2002; Wong, 2002; Cyr *et al.*, 2007).

Several studies have shown that environmental toxicants can alter epididymal function and affect sperm maturation (Klinefelter, 2002; Foster *et al.*, 2010). Few studies, however, have examined the presence of efflux transporters in the epididymis, and whether or not xenobiotics can be transported out of epididymal cells. The administration of phytoestrogens to the xenobiotic efflux transporter ABCG2 knockout mice resulted in elevated epididymal concentrations of phytoestrogens as compared to wild-type mice (Enokizono *et al.*, 2007) suggesting that efflux transporters may play a role in protecting the epididymis and maturing sperm from chemical insult.

Widely used industrial surfactants, such as alkylphenol ethoxylates, and their major degradation products, alkylphenols, have been proposed as male reproductive toxicants in vivo (Boockfor and Blake, 1997; Han *et al.*, 2004) and in vitro (Aravindakshan and Cyr, 2005). Recent in vivo studies however, suggest that environmentally relevant doses of certain alkylphenols, such 4-tert-octylphenol (OP) may be less toxic than originally reported (Gregory *et al.*, 2009). A possible reason for the lower reproductive toxicity of OP is that while it reaches the testis and epididymis, it is rapidly cleared from these tissues in adult rats (Hamelin *et al.*, 2009). This suggests that AP are either rapidly metabolized or transported out of the tissue.

The ATP-Binding Cassette (ABC) family of efflux transporters are plasma membrane proteins capable of transporting a wide array of chemically diverse substrates, including drugs, toxicants, lipids and other endogenous substrates, across cellular membranes. Several members of this family, including ABCB1 (P-glycoprotein, multidrug resistance-1), ABCG2 (breast cancer resistance protein, mitoxantrone resistance protein) and ABCC1 (multidrug resistance associated protein-1), were originally implicated in chemotherapeutic resistance, but were later found to be constitutively expressed in normal tissues, serving a barrier or excretory function. These transporters actively regulate the disposition of numerous drugs and toxicants. ABCB1 was the first ABC efflux transporter to be identified (Juliano and Ling, 1976). It is constitutively expressed in the intestine, kidneys, liver, pancreas, adrenals, placenta, blood-brain and blood-testis barriers (Thiebaut *et al.*, 1987; Staud *et al.*, 2010). ABCB1 is capable of translocating a wide range of primarily lipophilic and amphipathic substrates (Mizutani *et al.*, 2008; Klaassen and Aleksunes, 2010; Staud *et al.*, 2010). While humans possess one ABCB1 efflux gene, rodents have two ABCB1 efflux genes, ABCB1a and ABCB1b. Differences in the tissue distribution of ABCB1a/b have been reported, but the two genes are thought to collectively serve the same physiological function as human ABCB1 (Cui *et al.*, 2009). ABCB1 has been localized to the vasculature of the testis, interstitial steroidogenic Leydig cells, and was recently identified as an integrated component of the blood-testis barrier (Melaine *et al.*, 2002; Bart *et al.*, 2004; Su *et al.*, 2009). ABCB1a knockout mice are viable and fertile under control conditions, but display altered ABCB1a pharmacokinetic properties and are more susceptible to chemical injury (Schinkel *et al.*, 1994). In ABCB1-knockout mice, digoxin entry into the testis was significantly increased compared to wild-type mice (Schinkel *et al.*, 1995b).

Given the established role of ABCB1 in the blood-testis and other blood-tissue barriers, we hypothesized a role for ABCB1 in epididymal barrier function and protection of sperm. The objectives of the present study were to characterize the expression profile and functionality of ABCB1 in the adult rat epididymis and epididymal spermatozoa, as well as to assess ABCB1 functionality and the ability of an alkylphenol, nonylphenol (NP), to induce ABCB1 expression in rat epididymal cells.

MATERIALS & METHODS

Animals

Adult Sprague Dawley rats were purchased from Charles River Canada (St. Constant, QC, Canada) and acclimated for seven days prior to surgery. Rats were maintained under a consistent photoperiod of 12h light and 12h darkness and received food and water *ad libitum*. All animal protocols used in this study were approved by the university animal care committee.

Rats were euthanized with CO_2 at the time of sampling. Epididymides were dissected and subdivided into four anatomical regions (initial segment (IS), caput (CT), corpus (CS) and cauda (CA) epididymidis). Epididymal tissues were either flash-frozen in liquid nitrogen for RNA and protein analysis, prepared for immunolocalization studies, or used immediately for sperm extraction.

Real-time PCR

Total RNA was isolated from epididymal tissue and epididymal cells using the Illustra RNAspin Mini kit (GE Healthcare, Montreal, QC, Canada) according to manufacturer's instructions. RT-PCR was performed using a Rotor-Gene RG3000 (Corbett Life Science, Mortlake, NSW, Australia). All reactions were performed in triplicate. Reverse transcription was done using 500 ng RNA, oligo $(dT)_{16}$ primers (R&D Systems Inc., Minneapolis, MN, USA) and M-MLV reverse transcriptase (Sigma-Aldrich, Mississauga, ON, Canada). Real-time PCR reactions for ABCB1a, ABCB1b, ABCC1, ABCG2 and β-actin were done using primers listed in Table 1. PerfaCTa SYBR Green SuperMix (Quanta Biosciences Inc., Gaithersburg, MD, USA) was used according to the manufacturer's protocol. PCR reactions contained 2 µl of RT reaction, 1X PerfeCTa SYBR SuperMix and 0.9 µl (200 mM final concentration) of both forward and reverse primers (total volume of 15 µl). Standard curves using appropriate cDNA were created for all genes examined and used to calculate relative expression levels. Relative mRNA levels of target genes of interest were normalized to β-actin. Statistical analyses were performed using one-way analysis of variance ($P \leq 0.05$; ANOVA; Systat Software, Inc., Chicago, IL, USA).

Cell Culture and Treatments

Rat epididymal epithelial RCE cells (Dufresne *et al.*, 2005) were plated at an appropriate density in either 6-, 24-, or 96-well Cell-plus culture plates (Sarstedt Inc., Montreal, QC, Canada). Cells were cultured in Dulbecco modified Eagle medium/Ham nutrient mixture F12 (Sigma-Aldrich; containing 50 U/ml penicillin, 50 µg/ml streptomycin, 2 mM L-glutamine, 10 µg/ml insulin, 10 µg/ml transferrin, 80 ng/ml hydrocortisone, 10 ng/ml epidermal growth factor, 10 ng/ml cAMP, 5% fetal bovine serum (FBS), and 5 nM testosterone), in a humidified incubator at 32°C with 5% CO_2 until they reached confluency.

To assess the induction of ABCB1, cells were cultured 90-95% confluent as previously described, medium was removed and cells were washed 3 times in PBS. Fresh medium containing either ethanol (0.1%, vehicle), NP (1 to 20 µM; Pestanal, analytical standard, technical mixture, Sigma-Aldrich), or doxorubicin hydrochloride (50 or 500 ng/ml; DOX; Sigma-Aldrich), a known ABCB1 inducer (Mercier *et al.*, 2003), were added to the cells. Cells were treated for 24h under normal culture conditions. Culture medium was removed and cells were washed 3 times in PBS. For protein extraction, RIPA buffer (PBS, 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 10 µg/ml phenylmethylsulfonyl fluoride (PMSF), 100 µM sodium orthovanadate and a protease inhibitor cocktail (Sigma-Aldrich) was added to each of the wells. Cells were scraped from the wells, collected with a pipette and placed immediately on ice. After 30 min of shaking at 4°C, cells were placed at -80°C overnight. The next day, cells were centrifuged for 10 min at 10,000g. Aliquots of the supernatant were stored at -80°C. RNA extraction from RCE cells was done using the Illustra RNAspin Mini kit (GE Healthcare) according to the manufacturer's instructions.

Immunohistochemistry

Whole epididymides were excised, fixed by immersion in Bouin's fixative (Fisher Scientific, Ottawa, ON, Canada) for 24h, dehydrated, embedded in paraffin and sectioned (5 µm). For immunostaining, the tissue sections were rehydrated through a series of graded

ethanol (100-50%), including 70% alcohol with 1% lithium carbonate for 5 min, to remove residual picric acid. The sections were also incubated in 300 mM glycine for 5 min to block free aldehydes, and washed in TBS. Heat-induced epitope retrieval was performed in a microwave oven for 10 min in citrate buffer (1.8 mM citric acid, 8.2 mM sodium citrate). Immunolocalization was performed with the DAKO Catalyzed Signal Amplification System (DAKO, Carpenteria, CA, USA). An anti-ABCB1 mouse monoclonal antibody (1:50 dilution in DAKO blocking solution; Abcam, Cambridge, MA, USA) was incubated on tissue sections in a humidified chamber for 1h at room temperature. Pre-absorption of the primary antibody with a 200-fold excess of a commercially synthesized (Invitrogen, Burlington, ON, Canada) ABCB1 immunizing peptide (VVEAQLDKAREGRTC; (Georges *et al.*, 1990) was used as a negative control. Epididymal sections were counterstained for 5 min with 0.1% methylene blue, dehydrated in ethanol, immersed in Histoclear (Fisher Scientific), and mounted in Permount (Fisher Scientific). Sections were examined under a Leica DMRE microscope.

Immunofluoerescence

Whole epididymides were excised, frozen in OCT compound (Fisher Scientific) on dry ice and stored at -80°C until sectioning. Tissue sections (5 um) were cut using a cryotome and stored at -80°C until use. For immunostaining, the tissue sections were fixed in ice-cold methanol or ethanol for 20 min at -20°C and then washed in PBS. Tissue sections were blocked for one hr in PBS T.X(0.3%), 2 % Bovine Serum Albumin (BSA) and 2 % Normal Goat Serum (NGS) at room temperature. An anti-ABCB1 mouse monoclonal antibody (1:20 dilution in blocking solution; Abcam) was incubated on tissue sections in a humidified chamber for 1h at room temperature. Slides were washed in PBS and then incubated with Alexa 488 (1:500 dilution in blocking solution; Invitrogen, Carlsbad, CA) for one hour at room temperature. Following the secondary antibody, slides were washed in PBS and then counterstained with DAPI (Vector Laboratories, Burlington, ON). Pre-absorption of the primary antibody with a 200-fold excess of a commercially synthesized (Invitrogen, Burlington, ON, Canada) ABCB1 immunizing peptide (VVEAQLDKAREGRTC; (Georges et al., 1990) was used as a negative control. Sections were examined under a Leica DMRE microscope.

Sperm Extraction

Adult rat epididymides were excised and dissected into IS, CT, CS and CA. Tissue sections were immediately placed in pre-warmed PBS. For protein extraction, a protease inhibitor cocktail (Sigma-Aldrich) was added to the PBS solution. Epididymal tissue was placed in a Petri dish on a slide warmer at 37°C and a 30G needle used to perforate the tissue. The tissue was incubated for 10 min to allow sperm to exit the tissue. Sperm containing fluid was collected with a pipette and placed in microcentrifuge tubes. Spermatozoa were pelleted at 200g for 10 min and resuspended in lysis buffer for protein extraction or PBS for multidrug resistance (MDR) assays.

Western Blot Analysis

Frozen epididymal tissue was ground with a mortar and pestle in liquid nitrogen. Lysis buffer (1:3 wt/vol; 60 mM Tris-HCl (pH 6.8), 2 mM $CaCl_2$, 40 mM octyl β-D-glucopyranoside, 1 μg/mL Pepstatin A; Sigma-Aldrich) supplemented with a protease inhibitor cocktail (Sigma-Aldrich) was added to tissue samples prior to homogenization with a Polytron (PowerGen 500, Fisher Scientific). Homogenenized samples were transferred to microcentrifuge tubes and separated by centrifugation as previously described (DeBellefeuille *et al.*, 2003). Protein concentrations were determined using the Bradford Reagent (Sigma-Aldrich) according to the manufacturer's instructions.

Spermatozoa were resuspended in 100μl of buffer containing 1.0% Igepal CA-630, 154 mM NaCl, 0.4 M Tris and protease inhibitor cocktail (pH 8.0; Sigma-Aldrich; (Morales *et al.*, 2008). Samples were placed on a shaker at 4°C for 30 min and then centrifuged at 10,000 g for 10 min at 4°C. Aliquots of the supernatant, which contained the protein, were stored at -80°C for future analysis.

Proteins (50 μg) were diluted in 2X sample buffer and boiled at 95°C for 5 min prior to loading on a 6% polyacrylamide gel (5% stacking gel). Protein samples were separated by electrophoresis and transferred onto a nitrocellulose membrane (Bio-Rad Laboratories, Toronto, ON, Canada) at 400mA for 1h under cooling conditions. Protein transfer onto the

nitrocellulose membrane was verified with Ponceau red stain (0.6% wt/vol, 1% acetic acid). Membranes were washed with ddH_2O and subsequently blocked in TBST (0.25%) containing 5% powdered milk for 1h at room temperature. Membranes were incubated overnight at 4°C with a monoclonal anti-ABCB1 primary antibody (1:100, Abcam) diluted in blocking solution. The next day, blots were washed 3 times in TBST and incubated with horseradish peroxidise labelled goat anti-mouse secondary antibody (Santa Cruz, CA, USA) for 1h at room temperature. Membranes were washed with TBST and then incubated with Lumilight Western blotting substrate (Roche, Rockford, IL, USA) according to the manufacturer's instructions. Protein levels were normalized to α-tubulin (rabbit polyclonal anti-α-tubulin, Abcam) and subsequently quantified by densitometry with a Fluor Image analyzer (Bio-Rad Laboratories). Statistical analyses were done using one-way ANOVA (Statistical significance was set at $P \leq 0.05$; SigmaStat).

Multidrug Resistance Assays

MDR assays were performed on epididymal RCE cells and epididymal sperm extracted from proximal (IS, CT, CS) and distal (CA) regions of the adult rat epididymis using the Vybrant Multidrug Resistant Assay Kit (Invitrogen) according to manufacturer's instructions. Cells were incubated with calcein AM, an ABCB1 and ABCC1 substrate, in the presence or absence of specific inhibitors.

Cyclosporin A (CsA; ABCB1 inhibitor; Sigma-Aldrich), MK571 (ABCC1 inhibitor; Sigma-Aldrich) and verapamil (ABCB1 inhibitor, Invitrogen) were used as ABC inhibitors. Stock solutions of CsA (5 mg/ml), verapamil (10 mg/ml;) and MK571 (5 mg/ml) were prepared in 100% ethanol and stored at -20°C. Inhibitors were serially diluted to 4X final concentrations in PBS on the day of the experiment. A stock solution of 2 mM calcein in PBS was freshly prepared on the day of the experiment. Cell viability was determined using propidium iodide (PI; Sigma-Aldrich) according to procedures of (Dengler *et al.*, 1995).

RCE cells were platted in 96-well plates and grown until 90-95% confluent. Medium was removed and the cells washed in PBS. Fresh PBS (50 µl) was added to each well and additional PBS (50 µl), CsA (50ul; 1,15 or 30 µg/ml final) or MK571 (50 µl; 1, 15 or 30

μg/ml final) was added and incubated for 15 min at 37°C. Calcein stock solution (100 μl; 1 mM final concentration) was then added to the cells and incubated for an additional 15 min at 37°C. The solution was removed from each of the wells and cells washed in PBS. Fresh medium (100 μl) was added to each well and calcein fluorescence measured using a fluorometer (485 nm/530 nm). PI was then added to the culture medium (1 μg/ml, final concentration), incubated for 15 min at 37°C, and fluorescence measured (530 nm/590 nm). Plates were covered and frozen (in darkness) at -80°C for 24h and fluorescence was measured after thawing at room temperature. The relative number of viable cells was calculated by subtracting the initial PI measurement from PI measurement in cells following freezing and thawing (total cell number). Calcein fluorescence values were normalized relative total viable cell number. Statistical analyses were performed with one-way ANOVA ($P \leq 0.05$; SigmaStat).

Spermatozoa were extracted from epididymides of adult rats, as previously described, and re-suspended in 1 ml PBS. Spermatozoa concentration was determined with a hemocytometer and the suspension was diluted to 20 million cells/ml in PBS. Diluted sperm samples (250 μl) were added to microcentrifuge tubes and incubated with PBS (250 μl), MK571 (250 μl; 7.5 or 30 μg/ml final concentration) or verapamil (250 μl; 7.5 or 30 μg/ml final concentration). The tubes were gently mixed by inversion and incubated for 15 min at 37°C. At the end of the incubation, calcein stock solution (500 μl; 1 mM final concentration) was added, gently mixed by inversion and incubated for an additional 15 min at 37°C. Spermatozoa were washed 3 times in PBS and diluted to a final concentration of 10^6 cells/ml in PBS. Calcein fluorescence (geometric mean; 10,000 cellular events) was determined using a FACScan (Becton Dickinson, Oakville, ON). Sperm populations were gated during FACS analysis to exclude cells with altered physical characterstistics.

RESULTS

ABCB1 expression and localization in the adult rat epididymis

Relative mRNA levels of ABCB1a and ABCB1b were determined by real time-PCR. Transcripts for both orthologs were detected in all four regions of the adult rat epididymis

(Fig. 1A-B). ABCB1a mRNA levels in distal regions of the epididymis (CA), tended to be higher, but differences were not statistically significant. ABCB1b mRNA levels were similar in all epididymal regions (Fig. 1A-B). ABCB1a transcripts were more dominantly expressed than ABCB1b in the epididymis.

ABCB1 immunohistochemistry using a mouse monoclonal antibody revealed a localization gradient of the protein along the epididymis. No immunostaining was detected in the epithelium or lumen of the IS (Fig. 2A-B). Minimal immunostaining was observed in the epithelial cells or spermatozoa in the CT (Fig. 2E), and progressively increased in distal CT, CS (Fig. 2G-H) and CA (Fig. 2J-K). A strong apical immunoreaction was observed in principal cells of the CS (Fig. 2G-H) and CA (Fig. 2J-K) regions. ABCB1 was also detected in a subset of clear cells, the principal endocytic cell in the epididymal epithelium, in distal regions (CA) of the epididymis (Fig. 2K). The appearance of ABCB1 in the epididymal epithelium coincided with a strong increase in spermatozoal immunostaining, indicating that spermatozoa acquire the protein during epididymal transit. ABCB1 was detected in both the head and tail regions of epididymal sperm in CS (Fig. 2G-H) and CA (Fig. 2J-K). An immunoreaction of varying intensity in interstitial blood vessels was also observed throughout the epididymis (Fig. 2). Pre-absorption of the primary antibody with an immunizing peptide was used as a negative control (Fig. 2H).

Consistent with immunohistochemistry data, immunofluorescence revealed a similar gradient localization. No immunostaining was detected in the epithelium or lumen of the IS (Fig. 2C). Strong apical reaction was observed in the epithelium of distal CT, CS (Fig. 2I) and CA. ABCB1 was detected in individual clear cells in distal CT (Fig. 2F). A gradient in the sperm was also observed. A distinct immunopositive dot was observed on the midpiece of luminal spermatozoa under high magnification in CS and CA (Fig. 2L) regions. A variable immunostaining was also observed in interstitial blood-vessels throughout the epididymis (Fig. 2). Pre-absorption of the primary antibody with an immunizing peptide was used as a negative control (Fig. 2I).

Immunolocalization expression gradients were confirmed by western blot analysis. Significantly more ABCB1 protein was detected in CS and CA regions of the adult rat epididymis as compared to the IS and CT (Fig. 3A-B). Similarly, sperm extracted from the CA region of the epididymis expressed significantly greater ABCB1 protein as compared to sperm extracted from IS and CT (Fig. 3C-D). Pre-absorption of the primary antibody with excess peptide abolished all ABCB1 immunoreaction (data not shown).

ABCB1 functionality in a rat epididymal cell line and epididymal sperm

To determine if ABCB1 is functional in the rat epididymis and epididymal spermatozoa, MDR assays were done using epididymal RCE cells (Fig. 4A) as well as isolated epididymal sperm from adult rats (Fig. 4B-D). Both RCE and epididymal sperm demonstrated a MDR phenotype that could be inhibited under control conditions. The low- and mid-dose CsA significantly increased calcein fluorescence in RCE cells (Fig. 4A), demonstrating ABCB1 activity. The highest dose of CsA did not produce any significant changes (Fig. 4A). Further investigation is required to elucidate this phenomenon. The ABCC1 inhibitor, MK571, used as a negative control, also significantly increased RCE cell calcein fluorescence in a dose-dependent manner (Fig. 4A) suggesting that this transporter is also active in RCE cells (Fig. 4A).

Epididymal sperm extracted from proximal (Fig. 4B-D) and distal (Fig. 4C) regions of the adult epididymis also displayed an inhibitable MDR phenotype. A dose-dependent increase in calcein florescence was observed in both proximal (Fig. 4B-C) and distal (Fig. 4D) sperm treated with an ABCB1 inhibitor, verapamil. A rightward shift in the calcein fluorescence peak was observed in verapamil-treated sperm as compared to untreated cells (Fig. 4B). In contrast, sperm from the CA incubated with MK571 demonstrated a dose-dependent decrease in calcein fluorescence (Fig. 4D).

Induction of ABCB1 mRNA and protein in a rat epididymal cell line (RCE)

To determine if ABCB1 was inducible in the epididymal epithelium, relative mRNA and protein levels were measured in RCE cells following a 24h exposure to varying concentrations of the known ABCB1 inducer, DOX, and to NP (Fig. 5). There was a

tendency for ABCB1a mRNA to be increased by the highest DOX treatment (500 ng/ml), but this was not statistically significant (Fig. 5A). A greater than 6-fold increase in ABCB1b mRNA was observed in DOX (500 ng/ml)-treated cells (Fig. 5C). Exposure to 10 μM and 20 μM NP (Fig. 5B) resulted in 2- and 4-fold increases in ABCB1a mRNA levels. ABCB1b mRNA levels were also significantly increased in cells exposed to the highest NP concentration (20 μM; Fig. 5D). There were no significant changes in ABCC1 or ABCG2 mRNA levels following DOX or NP treatment (data not shown).

Consistent with the mRNA data, a significant up-regulation of ABCB1 protein was observed at the highest dose of DOX (Fig. 6). NP (10 μM and 20 μM) increased ABCB1 protein expression in a dose-dependent manner (Fig. 6). Pre-absorption with excess peptide abolished all ABCB1 staining at the expected molecular weight (data not shown).

Cell death varied in between 2.1 and 10.7 % in a dose- dependent manner for both DOX- and NP-treated cells (data not shown).

DISCUSSION

It has recently been reported that oral or intravenous administration of OP resulted in elevated OP levels in male reproductive tissues, which were rapidly cleared from the testis and the epididymis (Gregory *et al.*, 2009; Hamelin *et al.*, 2009). We hypothesized that cellular transporters such as ABCB1, a broad specificity xenobiotic efflux transporter, could play an important role in epididymal barrier function and spermatozoal protection and that it may be involved in the expulsion of alkylphenols from these tissues.

Our results demonstrate that ABCB1 is constitutively expressed and functional in both epididymal epithelial cells and in epididymal spermatozoa. ABCB1a mRNA levels were significantly higher in all regions of the epididymis relative to ABCB1b levels. Tissue-specific and gender-divergent expression of ABCB1 orthologs has been reported previously in mice (Cui *et al.*, 2009). Our results suggest ABCB1a is the dominant ABCB1 in the adult rat epididymis, but that both forms may play a role in epididymal barrier function.

As in other physiological systems, ABCB1 was detected in the capillary blood vessels of the epididymis. The presence of ABCB1 on the apical side of endothelial cells has been demonstrated previously to limit the penetration of circulating xenobiotics into sensitive organs and tissues (Schinkel *et al.*, 1994; Schinkel *et al.*, 1995b). ABCB1 immunostaining was also observed on the apical side of the epididymal epithelium in CS and CA epididymidis, suggesting a role in epididymal tissue defence. Although no directional efflux assays were conducted in the present study, directional studies in other tissues have suggested that ABCB1 pumps in a basal to apical direction, therefore, towards the epididymal lumen. Compounds capable of circumventing the endothelial barrier may therefore be actively transported out of the epididymal epithelium and into the lumen. Although not usually considered as such, the epididymis, like the kidney, liver and mammary gland ducts, can act as an excretory tissue. Toxic metabolites, including heavy metals, pesticides and industrial compounds have been previously detected in semen (Stachel *et al.*, 1989). Paradoxically, vulnerable transiting and maturing sperm are also present in the epididymal lumen. Although advantageous for the epididymal epithelium, directional efflux towards the epididymal lumen may inadvertently expose sperm to toxic substances. Since spermatozoa are transcriptionally incompetent cells, they may be more susceptible to chemical insult and require specific defence mechanisms.

A clear cell-specific localization of ABCB1 was observed in epithelium of the adult rat epididymis. Clear cells are the principle endocytic cells in the epididymal epithelium (Hermo *et al.*, 1988). It is possible that the ABCB1 immunoreaction of these cells resulted from the endocytosis of ABCB1-positive spermatozoal components and cellular debris from the epididymal lumen. Clear cells have also, however, been demonstrated to express several xenobiotic metabolizing enzymes, including CYP1A1 and CYP2E1 (Roman *et al.*, 1998; Forkert *et al.*, 2002). Interestingly, there is considerable overlap in substrate specificity between ABCB1 and certain cytochrome P450s (Staud *et al.*, 2010). Coordinated regulation of ABCB1 and cytochrome P450s has also been reported in other tissue systems (Xu *et al.*, 2005). An alternative hypothesis, therefore, may be that epididymal clear cells act in concert with drug metabolizing enzymes and xenobiotic efflux

transporters to uptake, metabolize and subsequently excrete luminal toxicants in more soluble forms.

Our findings demonstrated unique ABCB1 protein expression gradients in both the epididymal epithelium and in epididymal spermatozoa. Immunolocalization of ABCB1 was characterized by two different detection methods and further verified by western blot. Expression gradients were consistent in all three assays, but differences in spermatozoal localization were noted. Immunohistochemistry localized ABCB1 to both the head and tail of epididymal spermatozoa in distal CT, CS and CA, where as immunofluorence revealed a distinct immunopositive dot on the mid-piece of the sperm in CS and CA regions. These results are likely explained by differences in tissue preparation, fixation and ABCB1 detection protocols.

Constitutive expression and function of ABCB1 may indicate, in addition to a defence function, a normal physiological role in epididymal function and sperm maturation. An association between male infertility and an ABCB1 polymorphism was demonstrated recently, suggesting a role in the maintenance of male reproductive potential (Drozdzik *et al.*, 2009). ABCB1 may mediate the detrimental effects of xenobiotics on male reproductive function or play a more physiological role in male fertility; endogenous sterols are among the wide range of ABCB1 substrates (Garrigues *et al.*, 2002). Interestingly, the expression gradient in both the epididymal epithelium and epididymal spermatozoa coincides with changes in epithelial, luminal and sperm plasma membrane sterol composition. Cholesterol and lipid loss are among several important factors regulating sperm capacitation and the acrosome reaction, which usually occur in the female reproductive tract (de Lamirande *et al.*, 1997). Additionally, other ABC transporters implicated in sterol and phospholipid transport to apolipoproteins and high-density lipoprotein, have been localized to different portions of murine spermatozoa, including the mid-piece (Morales *et al.*, 2008).

The expression gradient in epididymal sperm was particularly interesting, given that spermatozoa are transcriptionally incompetent. Any transcripts that they possess must be

present in the testis, or acquired from the epithelium during epididymal transit. Transporters of carnitine (OCTN), an important biological molecule for sperm maturation and acquisition of motility, have been previously localized to epididymal sperm in a similar gradient pattern (Kobayashi *et al.*, 2007). Interestingly, several proteins, including both surface and GPI-anchored proteins, have been demonstrated previously to be transferred from the epithelium to sperm (Sullivan *et al.*, 2007). An alternative possibility is that a non-functional form of ABCB1 is already present in testicular sperm, but only unmasked to a functional form during epididymal transit: ABCB1 is a phospho-glycoprotein with several potential post-translational modification sites (Juliano and Ling, 1976). This notion is supported by the report of Oko *et al.* (1993) who identified the presence of a functional Golgi in the cytoplasmic droplet of epididymal spermatozoa. The Golgi may, for example, increase the glycosylation of ABCB1 allowing the protein to be recognized by the antibody. In any case, the presence of ABCB1 represents a unique mechanism by which spermatozoa may defend against certain classes of environmental toxicants.

ABCB1 activity was demonstrated in both sperm taken from proximal and distal regions of the epididymis. Proximal sperm populations included both sperm taken from IS and CT. Since ABCB1 expression begins in distal CT, the proximal sperm population likely containts both ABCB1 positive and ABCB1 negative cells. Consistent with the ABCB1 expression data, control calcein fluorescence levels were higher in proximal sperm compared to distal, suggesting lower ABCB1 activity.

Induction of ABCB1 mRNA and protein by DOX and NP, in dose-dependent manner, was demonstrated in RCE cells. As reported previously in mice, ABCB1b mRNA, but not ABCB1a was significantly induced by DOX exposure (Mercier *et al.*, 2003). Both ABCB1a and ABCB1b mRNA were significantly induced by NP exposure, indicating the ability of alkylphenolic compounds to regulate both for rodent forms of ABCB1. The fact that ABCB1 is inducible indicates the presence of a dynamic defence system that can respond to chemical insult. Although NPEs have been shown to modulate ABCB1-mediated efflux (Doo *et al.*, 2005), to the best of our knowledge, this is the first study to

demonstrate an induction of ABCB1 by an AP. These results may explain why OP is rapidly cleared from the epididymis of treated animals (Hamelin *et al.*, 2009).

MDR functional assays suggest that ABCC1 may also play a role in epididymal tissue defence. Additionally, although no induction was observed following DOX- and NP-treatment, mRNA transcripts for both ABCC1 and ABCG2 were detected in the RCE cells (data not shown). These findings suggest that several other members of the ABC transporters family may be implicated in epididymal function.

In summary, our results indicate that ABCB1 represents a toxicokinetic regulator of AP and other xenobiotics in the adult rat epididymis. The unique expression profile, localization and induction of ABCB1 suggest a role in epididymal barrier function. Furthermore, the presence of ABCB1 in epididymal spermatozoa represents a unique defence mechanism for vulnerable spermatozoa against chemical insult.

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REFERENCES

Aravindakshan, J., and Cyr, D. G. (2005). Nonylphenol alters connexin 43 levels and connexin 43 phosphorylation via an inhibition of the p38-mitogen-activated protein kinase pathway. *Biol Reprod* **72**, 1232-1240.

Bart, J., Hollema, H., Groen, H. J., de Vries, E. G., Hendrikse, N. H., Sleijfer, D. T., Wegman, T. D., Vaalburg, W., and van der Graaf, W. T. (2004). The distribution of drug-efflux pumps, P-gp, BCRP, MRP1 and MRP2, in the normal blood-testis barrier and in primary testicular tumours. *Eur J Cancer* **40**, 2064-2070.

Boockfor, F. R., and Blake, C. A. (1997). Chronic administration of 4-tert-octylphenol to adult male rats causes shrinkage of the testes and male accessory sex organs, disrupts spermatogenesis, and increases the incidence of sperm deformities. *Biol Reprod* **57**, 267-277.

Cui, Y. J., Cheng, X., Weaver, Y. M., and Klaassen, C. D. (2009). Tissue distribution, gender-divergent expression, ontogeny, and chemical induction of multidrug resistance transporter genes (Mdr1a, Mdr1b, Mdr2) in mice. *Drug Metab Dispos* **37**, 203-210.

Cyr, D. G., Finnson, K., Dufresne J., and Gregory, M. (2002). Cellular interactions and the blood-epididymal barrier. In B. Robaire and B. T. Hinton eds., The epididiymis: From molecules to clinical practice. Plenum Publisers, New York. Pp103-118.

Cyr, D. G., Gregory, M., Dube, E., Dufresne, J., Chan, P. T., and Hermo, L. (2007). Orchestration of occludins, claudins, catenins and cadherins as players involved in maintenance of the blood-epididymal barrier in animals and humans. *Asian J Androl* **9**, 463-475.

de Lamirande, E., Leclerc, P., and Gagnon, C. (1997). Capacitation as a regulatory event that primes spermatozoa for the acrosome reaction and fertilization. *Mol Hum Reprod* **3**, 175-194.

DeBellefeuille, S., Hermo, L., Gregory, M., Dufresne, J., and Cyr, D. G. (2003). Catenins in the rat epididymis: their expression and regulation in adulthood and during postnatal development. *Endocrinology* **144**, 5040-5049.

Dengler, W. A., Schulte, J., Berger, D. P., Mertelsmann, R., and Fiebig, H. H. (1995). Development of a propidium iodide fluorescence assay for proliferation and cytotoxicity assays. *Anticancer Drugs* **6**, 522-532.

Doo, M. H., Li, H., Jang, H. I., Song, I. S., Chung, S. J., and Shim, C. K. (2005). Effect of nonylphenol ethoxylates (NPEs) on barrier functions of epithelial cell membranes: opening of tight junctions and competitive inhibition of P-gp-mediated efflux. *Int J Pharm* **302**, 145-153.

Drozdzik, M., Stefankiewicz, J., Kurzawa, R., Gornik, W., Baczkowski, T., and Kurzawski, M. (2009). Association of the MDR1 (ABCB1) gene 3435C>T polymorphism with male infertility. *Pharmacol Rep* **61**, 690-696.

Dufresne, J., St-Pierre, N., Viger, R. S., Hermo, L., and Cyr, D. G. (2005). Characterization of a novel rat epididymal cell line to study epididymal function. *Endocrinology* **146**, 4710-4720.

Enokizono, J., Kusuhara, H., and Sugiyama, Y. (2007). Effect of breast cancer resistance protein (Bcrp/Abcg2) on the disposition of phytoestrogens. *Mol Pharmacol* **72**, 967-975.

Forkert, P. G., Lash, L. H., Nadeau, V., Tardif, R., and Simmonds, A. (2002). Metabolism and toxicity of trichloroethylene in epididymis and testis. *Toxicol Appl Pharmacol* **182**, 244-254.

Foster, W. G., Maharaj-Briceno, S., and Cyr, D. G. (2010). Dioxin-induced changes in epididymal sperm count and spermatogenesis. *Environ Health Perspect* **118**, 458-464.

Garrigues, A., Escargueil, A. E., and Orlowski, S. (2002). The multidrug transporter, P-glycoprotein, actively mediates cholesterol redistribution in the cell membrane. *Proc Natl Acad Sci U S A* **99**, 10347-10352.

Georges, E., Bradley, G., Gariepy, J., and Ling, V. (1990). Detection of P-glycoprotein isoforms by gene-specific monoclonal antibodies. *Proc Natl Acad Sci U S A* **87**, 152-156.

Gregory, M., Lacroix, A., Haddad, S., Devine, P., Charbonneau, M., Tardif, R., Krishnan, K., Cooke, G. M., Schrader, T., and Cyr, D. G. (2009). Effects of chronic exposure to octylphenol on the male rat reproductive system. *J Toxicol Environ Health A* **72**, 1553-1560.

Hamelin, G., Charest-Tardif, G., Krishnan, K., Cyr, D., Charbonneau, M., Devine, P. J., Haddad, S., Cooke, G. M., Schrader, T., and Tardif, R. (2009). Toxicokinetics of p-tert-octylphenol in male and female Sprague-Dawley rats after intravenous, oral, or subcutaneous exposures. *J Toxicol Environ Health A* **72**, 541-550.

Han, X. D., Tu, Z. G., Gong, Y., Shen, S. N., Wang, X. Y., Kang, L. N., Hou, Y. Y., and Chen, J. X. (2004). The toxic effects of nonylphenol on the reproductive system of male rats. *Reprod Toxicol* **19**, 215-221.

Hermo, L., Barin, K., and Robaire, B. (1992). Structural differentiation of the epithelial cells of the testicular excurrent duct system of rats during postnatal development. *Anat Rec* **233**, 205-228.

Hermo, L., Dworkin, J., and Oko, R. (1988). Role of epithelial clear cells of the rat epididymis in the disposal of the contents of cytoplasmic droplets detached from spermatozoa. *Am J Anat* **183**, 107-124.

Hinton, B. T., and Palladino, M. A. (1995). Epididymal epithelium: its contribution to the formation of a luminal fluid microenvironment. *Microsc Res Tech* **30**, 67-81.

Juliano, R. L., and Ling, V. (1976). A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. *Biochim Biophys Acta* **455**, 152-162.

Klaassen, C. D., and Aleksunes, L. M. (2010). Xenobiotic, bile acid, and cholesterol transporters: function and regulation. *Pharmacol Rev* **62**, 1-96.

Klinefelter, G. R. (2002). Actions of toxicants on the structure and function of the epididymis. In B. Robaire and B. T. Hinton eds., The epididiymis: From molecules to clinical practice. Plenum Publisers, New York. Pp353-370.

Kobayashi, D., Tamai, I., Sai, Y., Yoshida, K., Wakayama, T., Kido, Y., Nezu, J., Iseki, S., and Tsuji, A. (2007). Transport of carnitine and acetylcarnitine by carnitine/organic cation transporter (OCTN) 2 and OCTN3 into epididymal spermatozoa. *Reproduction* **134**, 651-658.

Melaine, N., Lienard, M. O., Dorval, I., Le Goascogne, C., Lejeune, H., and Jegou, B. (2002). Multidrug resistance genes and p-glycoprotein in the testis of the rat, mouse, Guinea pig, and human. *Biol Reprod* **67**, 1699-1707.

Mercier, C., Decleves, X., Masseguin, C., Fragner, P., Tardy, M., Roux, F., Gabrion, J., and Scherrmann, J. M. (2003). P-glycoprotein (ABCB1) but not multidrug resistance-

associated protein 1 (ABCC1) is induced by doxorubicin in primary cultures of rat astrocytes. *J Neurochem* **87**, 820-830.

Mizutani, T., Masuda, M., Nakai, E., Furumiya, K., Togawa, H., Nakamura, Y., Kawai, Y., Nakahira, K., Shinkai, S., and Takahashi, K. (2008). Genuine functions of P-glycoprotein (ABCB1). *Curr Drug Metab* **9**, 167-174.

Morales, C. R., Marat, A. L., Ni, X., Yu, Y., Oko, R., Smith, B. T., and Argraves, W. S. (2008). ATP-binding cassette transporters ABCA1, ABCA7, and ABCG1 in mouse spermatozoa. *Biochem Biophys Res Commun* **376**, 472-477.

Roman, B. L., Pollenz, R. S., and Peterson, R. E. (1998). Responsiveness of the adult male rat reproductive tract to 2,3,7,8-tetrachlorodibenzo-p-dioxin exposure: Ah receptor and ARNT expression, CYP1A1 induction, and Ah receptor down-regulation. *Toxicol Appl Pharmacol* **150**, 228-239.

Schinkel, A. H., Smit, J. J., van Tellingen, O., Beijnen, J. H., Wagenaar, E., van Deemter, L., Mol, C. A., van der Valk, M. A., Robanus-Maandag, E. C., te Riele, H. P., and et al. (1994). Disruption of the mouse mdr1a P-glycoprotein gene leads to a deficiency in the blood-brain barrier and to increased sensitivity to drugs. *Cell* **77**, 491-502.

Schinkel, A. H., Wagenaar, E., van Deemter, L., Mol, C. A., and Borst, P. (1995). Absence of the mdr1a P-Glycoprotein in mice affects tissue distribution and pharmacokinetics of dexamethasone, digoxin, and cyclosporin A. *J Clin Invest* **96**, 1698-1705.

Stachel, B., Dougherty, R. C., Lahl, U., Schlosser, M., and Zeschmar, B. (1989). Toxic environmental chemicals in human semen: analytical method and case studies. *Andrologia* **21**, 282-291.

Staud, F., Ceckova, M., Micuda, S., and Pavek, P. (2010). Expression and function of p-glycoprotein in normal tissues: effect on pharmacokinetics. *Methods Mol Biol* **596**, 199-222.

Su, L., Cheng, C. Y., and Mruk, D. D. (2009). Drug transporter, P-glycoprotein (MDR1), is an integrated component of the mammalian blood-testis barrier. *Int J Biochem Cell Biol* **41**, 2578-2587.

Sullivan, R., Frenette, G., and Girouard, J. (2007). Epididymosomes are involved in the acquisition of new sperm proteins during epididymal transit. *Asian J Androl* **9**, 483-491.

Thiebaut, F., Tsuruo, T., Hamada, H., Gottesman, M. M., Pastan, I., and Willingham, M. C. (1987). Cellular localization of the multidrug-resistance gene product P-glycoprotein in normal human tissues. *Proc Natl Acad Sci U S A* **84**, 7735-7738.

Wang, T., Chen, M., Yan, Y. E., Xiao, F. Q., Pan, X. L., and Wang, H. (2009). Growth retardation of fetal rats exposed to nicotine in utero: possible involvement of CYP1A1, CYP2E1, and P-glycoprotein. *Environ Toxicol* **24**, 33-42.

Wong, P. Y. D., Gong, X.D., Leung, G.P.H., and Cheukj, B.I.Y. (2002). Formation of the epididymal fluid microenvironment. In B. Robaire and B. T. Hinton eds., The epididymis: From molecules to clinical practice. Plenum Publisers, New York. Pp119-130.

Xu, C., Li, C. Y., and Kong, A. N. (2005). Induction of phase I, II and III drug metabolism/transport by xenobiotics. *Arch Pharm Res* **28**, 249-268.

Table 1: Real-time PCR primer sequences and amplification conditions used for amplifying ABC transporters and β-actin target genes.

Gene/Accession number	Primer Sequence (5`-3`)	Cycling Conditions	Product Size (bp)	Reference
ABCB1a NM_133401	F:GCATTCTGGTATGGGACTT R:GTCTTTTCGAGACGGGTA	95(°C) 5s 55(°C) 15s 72(°C) 15s	282	(Wang *et al.*, 2009)
ABCB1b NM_012623	F:CATTCTGCCGAGCGTTAC R:CCCGTGTAATAGTAGGCGTA	95(°C) 5s 55(°C) 15s 72(°C) 15s	107	(Wang *et al.*, 2009)
ABCC1 NM_022281	F:CCCTGAAGAGCAGTGACCTC R:TAGGCTTGGTGGGATCTTTG	95(°C) 15s 54(°C) 30s 30(°C) 30s	156	NCBI
ABCG2 NM_181381	F:TCTTCGCCTTCCAAAAGCTA R:AAACCAGTTGTGGGCTCATC	95(°C) 15s 54(°C) 30s 75(°C) 30s	213	NCBI
β-Actin NM_031144	F:TCTGTGTGGATTGGTGGCTCTA R:CTGCTTGCTGATCCACATCTG	95(°C) 15s 54(°C) 30s 75(°C) 30s	69	OLIGO 6

FIGURE LEGENDS

Figure 1: Relative mRNA levels of ABCB1a (A) and ABCB1b (B) in the four segments of the adult rat epididymis (IS, CT, CS and CA). Real time-PCR was done using gene specific ABCB1a (A) and ABCB1b (B) primers and the data expressed relative to β-actin levels. Data are expressed as the mean ±SEM (n=3).

Figure 2: Immunolocalization of ABCB1 in the four segments of the adult rat epididymis (IS (A-C), CT (D-F), CS (G-I) and CA (J-L). Arrows indicate positive immunostaining. Incubation of the ABCB1 antibody with excess specific immunizing peptide was used as a negative control (H-I; upper right panel). Blood Vessel, Bv; Clear Cell, C; Lumen, Lu; Principal Cell, P; Spermatozoa (S).

Figure 3: Western Blot analysis of ABCB1 protein expression in the four segments (IS, CT, CS and CA) of the adult epididymis (A) and epididymal spermatozoa extracted from proximal (P; IS/CT) and distal (D; CA) regions (C), of the adult rat epididymis. Protein loading was standardized using α-Tubulin as a protein loading control. Data are expressed as the mean percent of IS ±SEM (B) or mean percent of total ±SEM (D) of three different animals (n=3). Asterisks represent a significant difference from IS (B) or proximal sperm (D) ($P<0.05$; ANOVA). Incubation of the ABCB1 antibody with excess specific immunizing peptide abolished all ABCB1 immunoreaction (data not shown).

Figure 4: MDR assay using rat epididymal RCE cells (A) and epididymal sperm (B,C,D) extracted from the proximal (IS/CT/CS) and distal (CA) regions of the adult rat epididymis. RCE data is represented as mean calcein fluorescence (485 nm/530 nm) normalized to total cell number as determined by PI (n=3)(A). Asterisks represents a significant difference from control and vehicle treatment groups (ANOVA; $P<0.05$). Caclein fluorescence values of proximal (C) and distal (D) sperm are represented as geometric mean fluorescence of 10,000 events as determined by FACS analysis (B). FACS analysis histograms (B) were obtained for all sperm treatment conditions. The experiment was repeated three times with consistent results (n=3). Data from one experiment is represented above.

Figure 5: Relative mRNA expression levels of ABCB1a (A, B) and ABCB1b (C,D) in RCE cells following a 24h of exposure to medium alone, vehicle (0.1% ethanol), NP (1, 10, and 20 μM), or DOX (50 and 500 ng/ml). ABCB1a and ABC1b mRNA levels were determined be real-time PCR and normalized to β-actin mRNA levels (n=3). Data are expressed as the mean ± SEM. Asterisks indicate a significant difference from control and vehicle treatment groups (ANOVA; $P<0.05$).

Figure 6: Western Blot analysis of ABCB1 protein expression in RCE cells following a 24h of exposure to medium alone, vehicle (0.1% ethanol), NP (1, 10, and 20 μM), or DOX (50 and 500 ng/ml). Protein loading was standardized using α-Tubulin as a protein loading control. Incubation of the ABCB1 antibody with an excess of specific immunizing peptide abolished all ABCB1 staining (data not shown).

Figure 1

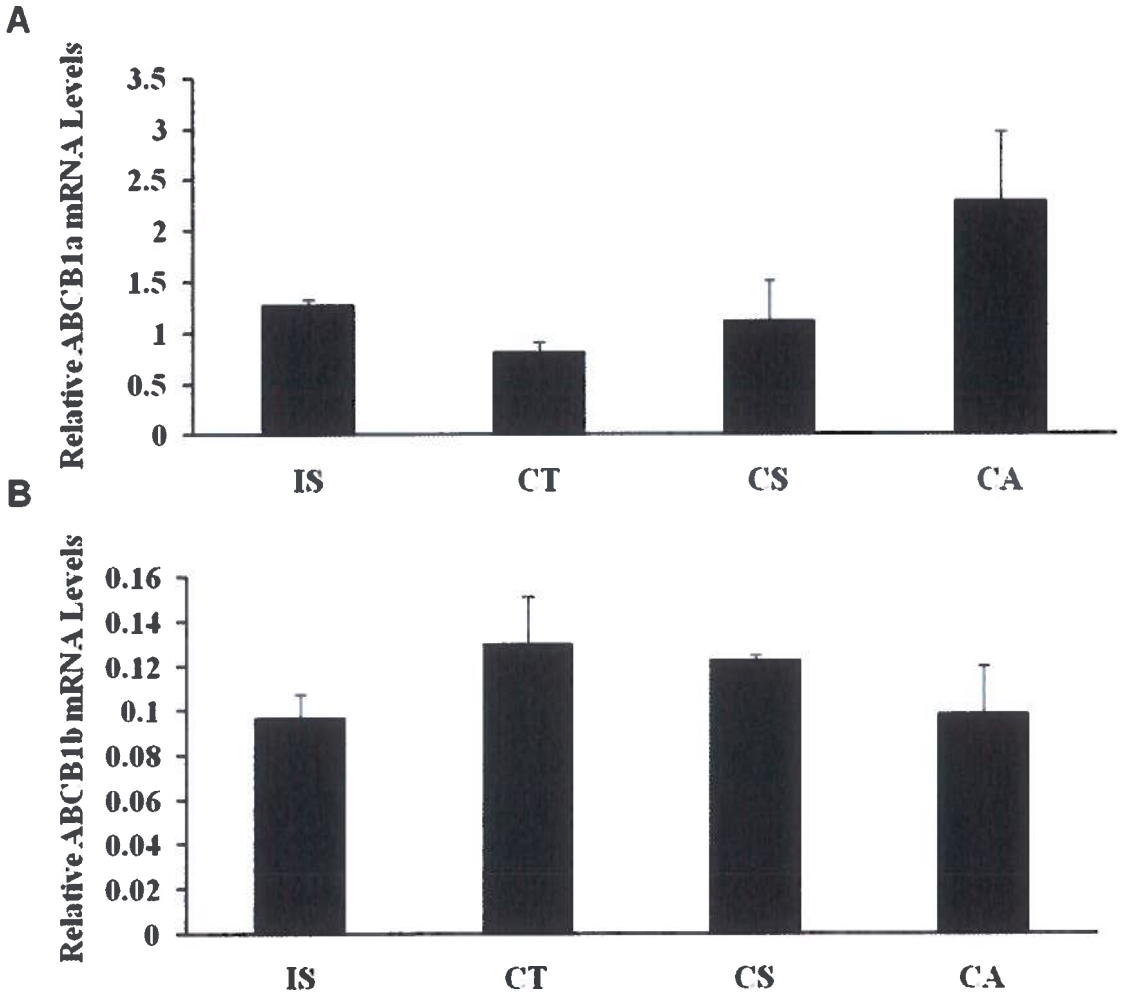

A
Relative ABCB1a mRNA Levels
3.5
3
2.5
2
1.5
1
0.5
0
IS
CT
CS
CA
B
Relative ABCB1b mRNA Levels
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
IS
CT
CS
CA

Figure 2

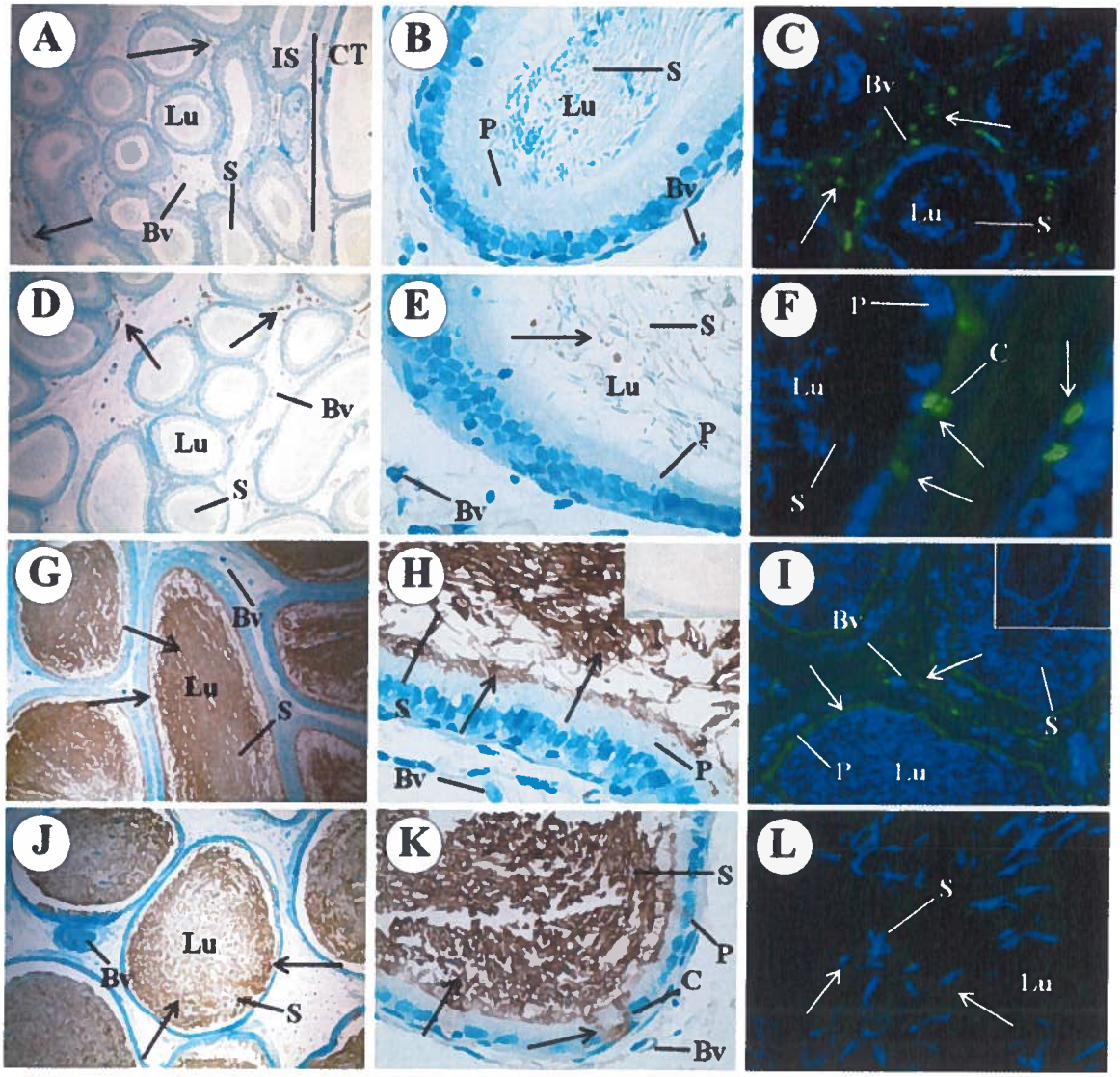

A
IS
CT
Lu
S
Bv
B
S
Lu
P
Bv
C
Bv
Lu
S
D
Bv
Lu
S
E
S
Lu
P
Bv
F
P
C
Lu
S
G
Bv
Lu
S
H
S
Bv
P
I
Bv
S
P
Lu
J
Lu
Bv
S
K
S
P
C
Bv
L
S
Lu

Figure 3

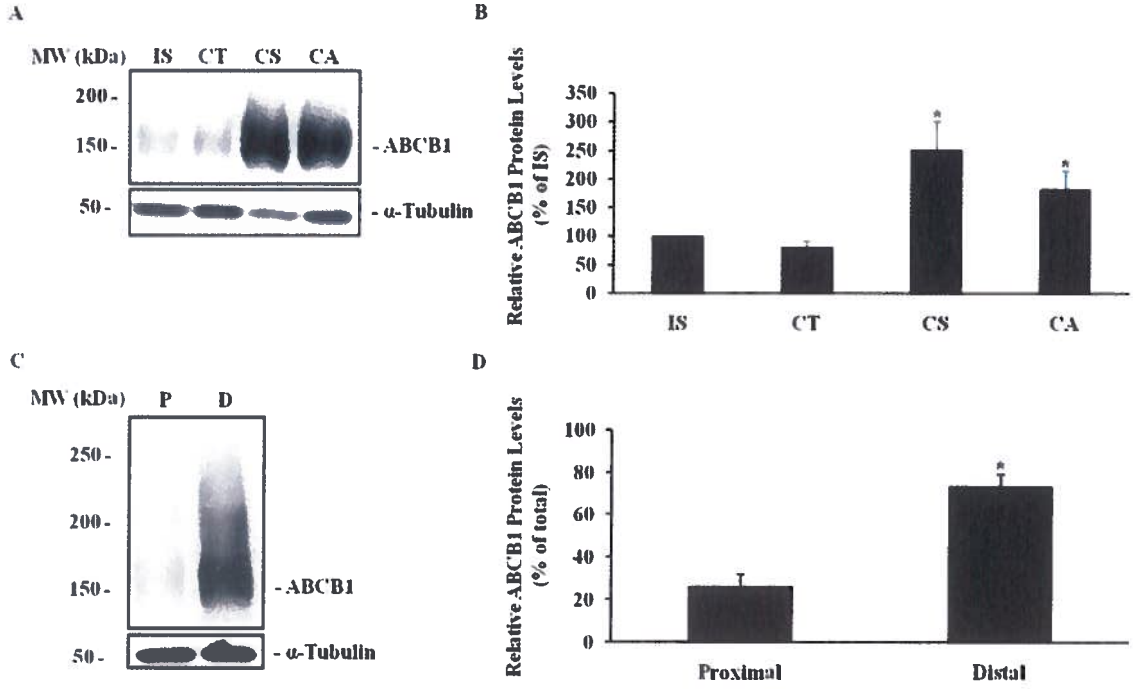

A
MW (kDa)
IS
CT
CS
CA
200 -
150 -
50 -
- ABCB1
- α-Tubulin
B
Relative ABCB1 Protein Levels (% of IS)
350
300
250
200
150
100
50
0
IS
CT
CS
CA
C
MW (kDa)
P
D
250 -
200 -
150 -
50 -
- ABCB1
- α-Tubulin
D
Relative ABCB1 Protein Levels (% of total)
100
80
60
40
20
0
Proximal
Distal

Figure 4

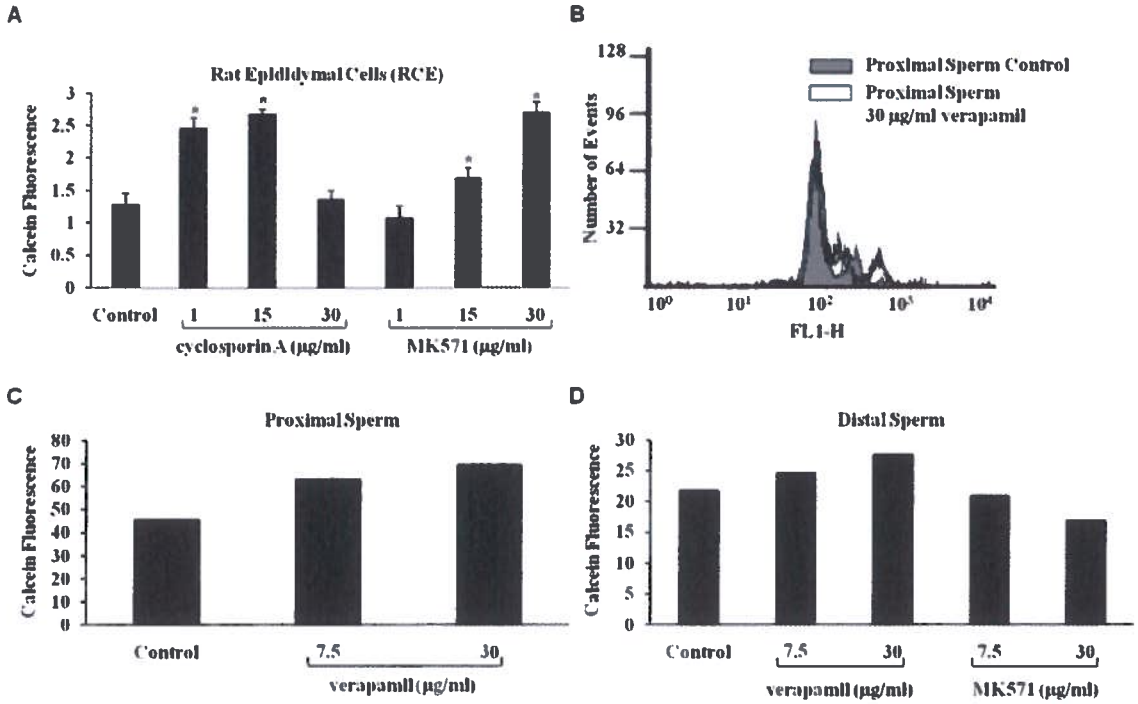

A
Rat Epididymal Cells (RCE)
Calcein Fluorescence
Control
1
15
30
1
15
30
cyclosporin A (μg/ml)
MK571 (μg/ml)
B
Proximal Sperm Control
Proximal Sperm
30 μg/ml verapamil
Number of Events
FL1-H
C
Proximal Sperm
Calcein Fluorescence
Control
7.5
30
verapamil (μg/ml)
D
Distal Sperm
Calcein Fluorescence
Control
7.5
30
7.5
30
verapamil (μg/ml)
MK571 (μg/ml)

Figure 5

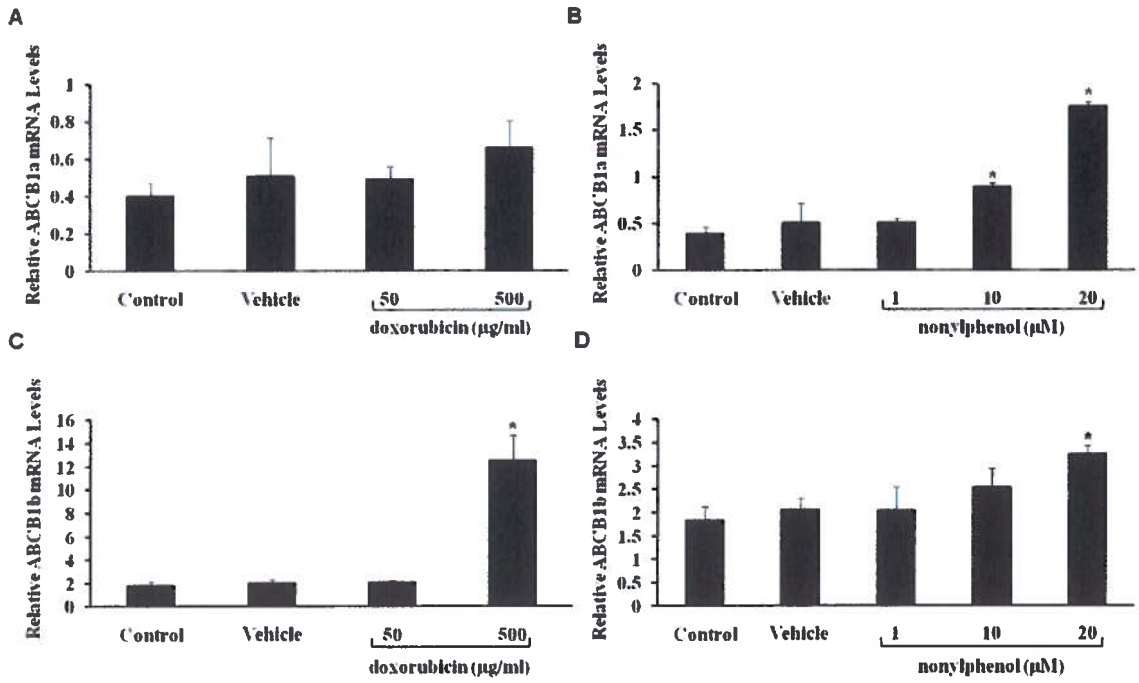
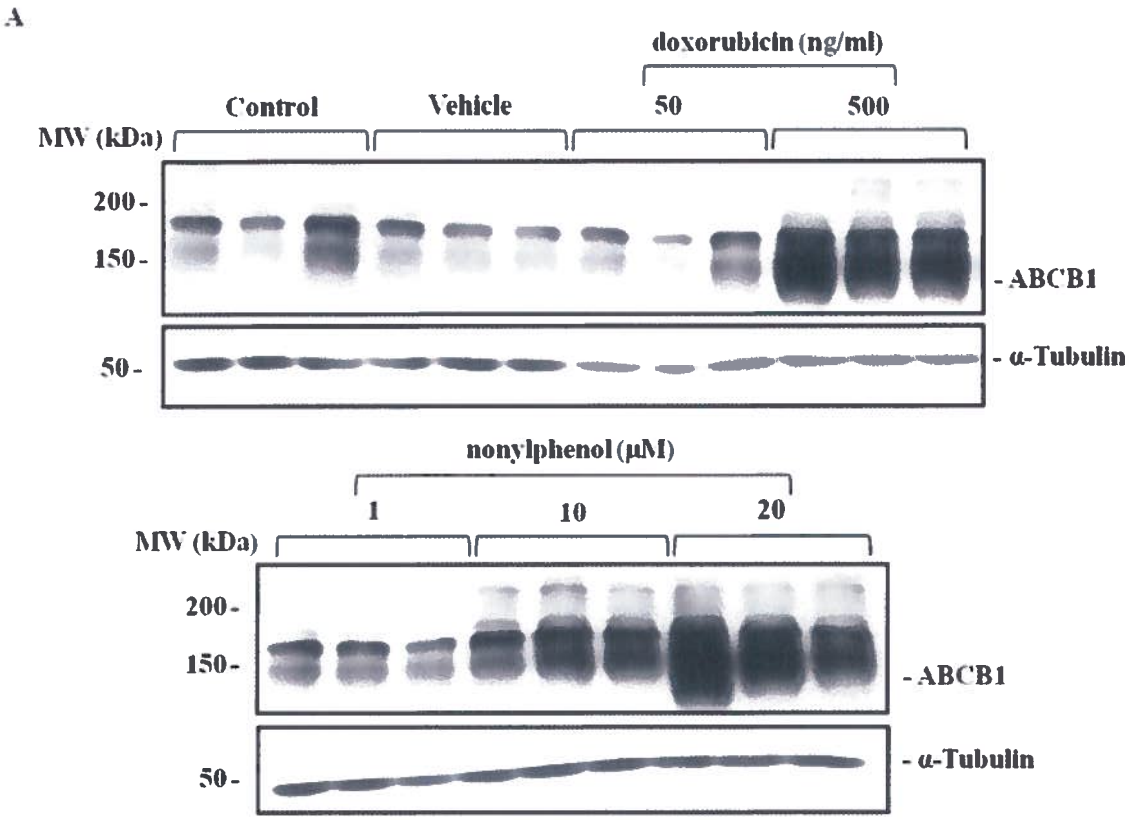
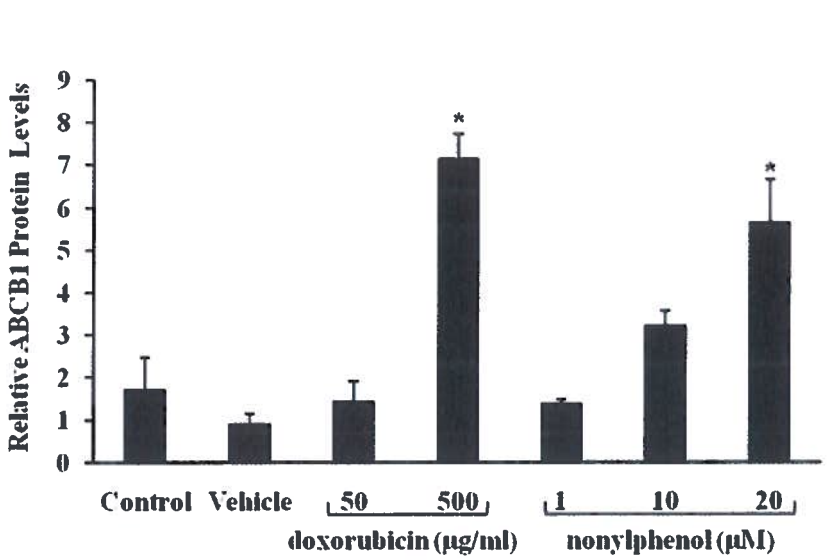

A
Relative ABCB1a mRNA Levels
1
0.8
0.6
0.4
0.2
0
Control
Vehicle
50
500
doxorubicin (μg/ml)
B
Relative ABCB1a mRNA Levels
2
1.5
1
0.5
0
Control
Vehicle
1
10
20
nonylphenol (μM)
C
Relative ABCB1b mRNA Levels
16
14
12
10
8
6
4
2
0
Control
Vehicle
50
500
doxorubicin (μg/ml)
D
Relative ABCB1b mRNA Levels
4
3.5
3
2.5
2
1.5
1
0.5
0
Control
Vehicle
1
10
20
nonylphenol (μM)

Figure 6


A
doxorubicin (ng/ml)
Control
Vehicle
50
500
MW (kDa)
200 -
150 -
- ABCB1
50 -
- α-Tubulin
nonylphenol (μM)
1
10
20
MW (kDa)
200 -
150 -
- ABCB1
50 -
- α-Tubulin


B
Relative ABCB1 Protein Levels
9
8
7
6
5
4
3
2
1
0
*
*
Control
Vehicle
50
500
1
10
20
doxorubicin (μg/ml)
nonylphenol (μM)

CHAPTER 3: DISCUSSION AND GENERAL CONCLUSIONS

This thesis investigated the possible involvement of ABC efflux transporters in epididymal barrier function and toxicokinetic regulation of xenobiotics. Xenobiotic efflux transporters, along with blood tissues and metabolic enzymes are important regulators of chemical disposition and toxicity (Klaasen, 2001; Klaassen and Aleksunes, 2010). It is known that the BEB provides a unique microenvironment that allows for sperm maturation, storage and protection and is distinct from circulating blood (Hinton and Palladino, 1995; Cyr, 2002; Cyr *et al.*, 2007; Cornwall, 2009). To date however, little is known about the role of xenobiotic efflux transporters in the epididymis. Despite the established importance of the epididymis in sperm maturation and protection, much of the research in this area has focused on the site of germ cell production (Melaine *et al.*, 2002; Bart *et al.*, 2004). Our research has characterized, for the first time, the unique expression profile, localization and functionality of a toxicologically, pharmacologically and physiologically important ABC-transporter, ABCB1, in the rat epididymis. These results indicate the existence of a plausible excretory defence mechanism against AP and other xenobiotics in the adult rat epididymis. This system may act in concert with physical and metabolic defence mechanisms to ensure normal physiological functioning of the rat epididymis and normal sperm maturation.

Our research indicates that ABCB1 provides several lines of defence in the adult rat epididymis. We have demonstrated that a functional ABCB1 is expressed along the length of the adult rat epididymis and epididymal spermatozoa in a unique gradient pattern (Fig. 1). The expression profile and functional MDR assay indicate that ABCB1 plays an active role in epididymal and spermatozoal detoxification.

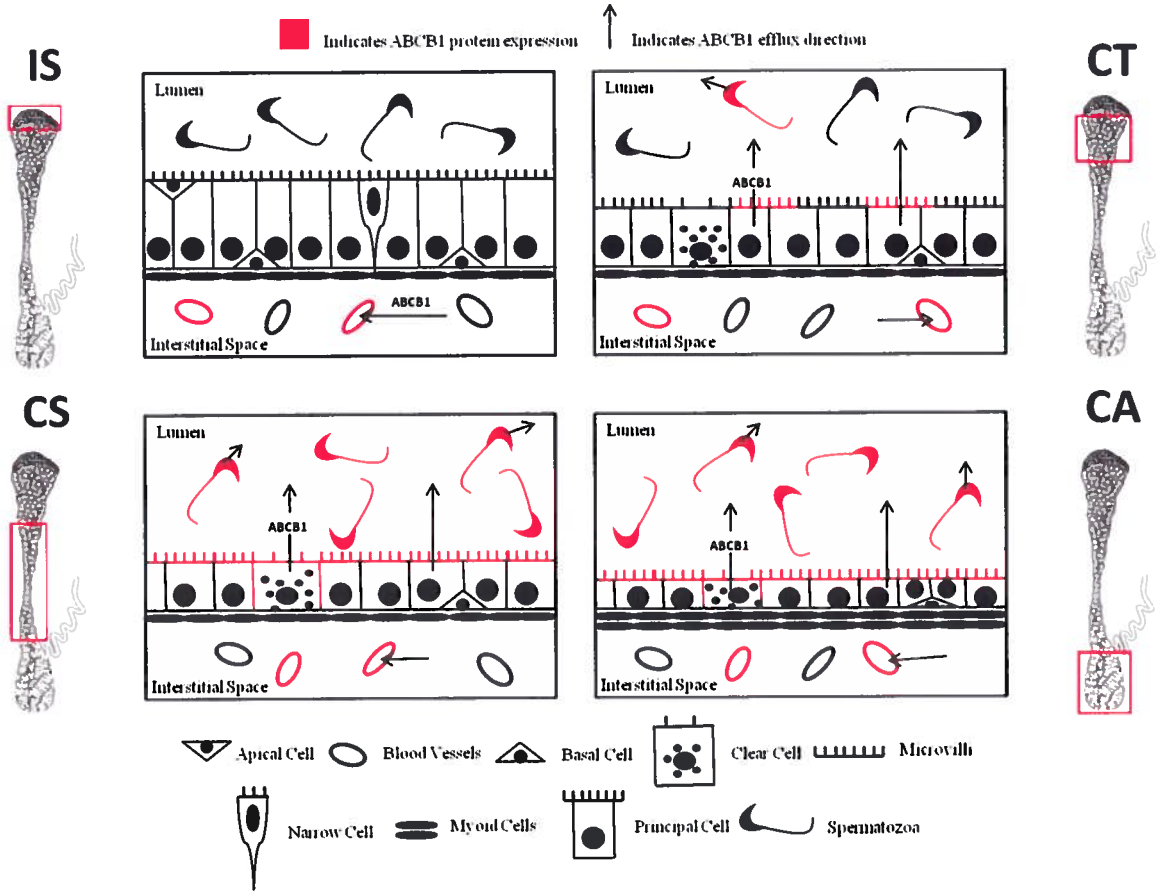


Figure 1: Schematic representation of ABCB1 expression and efflux in the adult rat epididymis.

Similar to other blood-tissue barriers, ABCB1 expression in the vasculature of the epididymis provides an initial line of defence against circulating xenobiotics (Schinkel *et al.*, 1994; Schinkel *et al.*, 1995a; Schinkel *et al.*, 1995b; Schinkel and Jonker, 2003). ABCB1 expression on the apical (luminal) edge of endothelial cells allows for active efflux of substrates towards the blood, attenuating the entry of potentially harmful substances into the tissue. Once a toxicant has successfully entered the tissue, expression along the apical surface of the epithelium in allows for active efflux from the epididymal epithelium towards the epididymal lumen. The toxicological relevance of ABCB1 expression in distal regions of the epididymis is currently unknown. The coordinated appearance of ABCB1 in the epididymal epithelium and epididymal spermatozoa may indicate a unique mechanism for ABCB1 transfer. Since sperm are incapable of protein synthesis, the epididymal

epithelium may be required for ABCB1 synthesis and transfer to the sperm. A unique protein transport system involving membrane bound vesicles, called epididymosomes, has been characterized (Sullivan *et al.*, 2007). Alternatively, ABCB1 may already be present in both the epithelium and spermatozoa, but only unmasked for antibody recognition by a protein modification. Golgi present in the epididymal epithelial cells or cytoplasmic droplet of sperm are capable of modifying protein epitopes through glycosylation (Oko *et al.*, 1993; Robaire *et al.*, 2006). Several important changes in sperm membrane composition and morphology also coincide with the appearance of ABCB1 expression (Cornwall, 2009; Hermo *et al.*, 2010c). Since ABCB1 is also capable of transporting a number of endogenous substrates, the expression gradient in the epididymal epithelium is also suggestive of a more physiological role for ABCB1 in male reproductive function (Mizutani *et al.*, 2008). Reinforcing this notion is recently demonstrated correlation between ABCB1 polymorphisms and male infertility (Drozdzik *et al.*, 2009).

ABCB1 was detected in maturing spermatozoa in distal CT, CS and CA, demonstrating the ability of sperm to assume their own defence mechanisms. Expression of ABCB1 on the apical surface of the sperm plasma membrane allows for active efflux of toxicants outside the sperm, protecting sensitive genetic information. Similar to the epididymal epithelium, ABCB1 may also serve a more direct physiological role in sperm maturation by regulating the composition of the sperm plasma membrane.

Both a pharmacologically relevant anti-cancer agent, DOX, and an environmental pollutant, NP, significantly induced ABCB1 mRNA and protein in dose dependent manner in a rat epididymal cell line. The inducibility of ABCB1 mRNA and protein suggests the existence of a dynamic defence system that can respond to chemical insult. Although NPEs have been previously demonstrated to modulate ABCB1 mediated efflux (Doo *et al.*, 2005), to the best of our knowledge, this is the first study to demonstrate an induction of ABCB1 by an AP in any mammalian organ system. These results may explain why AP were rapidly excluded from the epididymis of treated animals (Hamelin *et al.*, 2009).

How is ABCB1 being induced by AP?

This thesis work did not investigate the mechanism for ABCB1 regulation by AP. RCE cells exposed to NP however, demonstrated a dose dependent increase in cellular death. This is suggestive of NP inflicted cellular stress. Interestingly, oxidative stress can activate several signalling pathways that are involved in ABCB1 regulation (Callaghan *et al.*, 2008). NP has also been previously reported to decrease levels of antioxidant enzymes (catalase, glutathione peroxidise) and increase peroxide generation and lipid peroxidation, demonstrating the ability to induce oxidative stress (Chitra *et al.*, 2002). It therefore plausible that NP is capable up-regulating ABCB1 by generating a harsh intracellular environment similar to that observed in cancerous cells. The generation of ROS and altered bioenergetic metabolism are thought to be key contributors to the MDR phenotype observed in cancerous tissues (Callaghan *et al.*, 2008).

Alternatively, NP may be binding directly to xenosensor nuclear receptors such as CAR and PXR to stimulate ABCB1 expression and other genes involved in cellular defence mechanisms (Kretschmer and Baldwin, 2005; Xu *et al.*, 2005). Numerous classes of environmental estrogens, such as pesticides, plasticisers and industrial surfactants, including NP, have been demonstrated to activate PXR or PXR and CAR (Kretschmer and Baldwin, 2005).

How are xenobiotics reaching the male reproductive tract?

Further investigation is required to determine how orally administered toxicants, such as AP and APE, are surpassing numerous structural and biochemical barriers to reach the male reproductive tract. Interestingly, NPE have been previously demonstrated to modulate ABCB1 function (Doo *et al.*, 2005). It therefore possible that although AP successfully induce ABCB1 expression, they also impair efflux activity, allowing for increased penetration into sensitive tissues. AP have also been demonstrated to impact barrier function by altering structural integrity or cellular communication (Fiorini *et al.*, 2004; Han *et al.*, 2004; Salian *et al.*, 2009). GJIC, as well as the localization and expression of tight

junction proteins in the male reproductive tract have been previously demonstrated to be altered by AP and APE (Fiorini *et al.*, 2004; Han *et al.*, 2004; Aravindakshan and Cyr, 2005). Although ABCB1 is capable of transporting substrates against steep concentration gradients, efflux capacity is still largely dependent on the rate of passive diffusion (Staud *et al.*, 2010). If the rate of passive entry is accelerated, such as in the case of a structurally impaired barrier, ABCB1 is overwhelmed by passive diffusion and has little effect on substrate disposition.

Results from the MDR functional assay suggest ABCC1 may also be playing a role in epididymal tissue defence. Additionally, although no induction was observed following DOX and NP treatment, mRNA transcripts for both ABCC1 and ABCG2 were detected in the RCE cell line. These findings suggest that several other members of the ABC transporters family may also be playing an important role in epididymal barrier function and protection of spermatozoa. Further research is necessary to determine if other xenobiotic efflux transporters are regulators of AP disposition and toxicity in the epididymis.

It was previously accepted that the principle method for toxicant entry was by passive diffusion. The importance of specific xenobiotic importers, such as members of the solute carrier transporter (SLC) family, in toxicant disposition however, has been highlighted in recent literature (Klaassen and Aleksunes, 2010). It is likely that importers are playing an equally important role in the regulation of xenobiotic entry into biological systems (Klaassen and Aleksunes, 2010). Characterization of both import and export systems in the male reproductive tract will allow increasingly realistic toxicokinetic modeling of xenobiotic action.

Development of an in vitro model to study xenobiotic efflux in the rat epididymis

We have successfully created the first in vitro model for xenobiotic efflux in the rat epididymis. The model developed in the current study can be used as a unique tool for evaluating ABCB1 induction and modulation by xenobiotics in the rat epididymis.

Additionally, RCE cells may be used to evaluate the individual or coordinated expression and functional roles of other ABC transporters in the rat epididymis.

CONCLUSIONS

A functional form ABCB1 is expressed in a unique, cell specific pattern along the length of the adult rat epididymis.

Epididymal sperm express a functional form of ABCB1 and acquire ABCB1 protein expression during epididymal transit.

ABCB1 protein and mRNA is inducible by industrial surfactants in epididymal principal cells.

Our findings suggest an important role of ABCB1 in epididymal barrier function, protection of spermatozoa from xenobiotics and sperm maturation. ABCB1 may be an important toxicokinetic regulator of AP and other xenobiotics in the adult rat epididymis. The results also suggest a novel process where in maturing sperm acquire efflux potential during epididymal transit.

CHAPTER 4 : SYNTHÈSE DU MÉMOIRE REDIGÉ EN FRANÇAIS

1.0 Introduction

1.1.0 L'épididyme

Les cellules germinales sont produites dans le testicule, mais c'est lors de leur passage dans l'épididyme qu'elles deviennent matures c'est à dire motiles et capables de féconder l'ovocyte (Orgebin-Crist, 1969). L'épididyme est un long tubule enroulé sur lui-même reliant les canaux efférents au canal déférent. Ce tubule comporte un épithélium hautement spécialisé qui est composé de plusieurs types cellulaires dont la répartition varie d'une région à l'autre de l'épididyme (Hermo *et al.*, 1992; Hinton and Palladino, 1995). Chez les rongeurs, l'épididyme peut être divisé en quatre régions: le segment initial, la tête, le corps et la queue (Robaire and Hermo, 1988). Chaque segment fournit un microenvironnement intraluminal spécifique qui est essentiel à la maturation des spermatozoïdes. On retrouve également des jonctions serrées entre les cellules épithéliales adjacentes. L'ensemble de ces jonctions constitue la barrière hémato-épididymaire (BEB) (Cyr *et al.*, 2002; Cyr *et al.*, 2007). Cette barrière, ainsi que différentes protéines de transport spécialisées, permettent de réguler l'entrée de composés dans la lumière de l'épididyme créant ainsi un milieu intraluminal dont la composition diffère de celle de la circulation sanguine. De plus, des réseaux d'artères pénètrent dans l'épididyme par l'intermédiaire du tissu connectif et sont reliés à un réseau de capillaires sanguins assurant ainsi un débit sanguin constant et l'apport de substances exogènes et endogènes.

La maturation épididymaire des spermatozoïdes est un processus dynamique au cours duquel les spermatozoïdes interagissent avec l'épithélium épididymaire et l'environnement intraluminal. Les spermatozoïdes sont considérés comme étant inactifs d'un point de vue transcriptionnel. L'épithélium épididymaire, qui est androgéno-dépendant, est responsable de la sécrétion de protéines qui, en interagissant avec les spermatozoïdes, induisent des modifications biochimiques. Une fois matures, les spermatozoïdes sont entreposés dans la queue de l'épididyme jusqu'au moment de

l'éjaculation. Lorsqu'ils sont éjaculés, les spermatozoïdes pénètrent dans le canal déférent, le conduit éjaculateur puis dans les organes génitaux externes.

1.1.1 Histologie de l'épididyme – Types cellulaires, distribution et fonction

Le type cellulaire le plus abondant dans l'épididyme est la cellule principale qui représente 65 à 80% de l'ensemble des cellules (Robaire and Hinton, 2001). Les cellules principales jouent un rôle dans la création du milieu intraluminal et dans la maturation des spermatozoïdes. En effet, ces cellules ont des importantes fonctions endocytiques et sécrétoires. La majorité des protéines découvertes à ce jour sont d'ailleurs sécrétées par les cellules principales. Les cellules principales contiennent différents organelles impliqués dans l'endocytose tels que des dépressions membranaires appelées « coated pits », des vésicules, des endosomes, des corps multivésiculaires pâles et denses et des lysosomes. Les cellules principales sont présentent dans toutes les régions de l'épididyme mais leur morphologie varie d'une région à l'autre. L'épaisseur de l'épithélium diminue des régions proximales aux régions distales de l'épididyme alors que le diamètre de la lumière augmente. Ces changements sont en partie dus aux modifications morphologiques des cellules principales. De plus, on observe également des changements dans le contenu lipidique et dans la composition en organelles endocytiques et sécrétoires.

Les cellules basales sont le deuxième type cellulaire le plus fréquent dans l'épididyme. Elles se retrouvent dans les différentes régions de l'épididyme. Cependant le nombre de ces cellules est plus élevé dans les régions proximales que distales (Robaire and Hermo, 1988; Robaire and Hinton, 2001). Les cellules basales ont une forme hémisphérique avec des prolongations cytoplasmiques qui parfois rejoignent la lumière de l'épididyme. La forme des cellules basales semblerait être régulée par le volume et la pression luminale. De plus, même si ces cellules ne sont pas sous le contrôle des androgènes, la castration provoque un changement de morphologie puisque ces cellules prennent une forme en dôme avec très peu de projections cytoplasmiques (Hermo and Papp, 1996). Cependant, le rôle des cellules basales est encore inconnu. La présence de dépressions membranaires, appelées «coated pits», à la surface de leur membrane

plasmique suggère un rôle dans l'endocytose médiée par des récepteurs. Il a aussi été suggéré que ces cellules seraient impliquées dans la protection contre les réactifs dérivés de l'oxygène (ROS) puisque la sous-unité Yb de la glutathion S-transférase (GST) est exprimée par les cellules basales dans l'épididyme de rat adulte (Andonian and Hermo, 1999). Cette hypothèse est confirmée par leur expression des métallothionéines (MT). Les MT sont des protéines cytosoliques impliquées dans la réponse au stress cellulaire (Cyr *et al.*, 2001). De plus, les cellules basales, par l'intermédiaire de la biosynthèse des prostaglandines, pourraient jouer un rôle dans la régulation paracrine du transport de l'eau et des électrolytes par les cellules principales (Leung *et al.*, 2004). Il a récemment été découvert que les cellules basales expriment les récepteurs à l'angiotensine II suggérant également un rôle dans l'acidification luminale (Shum *et al.*, 2008).

Les cellules claires sont de larges cellules endocytiques cuboïdes caractérisées par la présence de dépressions membranaires, d'endosomes, de lysosomes, de corps multivésiculaires et de gouttelettes lipidiques. Ces cellules sont présentes au niveau de la tête, du corps et de la queue de l'épididyme. Les propriétés endocytiques des cellules claires ont pu être démontrées par l'emploi de traceurs injectés dans la lumière de l'épididyme (Hermo *et al.*, 1988). Les cellules claires internalisent par endocytose de nombreuses protéines ainsi que la gouttelette cytoplasmique libérée par les spermatozoïdes au cours de leur transit dans l'épididyme (Hermo *et al.*, 1988; Hermo *et al.*, 1992; Vierula *et al.*, 1995). De plus, la présence d'une ATPase membranaire à protons suggère un rôle dans l'acidification luminale (Brown *et al.*, 1992), ce qui permettrait de maintenir les spermatozoïdes dans un état latent au cours de leur passage et de leur entreposage dans l'épididyme. Les cellules claires seraient également impliquées dans la détoxification épididymaire et dans la protection des spermatozoïdes contre les xénobiotiques; plusieurs membres de la famille des cytochromes P450, incluant CYP1A1 et CYP2E1 sont en effet exprimés par les cellules claires (Roman *et al.*, 1998; Forkert *et al.*, 2002).

Les cellules apicales ne représentent qu'un faible pourcentage des cellules épididymaires et se retrouvent uniquement dans le segment initial (Adamali and Hermo, 1996). Ces cellules sont situées à la bordure de la lumière de l'épididyme, mais

contrairement aux cellules basales, les cellules apicales ne traversent pas la BEB. Les cellules apicales possèdent peu de vésicules apicales et d'endosomes ainsi qu'un noyau situé du côté apical. Le rôle de ces cellules est peu connu. La présence de sous-unités GST semble cependant suggérer que ces cellules seraient impliquées dans la protection des spermatozoïdes contre les ROS (Adamali and Hermo, 1996; Andonian and Hermo, 2003).

Les cellules étroites ne sont présentes que dans les régions proximales de l'épididyme. Les cellules étroites ont un cytoplasme qui s'étend jusqu'à la membrane basale et qui contient un noyau de forme allongée situé du côté apical de l'épithélium. Les cellules étroites sont caractérisées par des vésicules apicales impliquées dans l'endocytose de protéines luminales et dans la régulation du pH intraluminal par l'anhydrase carbonique et l'ATPase à protons (Adamali and Hermo, 1996; Hermo *et al.*, 2000; Hermo *et al.*, 2005). De plus , ces cellules expriment également des unités GST, suggérant ainsi un rôle dans la protection contre les ROS (Adamali and Hermo, 1996).

Finalement, les cellules en halo sont de petites cellules caractérisées par un noyau dense localisé du côté basal, un cytoplasme claire et des granules denses (Robaire *et al.*, 2006). Les cellules en halo sont présentes le long de l'épididyme et auraient une fonction immunologique, elles ont été décrites comme des monocytes, des lymphocytes T ou B ou des éosinophiles (Serre and Robaire, 1999).

1.1.2 Modifications des spermatozoïdes au cours du transit épididymaire

Des changements morphologiques importants des cellules germinales ont lieu pendant et après la spermatogenèse. Au cours de la spermatogenèse, les cellules germinales passent de cellules rondes à des cellules à l'apparence unique possédant un noyau très condensé et un flagelle (Gatti *et al.*, 2004; Hermo *et al.*, 2010c). Par la suite, le passage des spermatozoïdes dans les canaux efférents permet de les concentrer grâce à la réabsorption du fluide luminal (Robaire and Hermo, 1988). Les spermatozoïdes subissent aussi de nombreux changements morphologiques et biochimiques dans l'épididyme afin qu'ils deviennent matures. Le changement le plus important consiste en la migration de la

gouttelette lipidique le long du spermatozoïde (Gatti *et al.*, 2004; Hermo *et al.*, 2010c). Cette gouttelette est finalement libérée au moment de l'entreposage dans la queue de l'épididyme ou au moment de l'éjaculation. Cependant, la présence de cette gouttelette au moment de l'éjaculation a été associée à une baisse de fertilité. De plus, on retrouve dans cette gouttelette un appareil de Golgi, ce qui suggère un rôle dans la glycosylation des protéines spermatiques au cours du transit épididymaire (Oko *et al.*, 1993).

La maturation des spermatozoïdes implique également plusieurs modifications biochimiques, incluant des changements de la composition en lipides et en cholestérol de la membrane plasmique des spermatozoïdes, des modifications des protéines de surface (relocalisation, addition, élimination), une augmentation en ponts disulfures ou de la charge nette de surface négative (Sullivan *et al.*, 2007). La perte de cholestérol et de lipides au cours du transit épididymaire serait un élément-clé de la maturation et de la capacitation des spermatozoïdes (de Lamirande *et al.*, 1997). Plusieurs accepteurs de cholestérol, incluant les lipoprotéines de haute densité (HDL) et les apolipoprotéines A-I (apoA-1), ont été identifiés dans le tractus reproducteur mâle (Morales *et al.*, 2008). De plus, les spermatozoïdes et l'épithélium épididymaire expriment différents transporteurs qui sont impliqués dans le transfert de stérols et de phospholipides aux accepteurs apo-I et HDL. On peut entre autres citer les membres de la famille des transporteurs ABC (ATP binding cassette) (Morales *et al.*, 2008).

1.1.4 Mécanismes de défense épididymaires

L'épididyme fournit un microenvironnement spécifique aux spermatozoïdes afin qu'ils deviennent matures. Plusieurs mécanismes de défense existent pour protéger les spermatozoïdes au cours de ce processus, incluant la présence d'une barrière, de protéines de transport spécifiques et d'enzymes métaboliques régulant le milieu intraluminal.

L'épithélium épididymaire contient plusieurs familles de protéines jonctionnelles qui sont essentielles au maintien de la fertilité (Dufresne et al., 2003; Cyr et al., 2007). Les jonctions serrées présentes entre les cellules principales adjacentes forment la BEB qui

contrôle l'entrée et la sortie des composants dans le milieu intraluminal assurant un milieu propice à la maturation et à la survie des spermatozoïdes. On retrouve entre autres les claudines et l'occludine au niveau de ces jonctions (Cyr et al., 2007). De plus, l'identification de différentes connexines dans l'épididyme de rat adulte suggère un rôle important de la communication intercellulaire dans le développement épididymaire et dans la maturation des spermatozoïdes (Dufresne et al., 2003). Les connexines sont localisées le long de l'épididyme soient entre les cellules principales ou entre les cellules principales et basales, étroites et apicales (Cyr et al., 1996; Dufresne et al., 2003). Finalement, on retrouve aussi dans l'épididyme des jonctions adhérentes, qui sont nécessaires pour la formation des jonctions serrées et communicantes (Citi, 1993; Pincon-Raymond, 2004; Yan and Cheng, 2005). Parmi les protéines d'adhésion cellulaire, plusieurs cadhérines et caténines sont exprimées dans l'épididyme (Mruk and Cheng, 2004; Cyr et al., 2007).

Il existe aussi des transporteurs membranaires de xénobiotiques qui régulent le type et la quantité de composés entrant dans le tractus reproducteur mâle. Plusieurs membres de la famille des transporteurs ABC, incluant ABCB1, ABCC1 et ABCG2, ont été identifiés dans l'épididyme. Ces transporteurs seront discutés ultérieurement de manière plus détaillée.

Tel qu'il a déjà été mentionné, plusieurs types cellulaires dans l'épididyme expriment des GST et des MT, suggérant un rôle dans les mécanismes de défense contre le stress cellulaire et les ROS. En plus de réguler la composition du milieu intraluminal de manière endocytique, les cellules claires expriment différentes enzymes métabolisant les médicaments (par exemple les CYP450s) suggérant également un rôle dans la détoxification épididymaire.

2.0 Fertilité masculine et polluants environnementaux

Il existe une inquiétude grandissante quant à l'effet des polluants environnementaux sur la fertilité masculine. L'infertilité masculine affecte environ 20% des couples souffrant de problèmes de fertilité, et dans la majorité des cas l'infertilité est dite idiopathique, c'est à

dire de cause inconnue. Des les années 1940, il a été rapporté que des expositions occupationnelles au DDT diminuaient les comptes de sperme. Une méta-analyse portant sur 15000 hommes entre 1938 et 1990 a révélé une baisse du compte et du volume de sperme qui pourrait être due à des polluants environnementaux (Carlsen *et al.*, 1992). Il est de plus en plus évident que plusieurs polluants ont la capacité d'affecter la fonction reproductrice mâle par différents mécanismes (Phillips and Tanphaichitr, 2008). Les perturbateurs endocriniens (ED) sont des composés capables de mimer, bloquer ou perturber l'action d'une hormone endogène. Il a d'abord été pensé que les ED agissaient par l'intermédiaire des récepteurs stéroïdiens. Récemment, il a été démontré que les ED pouvaient également agir de manière non-génomique, comme par exemple, en interférant avec la biosynthèse des stéroïdes ou le métabolisme. À cause de son androgéno-dépendance, le système reproducteur mâle est particulièrement vulnérable aux ED ayant des propriétés anti-androgéniques ou estrogéniques. Des anomalies histologiques et ultra structurales, incluant des altérations des épithéliums testiculaires et épididymaires, un nombre réduit ou un dysfonctionnement de la fonction des cellules de Sertoli et de Leydig font partie des conséquences d'un problème au niveau de la synthèse ou du métabolisme des androgènes. Les xénoestrogènes sont des substances ayant un potentiel estrogénique qui constitueraient une menace importante pour la fonction reproductrice. Plusieurs polluants environnementaux, incluant des dérivés de plastiques, des peintures, des pesticides et différents produits industriels, ont une structure chimique similaire aux hormones stéroïdiennes et sont capables de se lier aux récepteurs à l'estrogène (Kuiper *et al.*, 1997; Kuiper *et al.*, 1998; McKinney and Waller, 1998).

2.1.0 Les alkylphénols (AP) et les alkylphénols polyéthoxylés (APE)

Les AP sont des produits chimiques à haut volume utilisés dans une variété de secteurs industriels dont le textile, l'industrie papetière, l'industrie de l'acier, la production de pesticides, de peintures et de résines (Soares *et al.*, 2008). Ils sont utilisés entre autres comme surfactants, agents de mouillage et plastifiants. Les APE sont acheminés avec les eaux domestiques dans les stations d'épuration, et par la suite peuvent se biodégrader en composés ayant une solubilité et une hydrophobicité plus grandes. Les AP sont plus stables

que les APE dans l'environnement. Plusieurs de ces composés, tels que les nonylphénols polyéthoxylés (NPE) et les octylphénols polyéthoxylés (OPE) et leur produits de dégradation, le 4- nonylphénol (NP), le 4-tert-octylphénol (OP), et le bisphénol-A (BPA) se retrouvent à faibles concentrations dans les eaux usées, l'eau, les sédiments et l'air ambiant. De plus, on observe une bioaccumulation des AP chez les poissons et mammifères (Ying *et al.*, 2002).

2.1.1 Toxicité reproductrice des AP et des APE

La toxicité reproductrice des AP et des APE est controversée. Certains modèles animaux ont démontré une panoplie d'anomalies reproductrices. Plusieurs études démontrent que des fortes doses de OP, de OPE, de NP, de NPE et de BPA administrées à des rats par voie orale entraînent un changement de poids des testicules, des épididymes et de la prostate, des anomalies histologiques (par exemple des cellules germinales et somatiques dans le testicule), des altérations de la motilité des spermatozoïdes et de la production journalière de sperme (DSP) (Boockfor and Blake, 1997; Han *et al.*, 2004; Hamelin *et al.*, 2009). Différents données suggèrent que les effets des AP sur la fonction reproductrice sont reliés à leur estrogénicité et à leur capacité à inhiber les gonadotrophines (Han *et al.*, 2004). En effet, les AP et les APE possèdent des caractéristiques chimiques similaires à l'œstradiol et sont capables d'induire des réponses estrogéniques dans différents organismes biologiques (Nimrod and Benson, 1996). D'autres études suggèrent que les effets endocriniens des AP pourraient impliquer la perturbation des jonctions cellulaires. En effet, la communication intercellulaire est affectée dans une lignée de cellules de Sertoli exposée au NP (Aravindakshan and Cyr, 2005). Des changements d'expression et de localisation de plusieurs protéines jonctionnelles ont également été observés dans une lignée de cellules de Sertoli exposée à l'OP et au BPA (Fiorini *et al.*, 2004).

Même si les surfactants industriels sont considérés comme des polluants environnementaux affectant la fonction reproductrice mâle in vivo (Boockfor and Blake, 1997; Han *et al.*, 2004) et in vitro (Aravindakshan and Cyr, 2005), des études in vivo

récentes utilisant des doses environnementales pertinentes suggèrent que certains alkylphénols tel que le OP seraient moins toxiques que ce qui a été proposé au départ (Gregory *et al.*, 2009). L'analyse toxicocinétique de rats adultes mâles a démontré une élimination rapide de cette substance dans l'épididyme (Hamelin *et al.*, 2009). De plus, contrairement à des études antérieures, peu de toxicité reproductrice a été observée à des doses environnementales pertinentes suggérant l'implication de mécanismes de défense dans l'épididyme (Gregory *et al.*, 2009; Hamelin *et al.*, 2009).

2.1.2 Le nonylphénol (NP)

NP est un composé organique synthétique qui sert de précurseur dans la fabrication des NPE. Les NPE sont biodégradés par des microorganismes en produits plus stables et plus toxiques. Les NPE sont largement utilisés comme surfactants dans l'industrie.

2.1.3 Le NP dans l'environnement

Le NP et les NPE sont libérés dans l'environnement par l'intermédiaire des eaux usées. Dans plusieurs pays, la concentration de NP a été déterminée dans les rivières, les lacs et sur le bord des côtes. De hauts niveaux de NP (654 µg/L (2.97 µM), 53 µg/L (0.24 µM) et 95 µg/L(0.43 µM)) ont été décelés en Espagne, au Royaume-Uni et aux États-Unis, respectivement (Vasquez-Duhalt *et al.*, 2005). Au Canada, des concentrations s'élevant jusqu'à 4.25 µg/L (19 nM), 0.06 µg/L (0.27 nM) et 0.98 µg/L (4.46 nM) ont été détectées dans les rivières, les lacs et les ports, respectivement (Soares *et al.*, 2008). De plus, des concentrations de NPE aussi élevées que 17.6 µg/L (0.079 µM) étaient présentes dans certaines rivières. Il n'existe cependant aucune donnée sur la concentration de NP et de NPE dans le sol.

2.1.4 Le NP- Exposition humaine

L'exposition humaine au NP et aux NPE peut arriver dans l'environnement mais aussi par l'utilisation de produits en contenant. Dans l'environnement, l'exposition peut se

faire par l'ingestion de substances contaminées, par inhalation ou par voie cutanée. Clark *et al.* (1992) ont rapporté des concentrations de NPE aussi élevées que 0.129 µg/L (0.5 nM) dans l'eau potable aux États-Unis. Il existe peu d'information sur la présence de NP et de NPE dans la nourriture. Une étude a cependant déterminé la présence de NP dans plusieurs produits alimentaires avec des concentrations variant entre 0.1-19.4 µg/Kg (Guenther *et al.*, 2002).

Des études se sont intéressées à la présence de NP et de NPE dans les tissus et les fluides humains. Une étude suisse portant sur 25 individus a décelé des taux de NP variant entre 19.8 ng/g et 84.4 ng/g dans les lipides alors que les taux de NPE étaient situés sous le seuil de détection (Muller 1997). La présence de plusieurs AP et APE a également été étudiée en Italie dans le lait maternel et le niveau de NP était deux fois supérieur à n'importe quel autre composé avec une concentration moyenne de 32 ng/ml (~0.145 µM) (Ademollo *et al.*, 2008). De plus, des taux de NP de 1.51 mg/L (6.85 µM) et de 1.12 mg/L (5.08 µM) ont été respectivement décelés dans le lait maternel et dans le cordon ombilical de nouveau-nés (Lin *et al.*, 2008).

3.0 Toxicologie et transporteurs

De nombreux facteurs peuvent affecter la réponse d'un individu à un agent thérapeutique. La toxicocinétique concerne le devenir d'une substance chimique avec le temps dans des organismes vivants. La toxicocinétique se base sur quatre grands principes: l'absorption, la distribution, la biotransformation et l'excrétion. Les études de toxicocinétique comprennent plusieurs variables incluant les caractéristiques chimiques du composé, la voie d'entrée et la variabilité interindividuelle. Les voies d'entrée et la lipophilicité du produit peuvent également affecter son absorption et sa distribution dans l'organisme vivant. En général, les composés liposolubles sont plus facilement absorbés par la peau, les systèmes digestif et respiratoire. D'autres substances vont être métabolisées dans le foie et vont alors devenir plus toxiques.

Cependant, chez les mammifères, il existe différents mécanismes de défense qui permettent d'atténuer l'absorption de ces substances et d'en faciliter l'excrétion. Ces lignes de défense incluent notamment les barrières cellulaires. En effet, les jonctions serrées présentes entre les entérocytes dans le petit intestin et dans le côlon, entre les cellules endothéliales dans les vaisseaux sanguins et entre les cellules épithéliales dans différents organes permettent de créer une barrière contre les produits toxiques. Les enzymes métabolisant les xénobiotiques dans le foie permettent aussi de diminuer la biodisponibilité des produits toxiques en augmentant leur hydrophilicité. Cependant, l'entrée par voie intraveineuse, intra péritonéale, intramusculaire et par inhalation permet de contourner ces défenses et de pénétrer directement dans le système sanguin. Les protéines de transport constituent alors une autre ligne de défense contre ces substances toxiques en travaillant de concert avec les barrières cellulaires pour réduire la toxicité des substances. Leur présence dans les vaisseaux sanguins et dans les épithéliums permet de minimiser le transfert de substances toxiques aux différents organes et tissus sensibles et, à l'intérieur des organes et de tissus, ces protéines facilitent leur élimination par sécrétion et par excrétion.

3.1.0 La famille des transporteurs ABC

Les transporteurs ABC (ATP-binding cassette) sont des protéines transmembranaires capables de réguler les concentrations intracellulaires de nombreux substrats, incluant des médicaments, des substances toxiques, des protéines, des peptides et des lipides, en utilisant l'énergie fournie par l'hydrolyse de l'ATP (Leslie *et al.*, 2001; Seeger and van Veen, 2009; Klaassen and Aleksunes, 2010). Ces transporteurs possèdent deux domaines transmembranaires (MD) et deux domaines de liaison nucléotidique (NBD) dont la séquence est très conservée par les membres de cette famille de transporteurs. Par contre, la séquence des MD varie et détermine ainsi la spécificité du substrat. Près de 51 membres de la famille des transporteurs ABC ont été identifiés (chez les rongeurs) et peuvent être classés selon l'homologie et la topologie de leur séquence (Table 1) en 7 sous-familles (ABCA-ABCG). Ces transporteurs sont impliqués dans différents processus physiologiques notamment au niveau des barrières cellulaires et de la détoxification par excrétion de médicaments et de composés toxiques.

3.1.1 ABCB1

ABCB1 (aussi connu sous le nom de P-glycoprotéine, P-gp, MDR1) est codée par le gène ABCB1. Il s'agit d'une protéine transmembranaire de la membrane plasmique (170 kDa) dont la localisation apicale permet d'expulser différents substrats. À l'origine, ABCB1 a été identifiée comme une phosphoglycoprotéine exprimée par des cellules ovariennes résistantes aux médicaments (Juliano and Ling, 1976). Sa surexpression permet une multi-résistance aux médicaments dans plusieurs types de cancers. Elle a par la suite été identifiée dans différents tissus normaux (Staud *et al.*, 2010) On retrouve un gène ABCB1 chez l'homme et deux chez les rongeurs, ABCB1a et ABCB1b. ABCB1a et ABCB1b présentent une homologie de séquence d'acides aminés de 85%, dépassant celle avec ABCB1 chez l'humain (80%) (Leslie *et al.*, 2001). ABCB1 est un important régulateur toxicocinétique d'une variété de xénobiotiques. Sa localisation limite leur absorption et en facilite l'excrétion.

3.1.2 Substrats, inhibiteurs et inducteurs

ABCB1 est capable de transporter divers substrats lipophiliques et amphipatiques. Il s'agit d'un important régulateur pharmacocinétique et toxicocinétique de substances pharmaceutiques et de polluants environnementaux. Il est intéressant de noter qu'ABCB1 partagent un large éventail de substrats avec les enzyme métabolisantes CYP3A suggérant une coordination dans leurs fonctions au niveau de la distribution et de la détoxification des xénobiotiques (Fromm, 2004). L'activité constitutive d'ATPase d'ABCB1 et sa capacité à transporter de nombreuses molécules endogènes, incluant des stérols, des lipides et des protéines, suggère également un rôle important dans le fonctionnement physiologique normal. De plus, la capacité d'ABCB1 à transporter plusieurs colorants, dont la calcéine, la rhodamine-123, le Hoescht 33342, et des drogues radioactives facilite son étude. Plusieurs essais commerciaux utilisent la calcéine et la rhodamine-123 pour évaluer la fonction d'ABCB1.

Plusieurs modulateurs d'ABCB1 ont été développés en vue d'une application clinique. Ces modulateurs ont été créés pour contourner le problème de la chimiorésistance ou pour moduler le profil pharmaco- ou toxicocinétique de certaines substances. Les inhibiteurs d'ABCB1 sont utilisés pour passer les barrières cellulaires et augmenter la biodisponibilité de différents composés (la ciclosporine (CsA), le vérapamil). De plus, plusieurs composés (substances thérapeutiques, polluants environnementaux, molécules endogènes) sont capables d'induire l'expression d'ABCB1 et d'en augmenter la fonctionnalité.

3.1.3 Régulation

L'induction d'ABCB1 dans des tissus normaux et cancéreux est un processus complexe et multifactoriel. L'induction d'ABCB1 implique l'activation de plusieurs facteurs de transcription, mais aussi des modifications épigénétiques et différentes voies de signalisation (Callaghan *et al.*, 2008). Les récepteurs nucléaires, CAR (constitutive androstane receptor) et PXR (Pregnane X receptor), sont capables d'induire la surexpression d'ABCB1 pour éliminer des substances potentiellement toxiques. Le stress cellulaire induit par l'environnement des cellules cancéreuses ou par la présence de fortes concentrations de composés toxiques induisent également l'expression d'ABCB1. Il a été démontré que le choc thermique, les UV et les dommages à l'ADN régulent la transcription d'ABCB1 par l'intermédiaire des facteurs de transcription HSF1, NF-kB et C/EBPb, et P53, respectivement. Le stress cellulaire engendré par les composés chimiothérapeutiques ou les polluants environnementaux engendre une hausse des niveaux endogènes de ROS, entraînant des changements compensatoires dans les systèmes antioxydants (Callaghan *et al.*, 2008). Le stress oxydatif peut provoquer l'activation de plusieurs voies de signalisation, incluant NF-kB, MAPK et PKC, et peut ainsi mener à la mort cellulaire. L'activation transitoire des voies de signalisation impliquées dans le stress cellulaire peut également contribuer à augmenter l'expression d'ABCB1. De nombreux polluants environnementaux, incluant des pesticides, des surfactants et des métaux provoquent la production de ROS *in vitro* et *in vivo* (Mena *et al.*, 2009). Il a d'ailleurs été suggéré qu'un débalancement dans le

statut oxydatif serait impliqué dans le déclin de la fertilité masculine (Sheweita *et al.*, 2005).

3.1.4 Expression et rôle dans les tissus normaux

ABCB1 est localisé du côté apical des cellules épithéliales, permettant la translocation des substrats du domaine basolatéral vers le domaine apical. Ce transporteur est impliqué dans la fonction de barrière et dans la sécrétion. En travaillant de concert avec les barrières cellulaires et certaines enzymes, ABCB1 régule l'entrée, la distribution et la biodisponibilité de plusieurs substances toxiques ou thérapeutiques. Les animaux déficients en ABCB1 permettent de mieux comprendre le rôle de ce transporteur. Même si ces animaux sont viables et fertiles, ils sont plus sensibles aux insultes chimiques et ont une biodisponibilité plus élevée des produits qui leur sont administrés. De plus, les souris déficientes en ABCB1a présentent des symptômes inflammatoires similaires à ceux retrouvés chez les patients souffrant de la maladie de Bowel, suggérant un rôle dans l'homéostasie intestinale (Panwala *et al.*, 1998). Cette inflammation serait le résultat de l'accumulation de toxines bactériennes qui sont normalement expulsées par ABCB1a.

Les cellules épithéliales sont liées par des jonctions serrées qui permettent de réguler le transport paracellulaire et de maintenir la polarité cellulaire. Les substrats d'ABCB1 sont généralement hydrophobiques et peuvent plus facilement traverser les membranes cellulaires. Des niveaux élevés d'ABCB1 permettent de limiter la diffusion passive des médicaments et des substances toxiques entre la circulation sanguine et les compartiments internes.

3.1.5 Expression dans le tractus reproducteur mâle

Plusieurs membres de la famille des transporteurs ABC sont exprimés dans le testicule et l'épididyme, notamment ABCB1, ABCC1 et ABCG2. ABCB1 a été originellement identifiée dans des testicules de hamsters chinois, suggérant un rôle dans le système reproducteur mâle (Baas and Borst, 1988). Plus tard, ABCB1 a été localisée dans

les vaisseaux sanguins du testicule chez l'homme et les rongeurs, ainsi que dans les cellules de Leydig et comme un élément de la barrière hémato-testiculaire (BTB) (Melaine *et al.*, 2002; Bart *et al.*, 2004; Su *et al.*, 2009). La localisation d'ABCB1 au cours de la spermatogenèse est cependant controversée à cause des différences de résultats obtenus avec différents anticorps et protocoles d'immuno-détection. Melaine *et al., (2002)* ont retrouvé ABCB1 au niveau des spermatides tardifs mais pas au niveau des cellules germinales mitotiques et méiotiques et de la BTB. Par contre, Su *et al.*, (2009) observent l'expression de ABCB1 à l'interface entre les cellules de Sertoli et les spermatides allongés et au niveau de la BTB. Le rôle protecteur de ABCB1 a été confirmé par la création d'animaux génétiquement modifiés (Schinkel *et al.*, 1995b). Il existe cependant très peu d'information sur son rôle dans l'épididyme adulte chez les mammifères.

3.1.6 ABCB1 et la fertilité masculine

La spermatogenèse et la maturation post-testiculaire nécessitent la présence de microenvironnements spécialisés. ABCB1 participe à ces processus en limitant l'exposition des spermatozoïdes à différentes substances potentiellement toxiques dans la lumière du testicule ou de l'épididyme. Les animaux déficients en ABCB1 ont d'ailleurs des niveaux testiculaires plus élevés des composés administrés et sont donc plus susceptibles à la toxicité (Schinkel *et al.*, 1995b). Le bon déroulement de la spermatogenèse dépend des cellules de Sertoli et de Leydig. La présence d'ABCB1 pourrait être nécessaire pour assurer la viabilité des cellules somatiques.

Récemment, un lien entre l'infertilité masculine et un polymorphisme d'ABCB1 a été démontré, suggérant un rôle pour ABCB1 dans le maintien de la fertilité en protégeant les spermatozoïdes des attaques de composés toxiques (Drozdzik *et al.*, 2009). ABCB1 limiterait l'entrée et l'accumulation des toxines environnementales, des carcinogènes et des métabolites dans le testicule. Les individus présentant un polymorphisme d'ABCB1 seraient plus susceptibles au stress environnemental et à la toxicité reproductrice. ABCB1 pourrait également jouer un rôle dans la régulation de la fonction reproductrice puisque les stérols font partie de ses substrats (Garrigues *et al.*, 2002).

3.1.7 Autres transporteurs ABC dans le tractus reproducteur mâle

ABCC1 (MRP1) est un orthologue du ABCB1 humain présent chez les rongeurs (Leslie *et al.*, 2001). Il est exprimé dans plusieurs tissus normaux dont les poumons, le tube digestif, le foie, le rein et le placenta. ABCC1 est localisé du côté basolatéral et transporte donc des substrats de l'épithélium vers le sang. ABCC1 transporte principalement des substances conjuguées au GSH, au glucuronate et au sulfate. Cela inclut des produits de la peroxydation des lipides, des pesticides, des dérivés du tabac, des métaux lourds et des substances pharmaceutiques. Il existe cependant des variations de substrats entre l'humain et les rongeurs.

De hauts niveaux d'expression d'ABCC1 ont été rapportés dans le testicule humain et chez les rongeurs, suggérant un rôle dans la fonction reproductrice mâle. ABCC1 est exprimé par les cellules de Leydig et de Sertoli (Bart *et al.*, 2004). Dans les cellules de Leydig, ABCC1 est entre autres co-localisé avec l'estrogène sulfotransférase suggérant un rôle dans le maintien des niveaux faibles en estrogène dans le testicule (Leslie *et al.*, 2001). Les souris déficientes en ABCC1 ont des dommages plus élevés reliés à l'étoposide dans la cavité oropharyngée et dans les tubules séminifères du testicule (Wijnholds *et al.*, 1998). Il n'existe aucune donnée sur la présence d'ABCC1 dans l'épididyme.

ABCG2 (breast cancer resistance protein, BCRP, MXR) a été originellement isolé à partir de cellules cancéreuses du sein résistantes aux médicaments. La surexpression d'ABCG2 confère la résistance à plusieurs substances thérapeutiques, dont les anthracyclines, le mitoxantrone et les inhibiteurs de la topoisomérase (Mao and Unadkat, 2005). ABCG2 est exprimé dans de nombreux organes, notamment par les cellules endothéliales de la barrière hémato-encéphalique, -testiculaire, -épididymaire et – placentaire, par les cellules épithéliales du petit intestin, du colon, du foie et du rein. Par contre, ABCG2 ne contient qu'un domaine de liaison nucléotidique et qu'un domaine transmembranaire. Il semblerait qu'ABCG2 forme des dimères (homo- ou hétéro-) avec les substrats. Ces substrats incluent des agents chimiothérapeutiques, mais aussi des anions organiques.

Dans le système reproducteur mâle, ABCG2 est localisé à la bordure de la lumière des vaisseaux sanguins du testicule et de l'épididyme, dans les cellules myoides entourant les tubules séminifères et à la surface de la lumière des régions proximales de l'épididyme. Une étude récente démontre sa présence sur l'acrosome de spermatozoïdes matures murins (Scharenberg *et al.*, 2009). L'administration de phytoestrogènes à des souris déficientes en ABCG2 entraîne des concentrations épididymaires élevées de phytoestrogènes comparativement à des souris normales suggérant un rôle dans la protection de l'épididyme et dans la maturation des spermatozoïdes (Enokizono *et al.*, 2007).

4.0 Hypothèse de recherche

Étant donné le rôle d'ABCB1 au niveau de différentes barrières cellulaires, dont la barrière hémato-testiculaire, nous avons émis l'hypothèse qu'ABCB1 jouerait un rôle au niveau de la barrière hémato-épididymaire et serait impliqué dans la protection des spermatozoïdes. Les objectifs de cette étude consistaient donc à établir le patron d'expression, le rôle et l'inductibilité d'ABCB1 dans l'épididyme du rat adulte tout en vérifiant si le NP, pouvait induire l'expression d'ABCB1 dans des cellules épididymaires de rat.

5.0 Résultats

Expression et localisation d'ABCB1 dans l'épididyme de rat adulte

Les niveaux d'expression relative des ARNm d'ABCB1a et d'ABCB1b ont été déterminés par RT-PCR en temps réel. Les transcrits des deux orthologues sont exprimés dans les quatre régions de l'épididyme du rat adulte. De plus, ABCB1a est plus fortement exprimé qu'ABC1b le long de l'épididyme. Même si l'expression d'ABCB1a semble plus élevée dans la queue de l'épididyme que dans les régions proximales, ces résultats ne sont pas statistiquement significatifs. Une étude d'immunolocalisation a démontré un gradient d'expression d'ABCB1 le long de l'épididyme. ABCB1 n'est pas exprimé dans le segment

initial, très peu dans la tête par les cellules épithéliales et par les spermatozoïdes. Son expression augmente progressivement dans la tête distale, le corps et la queue. Dans le corps et la queue, ABCB1 est majoritairement exprimé par les cellules principales. Une sous-population de cellules claires l'exprime aussi dans la queue. L'apparition de l'expression d'ABCB1 dans l'épithélium épididymaire est accompagnée d'une hausse d'expression par les spermatozoïdes (dans le noyau et le flagelle), suggérant qu'ABCB1 est acquis par les spermatozoïdes au cours du transit épididymaire. Ces résultats ont été confirmés par immunobuvardage de type western.

Étude de la fonctionnalité d'ABCB1 dans une lignée cellulaire et dans les spermatozoïdes de l'épididyme de rat

Pour déterminer si ABCB1 joue un rôle dans l'épididyme de rat, des essais MDR ont été effectués avec une lignée cellulaire d'épididyme de rat (RCE) et des spermatozoïdes des régions proximales et distales de l'épididyme de rat. Les deux types cellulaires présentent un phénotype MDR qui peut être inhibé dans des conditions contrôles. La faible et la moyenne dose de CsA augmente significativement la fluorescence de la calcéine dans les cellules RCE, démontrant une activité d'ABCB1. La plus haute dose de CsA n'a pas induit de changements significatifs. L'inhibiteur d'ABCC1, MK571 a significativement augmenté la fluorescence de la calcéine dans les cellules RCE de manière dose-dépendante suggérant que ce transporteur est aussi actif dans les cellules RCE. Une hausse significative et dose-dépendante de la fluorescence de la calcéine a également été observée dans les spermatozoïdes (provenant des régions proximales et distales de l'épididyme) traités avec l'inhibiteur d'ABCB1, le vérapamil. Par contre, les spermatozoïdes de la queue de l'épididyme traités avec MK571 démontrent une baisse dose-dépendante de fluorescence de la calcéine suggérant une perte de la viabilité cellulaire ou de la fonction.

Induction de l'ARNm et de la protéine d'ABCB1 dans les cellules RCE

Afin de déterminer si ABCB1 est inductible dans l'épithélium épididymaire, l'expression de l'ARNm et de la protéine a été vérifiée dans les cellules RCE exposées

pendant 24h à différentes concentrations de l'inducteur d'ABCB1, DOX, et au NP. L'expression de l'ARNm d'ABCB1a augmente à la plus forte dose de DOX (500 ng/ml), mais de manière non significative alors que l'ARNm d' ABCB1b augmente 6 fois plus dans les cellules traitées à la DOX (500 ng/ml), comparativement aux cellules non traitées. Une augmentation de l'ARNm d'ABCB1a et de la protéine ABCB1 a été observée respectivement dans les cellules traitées avec 10 µM et avec 20 µM NP. Une hausse d'expression de l'ARNm d'ABCB1b a également été observée à la plus forte dose de NP (20 µM). Aucun changement significatif n'a été observé pour ABCC1 ou ABCG2.

6.0 Discussion

Il a récemment été montré que l'administration d'OP par voie orale ou intraveineuse entraîne des niveaux élevés d'OP dans les tissus reproducteurs mâles, qui sont par la suite rapidement éliminés (Gregory *et al.*, 2009; Hamelin *et al.*, 2009). Notre hypothèse était que des transporteurs spécialisés, tel qu'ABCB1, étaient impliqués dans l'élimination des alkylphénols et dans la protection des spermatozoïdes dans l'épididyme. Nos résultats ont démontré qu'ABCB1 est exprimé et joue un rôle dans les cellules épithéliales et dans les spermatozoïdes de l'épididyme. Des différences d'expression entre les tissus et le sexe ont été observées chez la souris (Cui *et al.*, 2009). Nos résultats suggèrent qu'ABCB1a est la forme dominante dans l'épididyme du rat adulte, mais que les deux formes d'ABCB1 pourraient jouer un rôle dans la fonction de la BEB. Tel que démontré dans d'autres systèmes, ABCB1 est exprimé par les capillaires sanguins de l'épididyme. La présence apicale d'ABCB1 dans les cellules endothéliales permet de limiter l'entrée des xénobiotiques dans les tissus. ABCB1 était également présent du côté apical de l'épithélium épididymaire du corps et de la queue suggérant un rôle dans la défense épididymaire. D'autres études ont suggéré qu'ABCB1 permet de pomper du côté basal vers le côté apical donc en direction de la lumière de l'épididyme. Les composés capables de traverser la barrière endothéliale peuvent donc être activement transportés dans la lumière de l'épididyme. Les spermatozoïdes présents dans la lumière de l'épididyme peuvent alors être exposés à différentes substances toxiques. Des métaux lourds, des

pesticides et des substances industrielles ont d'ailleurs été détectés dans la semence (Stachel *et al.*, 1989).

ABCB1 est exprimé par les cellules claires dans l'épididyme du rat adulte. Il s'agit de cellules impliquées dans l'endocytose (Hermo *et al.*, 1988). L'expression d'ABCB1 pourrait être le résultat de l'endocytose de composants spermatiques et de débris cellulaires exprimant ABCB1. Cependant, les cellules claires expriment également plusieurs enzymes métabolisant des xénobiotiques dont CYP1A1 et CYP2E1 (Roman *et al.*, 1998; Forkert *et al.*, 2002). De plus, ABCB1 et les CYP450 partagent plusieurs substrats (Staud *et al.*, 2010). Une régulation coordonnée d'ABCB1 et des CYP450 a été observée dans d'autres tissus (Xu *et al.*, 2005). Les cellules claires pourraient donc travailler en concert avec des enzymes métabolisant des xénobiotiques pour transformer ces substances en formes plus solubles. De plus, il existe un gradient d'expression de la protéine ABCB1 dans l'épithélium et les spermatozoïdes de l'épididyme suggérant un rôle dans la défense épididymaire mais aussi dans les fonctions physiologiques de l'épididyme. De plus, les stérols endogènes font partie des substrats d'ABCB1 (Garrigues *et al.*, 2002). Il est intéressant que le gradient d'expression d'ABCB1 coïncide avec les changements de composition en stérols dans la lumière, les cellules épithéliales et les spermatozoïdes. Les pertes de cholestérol et de lipides font partie des modifications essentielles pour la capacitation et pour la réaction acrosomale des spermatozoïdes dans le tractus reproducteur femelle (de Lamirande *et al.*, 1997). ABCB1 pourrait donc être impliqué dans la régulation des concentrations de certains composés dans la lumière de l'épididyme et leurs interactions avec l'épithélium. Des niveaux élevés de métabolites de vitamine D3 ont été détectés dans la queue de l'épididyme du rat adulte (Kidroni *et al.*, 1983). ABCB1 module aussi l'efflux de dihydrotestostérone (DHT) et active l'expression génique de manière androgéno-dépendante dans des cellules cancéreuses de la prostate (Fedoruk *et al.*, 2004). L'expression d'ABCB1 pourrait également influencer l'activation de gênes régulés par les hormones de manière région-spécifique.

La présence d'ABCB1 au niveau des spermatozoïdes, des cellules transcriptionnellement inertes, est particulièrement intéressant. Des transporteurs de la

carnitine (OCTN), impliqués dans l'acquisition de la motilité, ont été détectés sur les spermatozoïdes épididymaires avec un gradient d'expression similaire à ABCB1 (Kobayashi *et al.*, 2007). Il est d'ailleurs connu que plusieurs protéines sont transférées de l'épididyme aux spermatozoïdes (Sullivan *et al.*, 2007). Il se pourrait aussi qu'une forme non fonctionnelle d'ABCB1 soit exprimée par les spermatozoïdes testiculaires, et que celle-ci est démasquée au moment du transit épididymaire. ABCB1 représente ainsi un moyen unique pour les spermatozoïdes pour se défendre contre certains polluants environnementaux.

L'induction de l'ARNm et de la protéine d'ABCB1 par la DOX et le NP, de façon dose-dépendante, suggère qu'il existe un mécanisme de défense en réponse à une insulte chimique. Même s'il a été montré le quels des NPE modulent l'efflux médié par ABCB1 (Doo *et al.*, 2005), il s'agit de la première étude qui prouve qu'un AP induit ABCB1. Ces données pourraient expliquer pourquoi l'OP est rapidement éliminé de la lumière de l'épididyme d'animaux traités (Hamelin *et al.*, 2009). De plus, les cellules RCE expriment également ABCC1 et ABCG2 au niveau de l'ARNm même si ces protéines n'ont pas été induites par un traitement à la DOX et au NP, ce qui suggère que d'autres membres de la famille des transporteurs ABC pourraient jouer un rôle dans l'épididyme.

7.0 Conclusions

Nos résultats indiquent qu'ABCB1 est un important régulateur toxicocinétique de l'AP et autres xénobiotiques dans l'épididyme du rat adulte. Il s'agit de la première étude à démontrer l'expression d'ABCB1 dans l'épididyme de rat adulte et son rôle potentiel dans la fonction de la BEB et dans la maturation des spermatozoïdes. Il s'agit pour les spermatozoïdes d'un mécanisme de défense unique contre les attaques chimiques. Des études additionnelles dans ce domaine permettraient de comprendre comment des substances thérapeutiques et toxiques peuvent pénétrer le tractus reproducteur mâle et affecter la fonction reproductrice.

BIBLIOGRAPHY

Adamali, H. I., and Hermo, L. (1996). Apical and narrow cells are distinct cell types differing in their structure, distribution, and functions in the adult rat epididymis. *J Androl* **17**, 208-222.

Ademollo, N., Ferrara, F., Delise, M., Fabietti, F., and Funari, E. (2008). Nonylphenol and octylphenol in human breast milk. *Environ Int* **34**, 984-987.

Agarwal, A., and Hoffer, A. P. (1989). Ultrastructural studies on the development of the blood-epididymis barrier in immature rats. *J Androl* **10**, 425-431.

Agarwal, A., and Saleh, R. A. (2002). Role of oxidants in male infertility: rationale, significance, and treatment. *Urol Clin North Am* **29**, 817-827.

Aitken, R. J. (2000). Possible redox regulation of sperm motility activation. *J Androl* **21**, 491-496.

Aitken, R. J., Nixon, B., Lin, M., Koppers, A. J., Lee, Y. H., and Baker, M. A. (2007). Proteomic changes in mammalian spermatozoa during epididymal maturation. *Asian J Androl* **9**, 554-564.

Aitken, R. J., Paterson, M., Fisher, H., Buckingham, D. W., and van Duin, M. (1995). Redox regulation of tyrosine phosphorylation in human spermatozoa and its role in the control of human sperm function. *J Cell Sci* **108 (Pt 5)**, 2017-2025.

Andonian, S., and Hermo, L. (1999). Immunocytochemical localization of the Ya, Yb1, Yc, Yf, and Yo subunits of glutathione S-transferases in the cauda epididymidis and vas deferens of adult rats. *J Androl* **20**, 145-157.

Andonian, S., and Hermo, L. (2003). Immunolocalization of the Yb1 subunit of glutathione S-transferase in the adult rat epididymis following orchidectomy and efferent duct ligation. *J Androl* **24**, 577-587.

Aravindakshan, J., and Cyr, D. G. (2005). Nonylphenol alters connexin 43 levels and connexin 43 phosphorylation via an inhibition of the p38-mitogen-activated protein kinase pathway. *Biol Reprod* **72**, 1232-1240.

Aydogan, M., Korkmaz, A., Barlas, N., and Kolankaya, D. (2010). Pro-oxidant effect of vitamin C coadministration with bisphenol A, nonylphenol, and octylphenol on the reproductive tract of male rats. *Drug Chem Toxicol* **33**, 193-203.

Baas, F., and Borst, P. (1988). The tissue dependent expression of hamster P-glycoprotein genes. *FEBS Lett* **229**, 329-332.

Baccetti, B., Strehler, E., Capitani, S., Collodel, G., De Santo, M., Moretti, E., Piomboni, P., Wiedeman, R., and Sterzik, K. (1997). The effect of follicle stimulating hormone therapy on human sperm structure (Notulae seminologicae 11). *Hum Reprod* **12**, 1955-1968.

Bart, J., Hollema, H., Groen, H. J., de Vries, E. G., Hendrikse, N. H., Sleijfer, D. T., Wegman, T. D., Vaalburg, W., and van der Graaf, W. T. (2004). The distribution of drug-efflux pumps, P-gp, BCRP, MRP1 and MRP2, in the normal blood-testis barrier and in primary testicular tumours. *Eur J Cancer* **40**, 2064-2070.

Baumert, C., and Hilgeroth, A. (2009). Recent advances in the development of P-gp inhibitors. *Anticancer Agents Med Chem* **9**, 415-436.

Boockfor, F. R., and Blake, C. A. (1997). Chronic administration of 4-tert-octylphenol to adult male rats causes shrinkage of the testes and male accessory sex organs,

disrupts spermatogenesis, and increases the incidence of sperm deformities. *Biol Reprod* **57**, 267-277.

Borst, P., Evers, R., Kool, M., and Wijnholds, J. (1999). The multidrug resistance protein family. *Biochim Biophys Acta* **1461**, 347-357.

Borst, P., Evers, R., Kool, M., and Wijnholds, J. (2000). A family of drug transporters: the multidrug resistance-associated proteins. *J Natl Cancer Inst* **92**, 1295-1302.

Breucker, H., Schafer, E., and Holstein, A. F. (1985). Morphogenesis and fate of the residual body in human spermiogenesis. *Cell Tissue Res* **240**, 303-309.

Brown, D., Lui, B., Gluck, S., and Sabolic, I. (1992). A plasma membrane proton ATPase in specialized cells of rat epididymis. *Am J Physiol* **263**, C913-916.

Brown, P. D., and Waites, G. M. (1972). Regional blood flow in the epididymis of the rat and rabbit: effect of efferent duct ligation and orchidectomy. *J Reprod Fertil* **28**, 221-233.

Callaghan, R., Crowley, E., Potter, S., and Kerr, I. D. (2008). P-glycoprotein: so many ways to turn it on. *J Clin Pharmacol* **48**, 365-378.

Callewaert, F., Boonen, S., and Vanderschueren, D. (2010). Sex steroids and the male skeleton: a tale of two hormones. *Trends Endocrinol Metab* **21**, 89-95.

Carlsen, E., Giwercman, A., Keiding, N., and Skakkebaek, N. E. (1992). Evidence for decreasing quality of semen during past 50 years. *BMJ* **305**, 609-613.

Carr, D. W., Usselman, M. C., and Acott, T. S. (1985). Effects of pH, lactate, and viscoelastic drag on sperm motility: a species comparison. *Biol Reprod* **33**, 588-595.

Carreau, S. (1996). Paracrine control of human Leydig cell and Sertoli cell functions. *Folia Histochem Cytobiol* **34**, 111-119.

Charuk, M. H., Grey, A. A., and Reithmeier, R. A. (1998). Identification of the synthetic surfactant nonylphenol ethoxylate: a P-glycoprotein substrate in human urine. *Am J Physiol* **274**, F1127-1139.

Chausiaux, O. E., Abel, M. H., Baxter, F. O., Khaled, W. T., Ellis, P. J., Charlton, H. M., and Affara, N. A. (2008). Hypogonadal mouse, a model to study the effects of the endogenous lack of gonadotropins on apoptosis. *Biol Reprod* **78**, 77-90.

Cheng, C. Y., and Mruk, D. D. (2002). Cell junction dynamics in the testis: Sertoli-germ cell interactions and male contraceptive development. *Physiol Rev* **82**, 825-874.

Chin, K. V., Tanaka, S., Darlington, G., Pastan, I., and Gottesman, M. M. (1990). Heat shock and arsenite increase expression of the multidrug resistance (MDR1) gene in human renal carcinoma cells. *J Biol Chem* **265**, 221-226.

Chitra, K. C., Latchoumycandane, C., and Mathur, P. P. (2002). Effect of nonylphenol on the antioxidant system in epididymal sperm of rats. *Arch Toxicol* **76**, 545-551.

Citi, S. (1993). The molecular organization of tight junctions. *J Cell Biol* **121**, 485-489.

Clermont, Y. (1962). Quantitative analysis of spermatogenesis of the rat: a revised model for the renewal of spermatogonia. *Am J Anat* **111**, 111-129.

Clermont, Y. (1972). Kinetics of spermatogenesis in mammals: seminiferous epithelium cycle and spermatogonial renewal. *Physiol Rev* **52**, 198-236.

Clermont, Y., and Leblond, C. (1955). Spermiogenesis of man, monkey, ram and other mammals as shown by the periodic acid-Schiff technique. *Ann NY Acad Sci* **647**, 17-25.

Colabufo, N. A., Berardi, F., Perrone, M. G., Capparelli, E., Cantore, M., Inglese, C., and Perrone, R. (2010). Substrates, Inhibitors and Activators of P-Glycoprotein: Candidates for Radiolabeling and Imaging Perspectives. *Curr Top Med Chem.*

Cole, S. P., Bhardwaj, G., Gerlach, J. H., Mackie, J. E., Grant, C. E., Almquist, K. C., Stewart, A. J., Kurz, E. U., Duncan, A. M., and Deeley, R. G. (1992). Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. *Science (New York, N.Y* **258**, 1650-1654.

Cooke, P. S., Hess, R. A., Porcelli, J., and Meisami, E. (1991). Increased sperm production in adult rats after transient neonatal hypothyroidism. *Endocrinology* **129**, 244-248.

Cooke, P. S., and Meisami, E. (1991). Early hypothyroidism in rats causes increased adult testis and reproductive organ size but does not change testosterone levels. *Endocrinology* **129**, 237-243.

Cooper, T. G. (1998). Interactions between epididymal secretions and spermatozoa. *Journal of reproduction and fertility* **53**, 119-136.

Cooper, T. G., and Yeung, C. H. (2003). Acquisition of volume regulatory response of sperm upon maturation in the epididymis and the role of the cytoplasmic droplet. *Microsc Res Tech* **61**, 28-38.

Cooray, H. C., Blackmore, C. G., Maskell, L., and Barrand, M. A. (2002). Localisation of breast cancer resistance protein in microvessel endothelium of human brain. *Neuroreport* **13**, 2059-2063.

Cornwall, G. A. (2009). New insights into epididymal biology and function. *Human Reproduction Update* **15**, 213-227.

Cornwall, G. A., and von Horsten, H. H. (2007). Sperm Maturation in the Epididymis. Role of Segment-Specific Microenvironments. In *The Genetics of Male Fertility*, pp. 211-231. Humana Press Inc, Totowa, NJ.

Costa, Y., and Cooke, H. J. (2007). Dissecting the mammalian synaptonemal complex using targeted mutations. *Chromosome Res* **15**, 579-589.

Cotton, L. M., Rodriguez, C. M., Suzuki, K., Orgebin-Crist, M. C., and Hinton, B. T. (2010). Organic Cation/Carnitine Transporter, OCTN2, Transcriptional Activity Is Regulated by Osmotic Stress in Epididymal Cells. *Molecular Reproduction and Development* **77**, 114-125.

Cui, Y. J., Cheng, X., Weaver, Y. M., and Klaassen, C. D. (2009). Tissue distribution, gender-divergent expression, ontogeny, and chemical induction of multidrug resistance transporter genes (Mdr1a, Mdr1b, Mdr2) in mice. *Drug Metab Dispos* **37**, 203-210.

Cyr, D. G., Dufresne, J., Pillet, S., Alfieri, T. J., and Hermo, L. (2001). Expression and regulation of metallothioneins in the rat epididymis. *J Androl* **22**, 124-135.

Cyr, D. G., Finnson, K., Dufresne, J., and Gregory, M. (2002). Cellular Interactions and the Blood-Epididymal Barrier. In *The Epididymis: From Molecules to Clinical Practice* (B. Robaire, and B. T. Hinton, Eds.), pp. 103-118. Kluwer Academic/Plenum Publishers, New York.

Cyr, D. G., Finnson, K., Dufresne J., and Gregory, M. (2002). Cellular interactions and the blood-epididymal barrier. In B. Robaire and B. T. Hinton eds., The epididiymis: From molecules to clinical practice. Plenum Publisers, New York. Pp103-118.

Cyr, D. G., Gregory, M., Dube, E., Dufresne, J., Chan, P. T., and Hermo, L. (2007). Orchestration of occludins, claudins, catenins and cadherins as players involved in maintenance of the blood-epididymal barrier in animals and humans. *Asian J Androl* **9**, 463-475.

Cyr, D. G., Hermo, L., and Laird, D. W. (1996). Immunocytochemical localization and regulation of connexin43 in the adult rat epididymis. *Endocrinology* **137**, 1474-1484.

Cyr, D. G., Robaire, B., and Hermo, L. (1995). Structure and turnover of junctional complexes between principal cells of the rat epididymis. *Microsc Res Tech* **30**, 54-66.

Dacheux, J. L., Belleannee, C., Jones, R., Labas, V., Belghazi, M., Guyonnet, B., Druart, X., Gatti, J. L., and Dacheux, F. (2009). Mammalian epididymal proteome. *Molecular and cellular endocrinology* **306**, 45-50.

Darendeliler, F., Fournet, J. C., Bas, F., Junien, C., Gross, M. S., Bundak, R., Saka, N., and Gunoz, H. (2002). ABCC8 (SUR1) and KCNJ11 (KIR6.2) mutations in persistent hyperinsulinemic hypoglycemia of infancy and evaluation of different therapeutic measures. *J Pediatr Endocrinol Metab* **15**, 993-1000.

De Kretser, D. M., Catt, K. J., and Paulsen, C. A. (1971). Studies on the in vitro testicular binding of iodinated luteinizing hormone in rats. *Endocrinology* **88**, 332-337.

de Kretser, D. M., and Kerr, J. B. (1988). The cytology of the testis. In *The physiology of reproduction* (E. Knobil, and J. Neill, Eds.), pp. 837-932. Raven Press, Ltd., New York.

de Kretser, D. M., Loveland, K. L., Meinhardt, A., Simorangkir, D., and Wreford, N. (1998). Spermatogenesis. *Hum Reprod* **13 Suppl 1**, 1-8.

de Lamirande, E., and Gagnon, C. (1993). Human sperm hyperactivation and capacitation as parts of an oxidative process. *Free Radic Biol Med* **14**, 157-166.

de Lamirande, E., Leclerc, P., and Gagnon, C. (1997). Capacitation as a regulatory event that primes spermatozoa for the acrosome reaction and fertilization. *Mol Hum Reprod* **3**, 175-194.

De, S. K., Chen, H. L., Pace, J. L., Hunt, J. S., Terranova, P. F., and Enders, G. C. (1993). Expression of tumor necrosis factor-alpha in mouse spermatogenic cells. *Endocrinology* **133**, 389-396.

Dean, M., Hamon, Y., and Chimini, G. (2001). The human ATP-binding cassette (ABC) transporter superfamily. *J Lipid Res* **42**, 1007-1017.

DeBellefeuille, S., Hermo, L., Gregory, M., Dufresne, J., and Cyr, D. G. (2003). Catenins in the rat epididymis: their expression and regulation in adulthood and during postnatal development. *Endocrinology* **144**, 5040-5049.

Dengler, W. A., Schulte, J., Berger, D. P., Mertelsmann, R., and Fiebig, H. H. (1995). Development of a propidium iodide fluorescence assay for proliferation and cytotoxicity assays. *Anticancer Drugs* **6**, 522-532.

Dohle, G. R., Smit, M., and Weber, R. F. (2003). Androgens and male fertility. *World J Urol* **21**, 341-345.

Doo, M. H., Li, H., Jang, H. I., Song, I. S., Chung, S. J., and Shim, C. K. (2005). Effect of nonylphenol ethoxylates (NPEs) on barrier functions of epithelial cell membranes: opening of tight junctions and competitive inhibition of P-gp-mediated efflux. *Int J Pharm* **302**, 145-153.

Drevet, J. R. (2006). The antioxidant glutathione peroxidase family and spermatozoa: a complex story. *Mol Cell Endocrinol* **250**, 70-79.

Drozdzik, M., Stefankiewicz, J., Kurzawa, R., Gornik, W., Baczkowski, T., and Kurzawski, M. (2009). Association of the MDR1 (ABCB1) gene 3435C>T polymorphism with male infertility. *Pharmacol Rep* **61**, 690-696.

Dufresne, J., Finnson, K. W., Gregory, M., and Cyr, D. G. (2003). Expression of multiple connexins in the rat epididymis indicates a complex regulation of gap junctional communication. *Am J Physiol Cell Physiol* **284**, C33-43.

Dufresne, J., St-Pierre, N., Viger, R. S., Hermo, L., and Cyr, D. G. (2005). Characterization of a novel rat epididymal cell line to study epididymal function. *Endocrinology* **146**, 4710-4720.

Dupuy, J., Lespine, A., Sutra, J. F., and Alvinerie, M. (2006). Fumagillin, a new P-glycoprotein-interfering agent able to modulate moxidectin efflux in rat hepatocytes. *J Vet Pharmacol Ther* **29**, 489-494.

Eddy, E. M., Washburn, T. F., Bunch, D. O., Goulding, E. H., Gladen, B. C., Lubahn, D. B., and Korach, K. S. (1996). Targeted disruption of the estrogen receptor gene in male mice causes alteration of spermatogenesis and infertility. *Endocrinology* **137**, 4796-4805.

Eickhoff, R., Wilhelm, B., Renneberg, H., Wennemuth, G., Bacher, M., Linder, D., Bucala, R., Seitz, J., and Meinhardt, A. (2001). Purification and characterization of macrophage migration inhibitory factor as a secretory protein from rat epididymis: evidences for alternative release and transfer to spermatozoa. *Mol Med* **7**, 27-35.

Enokizono, J., Kusuhara, H., and Sugiyama, Y. (2007). Effect of breast cancer resistance protein (Bcrp/Abcg2) on the disposition of phytoestrogens. *Mol Pharmacol* **72**, 967-975.

Erkkila, K., Henriksen, K., Hirvonen, V., Rannikko, S., Salo, J., Parvinen, M., and Dunkel, L. (1997). Testosterone regulates apoptosis in adult human seminiferous tubules in vitro. *The Journal of clinical endocrinology and metabolism* **82**, 2314-2321.

Fawcett, D. W. (1975). The mammalian spermatozoon. *Dev Biol* **44**, 394-436.

Fazlina, N., Maha, A., Zarina, A. L., Hamidah, A., Zulkifli, S. Z., Cheong, S. K., Ainoon, O., Jamal, R., and Hamidah, N. H. (2008). Assessment of P-gp and MRP1 activities using MultiDrugQuant Assay Kit: a preliminary study of correlation between protein expressions and its functional activities in newly diagnosed acute leukaemia patients. *Malays J Pathol* **30**, 87-93.

Fetsch, P. A., Abati, A., Litman, T., Morisaki, K., Honjo, Y., Mittal, K., and Bates, S. E. (2006). Localization of the ABCG2 mitoxantrone resistance-associated protein in normal tissues. *Cancer Lett* **235**, 84-92.

Fiorini, C., Tilloy-Ellul, A., Chevalier, S., Charuel, C., and Pointis, G. (2004). Sertoli cell junctional proteins as early targets for different classes of reproductive toxicants. *Reprod Toxicol* **18**, 413-421.

Flens, M. J., Zaman, G. J., van der Valk, P., Izquierdo, M. A., Schroeijers, A. B., Scheffer, G. L., van der Groep, P., de Haas, M., Meijer, C. J., and Scheper, R. J. (1996). Tissue distribution of the multidrug resistance protein. *Am J Pathol* **148**, 1237-1247.

Forkert, P. G., Lash, L. H., Nadeau, V., Tardif, R., and Simmonds, A. (2002). Metabolism and toxicity of trichloroethylene in epididymis and testis. *Toxicol Appl Pharmacol* **182**, 244-254.

Foster, W. G., Maharaj-Briceno, S., and Cyr, D. G. (2010). Dioxin-induced changes in epididymal sperm count and spermatogenesis. *Environ Health Perspect* **118**, 458-464.

Frenette, G., Lessard, C., Madore, E., Fortier, M. A., and Sullivan, R. (2003). Aldose reductase and macrophage migration inhibitory factor are associated with

epididymosomes and spermatozoa in the bovine epididymis. *Biol Reprod* **69**, 1586-1592.

Frenette, G., and Sullivan, R. (2001). Prostasome-like particles are involved in the transfer of P25b from the bovine epididymal fluid to the sperm surface. *Mol Reprod Dev* **59**, 115-121.

Fromm, M. F. (2004). Importance of P-glycoprotein at blood-tissue barriers. *Trends Pharmacol Sci* **25**, 423-429.

Garrigues, A., Escargueil, A. E., and Orlowski, S. (2002). The multidrug transporter, P-glycoprotein, actively mediates cholesterol redistribution in the cell membrane. *Proc Natl Acad Sci U S A* **99**, 10347-10352.

Gatti, J. L., Castella, S., Dacheux, F., Ecroyd, H., Metayer, S., Thimon, V., and Dacheux, J. L. (2004). Post-testicular sperm environment and fertility. *Anim Reprod Sci* **82-83**, 321-339.

Georges, E., Bradley, G., Gariepy, J., and Ling, V. (1990). Detection of P-glycoprotein isoforms by gene-specific monoclonal antibodies. *Proc Natl Acad Sci U S A* **87**, 152-156.

Gilleron, J., Nebout, M., Scarabelli, L., Senegas-Balas, F., Palmero, S., Segretain, D., and Pointis, G. (2006). A potential novel mechanism involving connexin 43 gap junction for control of sertoli cell proliferation by thyroid hormones. *J Cell Physiol* **209**, 153-161.

Gilula, N. B., Fawcett, D. W., and Aoki, A. (1976). The Sertoli cell occluding junctions and gap junctions in mature and developing mammalian testis. *Dev Biol* **50**, 142-168.

Gregory, M., Lacroix, A., Haddad, S., Devine, P., Charbonneau, M., Tardif, R., Krishnan, K., Cooke, G. M., Schrader, T., and Cyr, D. G. (2009). Effects of chronic exposure to octylphenol on the male rat reproductive system. *J Toxicol Environ Health A* **72**, 1553-1560.

Griffin, J., Fletcher, N., Clemence, R., Blanchflower, S., and Brayden, D. J. (2005). Selamectin is a potent substrate and inhibitor of human and canine P-glycoprotein. *J Vet Pharmacol Ther* **28**, 257-265.

Griswold, M. D. (1998). The central role of Sertoli cells in spermatogenesis. *Seminars in cell & developmental biology* **9**, 411-416.

Griveau, J. F., Renard, P., and Le Lannou, D. (1995). Superoxide anion production by human spermatozoa as a part of the ionophore-induced acrosome reaction process. *Int J Androl* **18**, 67-74.

Guenther, K., Heinke, V., Thiele, B., Kleist, E., Prast, H., and Raecker, T. (2002). Endocrine disrupting nonylphenols are ubiquitous in food. *Environ Sci Technol* **36**, 1676-1680.

Gupta, P., and Bhatia, V. (2008). Corticosteroid physiology and principles of therapy. *Indian J Pediatr* **75**, 1039-1044.

Hamelin, G., Charest-Tardif, G., Krishnan, K., Cyr, D., Charbonneau, M., Devine, P. J., Haddad, S., Cooke, G. M., Schrader, T., and Tardif, R. (2009). Toxicokinetics of p-tert-octylphenol in male and female Sprague-Dawley rats after intravenous, oral, or subcutaneous exposures. *J Toxicol Environ Health A* **72**, 541-550.

Han, X. D., Tu, Z. G., Gong, Y., Shen, S. N., Wang, X. Y., Kang, L. N., Hou, Y. Y., and Chen, J. X. (2004). The toxic effects of nonylphenol on the reproductive system of male rats. *Reprod Toxicol* **19**, 215-221.

Hardy, M. P., Kirby, J. D., Hess, R. A., and Cooke, P. S. (1993). Leydig cells increase their numbers but decline in steroidogenic function in the adult rat after neonatal hypothyroidism. *Endocrinology* **132**, 2417-2420.

Hazzouri, M., Pivot-Pajot, C., Faure, A. K., Usson, Y., Pelletier, R., Sele, B., Khochbin, S., and Rousseaux, S. (2000). Regulated hyperacetylation of core histones during mouse spermatogenesis: involvement of histone deacetylases. *Eur J Cell Biol* **79**, 950-960.

Hecht, N. B. (1998). Molecular mechanisms of male germ cell differentiation. *Bioessays* **20**, 555-561.

Heckert, L. L., and Griswold, M. D. (2002). The expression of the follicle-stimulating hormone receptor in spermatogenesis. *Recent Prog Horm Res* **57**, 129-148.

Hermo, L., Adamali, H. I., and Andonian, S. (2000). Immunolocalization of CA II and H+ V-ATPase in epithelial cells of the mouse and rat epididymis. *J Androl* **21**, 376-391.

Hermo, L., Barin, K., and Robaire, B. (1992). Structural differentiation of the epithelial cells of the testicular excurrent duct system of rats during postnatal development. *Anat Rec* **233**, 205-228.

Hermo, L., Chong, D. L., Moffatt, P., Sly, W. S., Waheed, A., and Smith, C. E. (2005). Region- and cell-specific differences in the distribution of carbonic anhydrases II, III, XII, and XIV in the adult rat epididymis. *J Histochem Cytochem* **53**, 699-713.

Hermo, L., Dworkin, J., and Oko, R. (1988). Role of epithelial clear cells of the rat epididymis in the disposal of the contents of cytoplasmic droplets detached from spermatozoa. *Am J Anat* **183**, 107-124.

Hermo, L., and Papp, S. (1996). Effects of ligation, orchidectomy, and hypophysectomy on expression of the Yf subunit of GST-P in principal and basal cells of the adult rat epididymis and on basal cell shape and overall arrangement. *Anat Rec* **244**, 59-69.

Hermo, L., Pelletier, R. M., Cyr, D. G., and Smith, C. E. (2010a). Surfing the wave, cycle, life history, and genes/proteins expressed by testicular germ cells. Part 1: background to spermatogenesis, spermatogonia, and spermatocytes. *Microsc Res Tech* **73**, 241-278.

Hermo, L., Pelletier, R. M., Cyr, D. G., and Smith, C. E. (2010b). Surfing the wave, cycle, life history, and genes/proteins expressed by testicular germ cells. Part 2: changes in spermatid organelles associated with development of spermatozoa. *Microsc Res Tech* **73**, 279-319.

Hermo, L., Pelletier, R. M., Cyr, D. G., and Smith, C. E. (2010c). Surfing the wave, cycle, life history, and genes/proteins expressed by testicular germ cells. Part 3: developmental changes in spermatid flagellum and cytoplasmic droplet and interaction of sperm with the zona pellucida and egg plasma membrane. *Microsc Res Tech* **73**, 320-363.

Hernandez-Hernandez, A., Vazquez-Nin, G. H., Echeverria, O. M., and Recillas-Targa, F. (2009). Chromatin structure contribution to the synaptonemal complex formation. *Cell Mol Life Sci* **66**, 1198-1208.

Hess, R. A. (1998). Effects of environmental toxicants on the efferent ducts, epididymis and fertility. *Journal of reproduction and fertility* **53**, 247-259.

Hess, R. A., Cooke, P. S., Bunick, D., and Kirby, J. D. (1993). Adult testicular enlargement induced by neonatal hypothyroidism is accompanied by increased Sertoli and germ cell numbers. *Endocrinology* **132**, 2607-2613.

Hinton, B. T. (1990). The testicular and epididymal luminal amino acid microenvironment in the rat. *J Androl* **11**, 498-505.

Hinton, B. T., and Howards, S. S. (1988). Permeability characteristics of the epithelium in the rat caput epididymidis. *J Reprod Fertil* **63**, 95-99.

Hinton, B. T., and Palladino, M. A. (1995). Epididymal epithelium: its contribution to the formation of a luminal fluid microenvironment. *Microsc Res Tech* **30**, 67-81.

Holdcraft, R. W., and Braun, R. E. (2004). Hormonal regulation of spermatogenesis. *Int J Androl* **27**, 335-342.

Holsberger, D. R., and Cooke, P. S. (2005). Understanding the role of thyroid hormone in Sertoli cell development: a mechanistic hypothesis. *Cell Tissue Res* **322**, 133-140.

Holsberger, D. R., Jirawatnotai, S., Kiyokawa, H., and Cooke, P. S. (2003). Thyroid hormone regulates the cell cycle inhibitor p27Kip1 in postnatal murine Sertoli cells. *Endocrinology* **144**, 3732-3738.

Huckins, C. (1971a). The spermatogonial stem cell population in adult rats. 3. Evidence for a long-cycling population. *Cell Tissue Kinet* **4**, 335-349.

Huckins, C. (1971b). The spermatogonial stem cell population in adult rats. I. Their morphology, proliferation and maturation. *Anat Rec* **169**, 533-557.

Huckins, C. (1971c). The spermatogonial stem cell population in adult rats. II. A radioautographic analysis of their cell cycle properties. *Cell Tissue Kinet* **4**, 313-334.

Huckins, C. (1978). The morphology and kinetics of spermatogonial degeneration in normal adult rats: an analysis using a simplified classification of the germinal epithelium. *Anat Rec* **190**, 905-926.

Huckins, C., and Oakberg, E. F. (1978). Morphological and quantitative analysis of spermatogonia in mouse testes using whole mounted seminiferous tubules. II. The irradiated testes. *Anat Rec* **192**, 529-542.

Hughes, I. A., Lim, H. N., Martin, H., Mongan, N. P., Dovey, L., Ahmed, S. F., and Hawkins, J. R. (2001). Developmental aspects of androgen action. *Molecular and cellular endocrinology* **185**, 33-41.

Hugues, J. N., and Durnerin, I. C. (2005). Impact of androgens on fertility - physiological, clinical and therapeutic aspects. *Reprod Biomed Online* **11**, 570-580.

Ilio, K. Y., and Hess, R. A. (1994). Structure and function of the ductuli efferentes: a review. *Microsc Res Tech* **29**, 432-467.

Jedlitschky, G., Leier, I., Buchholz, U., Barnouin, K., Kurz, G., and Keppler, D. (1996). Transport of glutathione, glucuronate, and sulfate conjugates by the MRP gene-encoded conjugate export pump. *Cancer research* **56**, 988-994.

Jiang, F. X. (1998). Behaviour of spermatogonia following recovery from busulfan treatment in the rat. *Anat Embryol (Berl)* **198**, 53-61.

Jin, H., and Audus, K. L. (2005). Effect of bisphenol A on drug efflux in BeWo, a human trophoblast-like cell line. *Placenta* **26 Suppl A**, S96-S103.

Johnston, D. S., Jelinsky, S. A., Bang, H. J., DiCandeloro, P., Wilson, E., Kopf, G. S., and Turner, T. T. (2005). The mouse epididymal transcriptome: transcriptional profiling of segmental gene expression in the epididymis. *Biol Reprod* **73**, 404-413.

Jones, R. (1986). Preparation of a plasma membrane-rich fraction from rat spermatozoa. *J Reprod Fertil* **77**, 435-449.

Jones, R., Hamilton, D. W., and Fawcett, D. W. (1979a). Morphology of the epithelium of the extratesticular rete testis, ductuli efferentes and ductus epididymidis of the adult male rabbit. *Am J Anat* **156**, 373-400.

Jones, R., James, P. S., Howes, L., Bruckbauer, A., and Klenerman, D. (2007). Supramolecular organization of the sperm plasma membrane during maturation and capacitation. *Asian J Androl* **9**, 438-444.

Jones, R., Mann, T., and Sherins, R. (1979b). Peroxidative breakdown of phospholipids in human spermatozoa, spermicidal properties of fatty acid peroxides, and protective action of seminal plasma. *Fertil Steril* **31**, 531-537.

Joseph, A., Hess, R. A., Schaeffer, D. J., Ko, C., Hudgin-Spivey, S., Chambon, P., and Shur, B. D. (2010). Absence of estrogen receptor alpha leads to physiological alterations in the mouse epididymis and consequent defects in sperm function. *Biol Reprod* **82**, 948-957.

Juliano, R. L., and Ling, V. (1976). A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. *Biochim Biophys Acta* **455**, 152-162.

Jutte, N. H., Jansen, R., Grootegoed, J. A., Rommerts, F. F., and van der Molen, H. J. (1983). FSH stimulation of the production of pyruvate and lactate by rat Sertoli cells may be involved in hormonal regulation of spermatogenesis. *J Reprod Fertil* **68**, 219-226.

Kadota, T., Chikazawa, H., and Takahashi, N. (1989). Toxicological study of etoposide (VP-16) in rats with special emphasis on testicular alteration. *Toxicol Lett* **45**, 185-194.

Kaitu'u-Lino, T. J., Sluka, P., Foo, C. F., and Stanton, P. G. (2007). Claudin-11 expression and localisation is regulated by androgens in rat Sertoli cells in vitro. *Reproduction* **133**, 1169-1179.

Kato, S., Yasui, T., and Kanda, S. (1983). Migration of the cytoplasmic droplet in goat spermatozoa by centrifugation. *Jap J Anim AI Rex* **29**, 214-216.

Kato, S., Yasui, T., Nano, I., and Kanda, S. (1984). Migration of the cytoplasmic droplet in boar spermatozoa by centrifugation. *Jap J Anim AI Res* **6**.

Kiki-Mvouaka, S., Menez, C., Borin, C., Lyazrhi, F., Foucaud-Vignault, M., Dupuy, J., Collet, X., Alvinerie, M., and Lespine, A. (2010). Role of P-glycoprotein in the disposition of macrocyclic lactones: A comparison between ivermectin, eprinomectin, and moxidectin in mice. *Drug Metab Dispos* **38**, 573-580.

Kim, W. Y., and Benet, L. Z. (2004). P-glycoprotein (P-gp/MDR1)-mediated efflux of sex-steroid hormones and modulation of P-gp expression in vitro. *Pharm Res* **21**, 1284-1293.

Kimmins, S., Kotaja, N., Davidson, I., and Sassone-Corsi, P. (2004). Testis-specific transcription mechanisms promoting male germ-cell differentiation. *Reproduction* **128**, 5-12.

Kimmins, S., and Sassone-Corsi, P. (2005). Chromatin remodelling and epigenetic features of germ cells. *Nature* **434**, 583-589.

Kimura, Y., Kioka, N., Kato, H., Matsuo, M., and Ueda, K. (2007). Modulation of drug-stimulated ATPase activity of human MDR1/P-glycoprotein by cholesterol. *The Biochemical journal* **401**, 597-605.

Kirchhoff, C., and Hale, G. (1996). Cell-to-cell transfer of glycosylphosphatidylinositol-anchored membrane proteins during sperm maturation. *Mol Hum Reprod* **2**, 177-184.

Kistler, M. K., Sassone-Corsi, P., and Kistler, W. S. (1994). Identification of a functional cyclic adenosine 3',5'-monophosphate response element in the 5'-flanking region of the gene for transition protein 1 (TP1), a basic chromosomal protein of mammalian spermatids. *Biol Reprod* **51**, 1322-1329.

Klaasen, C. D. (2001). *Casarett and Doull's Toxicology - The Basic Science of Poisons (6th Edition)*. McGraw-Hill

Klaassen, C. D., and Aleksunes, L. M. (2010). Xenobiotic, bile acid, and cholesterol transporters: function and regulation. *Pharmacol Rev* **62**, 1-96.

Klinefelter, G. R. (2002). Actions of toxicants on the structure and function of the epididymis. In B. Robaire and B. T. Hinton eds., The epididiymis: From molecules to clinical practice. Plenum Publisers, New York. Pp353-370.

Kobayashi, D., Tamai, I., Sai, Y., Yoshida, K., Wakayama, T., Kido, Y., Nezu, J., Iseki, S., and Tsuji, A. (2007). Transport of carnitine and acetylcarnitine by carnitine/organic cation transporter (OCTN) 2 and OCTN3 into epididymal spermatozoa. *Reproduction* **134**, 651-658.

Komori, S., Kasumi, H., Sakata, K., and Koyama, K. (2007). The role of androgens in spermatogenesis. *Soc Reprod Fertil Suppl* **63**, 25-30.

Kopera, I. A., Bilinska, B., Cheng, C. Y., and Mruk, D. D. (2010). Sertoli-germ cell junctions in the testis: a review of recent data. *Philos Trans R Soc Lond B Biol Sci* **365**, 1593-1605.

Kotaja, N., and Sassone-Corsi, P. (2007). The chromatoid body: a germ-cell-specific RNA-processing centre. *Nature reviews* **8**, 85-90.

Kretschmer, X. C., and Baldwin, W. S. (2005). CAR and PXR: xenosensors of endocrine disrupters? *Chem Biol Interact* **155**, 111-128.

Kuiper, G. G., Carlsson, B., Grandien, K., Enmark, E., Haggblad, J., Nilsson, S., and Gustafsson, J. A. (1997). Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. *Endocrinology* **138**, 863-870.

Kuiper, G. G., Lemmen, J. G., Carlsson, B., Corton, J. C., Safe, S. H., van der Saag, P. T., van der Burg, B., and Gustafsson, J. A. (1998). Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. *Endocrinology* **139**, 4252-4263.

Kujala, M., Hihnala, S., Tienari, J., Kaunisto, K., Hastbacka, J., Holmberg, C., Kere, J., and Hoglund, P. (2007). Expression of ion transport-associated proteins in human efferent and epididymal ducts. *Reproduction* **133**, 775-784.

Lambard, S., Galeraud-Denis, I., Bouraima, H., Bourguiba, S., Chocat, A., and Carreau, S. (2003). Expression of aromatase in human ejaculated spermatozoa: a putative marker of motility. *Mol Hum Reprod* **9**, 117-124.

Lankas, G. R., Wise, L. D., Cartwright, M. E., Pippert, T., and Umbenhauer, D. R. (1998). Placental P-glycoprotein deficiency enhances susceptibility to chemically induced birth defects in mice. *Reprod Toxicol* **12**, 457-463.

Law, G. L., McGuinness, M. P., Linder, C. C., and Griswold, M. D. (1997). Expression of apolipoprotein E mRNA in the epithelium and interstitium of the testis and the epididymis. *J Androl* **18**, 32-42.

Lecoeur, S., Videmann, B., and Mazallon, M. (2006). Effect of organophosphate pesticide diazinon on expression and activity of intestinal P-glycoprotein. *Toxicol Lett* **161**, 200-209.

Legare, C., Berube, B., Boue, F., Lefievre, L., Morales, C. R., El-Alfy, M., and Sullivan, R. (1999). Hamster sperm antigen P26h is a phosphatidylinositol-anchored protein. *Mol Reprod Dev* **52**, 225-233.

Leier, I., Jedlitschky, G., Buchholz, U., Center, M., Cole, S. P., Deeley, R. G., and Keppler, D. (1996). ATP-dependent glutathione disulphide transport mediated by the MRP gene-encoded conjugate export pump. *The Biochemical journal* **314 (Pt 2)**, 433-437.

Leier, I., Jedlitschky, G., Buchholz, U., Cole, S. P., Deeley, R. G., and Keppler, D. (1994). The MRP gene encodes an ATP-dependent export pump for leukotriene C4 and structurally related conjugates. *J Biol Chem* **269**, 27807-27810.

Leslie, E. M., Deeley, R. G., and Cole, S. P. (2001). Toxicological relevance of the multidrug resistance protein 1, MRP1 (ABCC1) and related transporters. *Toxicology* **167**, 3-23.

Letrent, S. P., Polli, J. W., Humphreys, J. E., Pollack, G. M., Brouwer, K. R., and Brouwer, K. L. (1999). P-glycoprotein-mediated transport of morphine in brain capillary endothelial cells. *Biochem Pharmacol* **58**, 951-957.

Leung, G. P., Cheung, K. H., Leung, C. T., Tsang, M. W., and Wong, P. Y. (2004). Regulation of epididymal principal cell functions by basal cells: role of transient receptor potential (Trp) proteins and cyclooxygenase-1 (COX-1). *Mol Cell Endocrinol* **216**, 5-13.

Li, M. W., Mruk, D. D., Lee, W. M., and Cheng, C. Y. (2009). Cytokines and junction restructuring events during spermatogenesis in the testis: an emerging concept of regulation. *Cytokine Growth Factor Rev* **20**, 329-338.

Lin, L., Zheng, L. X., Gu, Y. P., Wang, J. Y., Zhang, Y. H., and Song, W. M. (2008). [Levels of environmental endocrine disruptors in umbilical cord blood and maternal blood of low-birth-weight infants]. *Zhonghua Yu Fang Yi Xue Za Zhi* **42**, 177-180.

Liu, T. Y., Brownell, K. A., and Hartman, F. A. (1955). Production of androgenic hormone by the adrenal. *Am J Physiol* **180**, 50-52.

Lockshin, R. A., and Zakeri, Z. (2004). Caspase-independent cell death? *Oncogene* **23**, 2766-2773.

Lombardo, F., Sgro, P., Salacone, P., Gilio, B., Gandini, L., Dondero, F., Jannini, E. A., and Lenzi, A. (2005). Androgens and fertility. *J Endocrinol Invest* **28**, 51-55.

Loss, E. S., Jacobus, A. P., and Wassermann, G. F. (2007). Diverse FSH and testosterone signaling pathways in the Sertoli cell. *Horm Metab Res* **39**, 806-812.

Lui, W. Y., Mruk, D., Lee, W. M., and Cheng, C. Y. (2003). Sertoli cell tight junction dynamics: their regulation during spermatogenesis. *Biol Reprod* **68**, 1087-1097.

Main, K. M., Skakkebaek, N. E., Virtanen, H. E., and Toppari, J. (2010). Genital anomalies in boys and the environment. *Best Pract Res Clin Endocrinol Metab* **24**, 279-289.

Maliepaard, M., Scheffer, G. L., Faneyte, I. F., van Gastelen, M. A., Pijnenborg, A. C., Schinkel, A. H., van De Vijver, M. J., Scheper, R. J., and Schellens, J. H. (2001). Subcellular localization and distribution of the breast cancer resistance protein transporter in normal human tissues. *Cancer research* **61**, 3458-3464.

Mao, Q., and Unadkat, J. D. (2005). Role of the breast cancer resistance protein (ABCG2) in drug transport. *AAPS J* **7**, E118-133.

McKinney, J. D., and Waller, C. L. (1998). Molecular determinants of hormone mimicry: halogenated aromatic hydrocarbon environmental agents. *J Toxicol Environ Health B Crit Rev* **1**, 27-58.

McLachlan, R. I., and de Kretser, D. M. (2001). Male infertility: the case for continued research. *Med J Aust* **174**, 116-117.

Melaine, N., Lienard, M. O., Dorval, I., Le Goascogne, C., Lejeune, H., and Jegou, B. (2002). Multidrug resistance genes and p-glycoprotein in the testis of the rat, mouse, Guinea pig, and human. *Biol Reprod* **67**, 1699-1707.

Mena, S., Ortega, A., and Estrela, J. M. (2009). Oxidative stress in environmental-induced carcinogenesis. *Mutat Res* **674**, 36-44.

Mendis-Handagama, S. M., and Siril Ariyaratne, H. B. (2005). Leydig cells, thyroid hormones and steroidogenesis. *Indian J Exp Biol* **43**, 939-962.

Meng, J., Holdcraft, R. W., Shima, J. E., Griswold, M. D., and Braun, R. E. (2005). Androgens regulate the permeability of the blood-testis barrier. *Proc Natl Acad Sci U S A* **102**, 16696-16700.

Meng, W., and Takeichi, M. (2009). Adherens junction: molecular architecture and regulation. *Cold Spring Harb Perspect Biol* **1**, a002899.

Mercier, C., Decleves, X., Masseguin, C., Fragner, P., Tardy, M., Roux, F., Gabrion, J., and Scherrmann, J. M. (2003). P-glycoprotein (ABCB1) but not multidrug resistance-associated protein 1 (ABCC1) is induced by doxorubicin in primary cultures of rat astrocytes. *J Neurochem* **87**, 820-830.

Merton, H. (1939). Studies on reproduction in the albino mouse. II. Contributions on the maturation of the sperm cells. *Proc Roy Soc* **59**, 145-152.

Miller, K. K. (2009). Androgen deficiency: effects on body composition. *Pituitary* **12**, 116-124.

Mizutani, T., Masuda, M., Nakai, E., Furumiya, K., Togawa, H., Nakamura, Y., Kawai, Y., Nakahira, K., Shinkai, S., and Takahashi, K. (2008). Genuine functions of P-glycoprotein (ABCB1). *Curr Drug Metab* **9**, 167-174.

Moffatt, P., and Seguin, C. (1998). Expression of the gene encoding metallothionein-3 in organs of the reproductive system. *DNA Cell Biol* **17**, 501-510.

Moore, K., Lovercamp, K., Feng, D., Antelman, J., Sutovsky, M., Manandhar, G., van Leyen, K., Safranski, T., and Sutovsky, P. (2010). Altered epididymal sperm maturation and cytoplasmic droplet migration in subfertile male Alox15 mice. *Cell Tissue Res* **340**, 569-581.

Morales, C. R., Marat, A. L., Ni, X., Yu, Y., Oko, R., Smith, B. T., and Argraves, W. S. (2008). ATP-binding cassette transporters ABCA1, ABCA7, and ABCG1 in mouse spermatozoa. *Biochem Biophys Res Commun* **376**, 472-477.

Mruk, D. D., and Cheng, C. Y. (2004). Sertoli-Sertoli and Sertoli-germ cell interactions and their significance in germ cell movement in the seminiferous epithelium during spermatogenesis. *Endocr Rev* **25**, 747-806.

Muller, H., Klinkhammer, W., Globisch, C., Kassack, M. U., Pajeva, I. K., and Wiese, M. (2007). New functional assay of P-glycoprotein activity using Hoechst 33342. *Bioorg Med Chem* **15**, 7470-7479.

Nashan, D., Malorny, U., Sorg, C., Cooper, T., and Nieschlag, E. (1989). Immuno-competent cells in the murine epididymis. *Int J Androl* **12**, 85-94.

Nimrod, A. C., and Benson, W. H. (1996). Environmental estrogenic effects of alkylphenol ethoxylates. *Crit Rev Toxicol* **26**, 335-364.

O'Donnell, L., Meachem, S., Stanton, P., and McLachlan, R. (2006). Endocrine regulation of spermatogenesis. In *Knobil and Neill's physiology of reproduction* (J. Neill, Ed.), pp. 1017-1070. Elsevier Academic Press, San Diego.

Oko, R., Hermo, L., Chan, P. T., Fazel, A., and Bergeron, J. J. (1993). The cytoplasmic droplet of rat epididymal spermatozoa contains saccular elements with Golgi characteristics. *J Cell Biol* **123**, 809-821.

Olson, L. M., Zhou, X., and Schreiber, J. R. (1995). Cell-specific localization of apolipoprotein E messenger ribonucleic acid in the testis and epididymis of the rat. *Biol Reprod* **52**, 1003-1011.

Orgebin-Crist, M. C. (1969). Studies on the function of the epididymis. *Biol Reprod* **1**, Suppl 1:155-175.

Padowski, J. M., and Pollack, G. M. (2010). Pharmacokinetic and pharmacodynamic implications of P-glycoprotein modulation. *Methods Mol Biol* **596**, 359-384.

Paintrand, M., Moudjou, M., Delacroix, H., and Bornens, M. (1992). Centrosome organization and centriole architecture: their sensitivity to divalent cations. *J Struct Biol* **108**, 107-128.

Panwala, C. M., Jones, J. C., and Viney, J. L. (1998). A novel model of inflammatory bowel disease: mice deficient for the multiple drug resistance gene, mdr1a, spontaneously develop colitis. *J Immunol* **161**, 5733-5744.

Payne, A. H., Kelch, R. P., Musich, S. S., and Halpern, M. E. (1976). Intratesticular site of aromatization in the human. *The Journal of clinical endocrinology and metabolism* **42**, 1081-1087.

Peng, K. C., Cluzeaud, F., Bens, M., Duong Van Huyen, J. P., Wioland, M. A., Lacave, R., and Vandewalle, A. (1999). Tissue and cell distribution of the multidrug resistance-associated protein (MRP) in mouse intestine and kidney. *J Histochem Cytochem* **47**, 757-768.

Phelps, B., Koppel, D., Primakoff, P., and Myles, D. (1990). Evidence that proteolysis of the surface is an initial step in the mechanism of formation of sperm cell surface domains. *J Cell Biol* **5 Pt 1**, 1839-1847.

Phillips, K. P., and Tanphaichitr, N. (2008). Human exposure to endocrine disrupters and semen quality. *J Toxicol Environ Health B Crit Rev* **11**, 188-220.

Pincon-Raymond, M. (2004). [Cadherins: structure and signalisation protein of cell-cell junctions. Introduction and conclusions]. *J Soc Biol* **198**, 353-356.

Pointis, G., Fiorini, C., Defamie, N., and Segretain, D. (2005). Gap junctional communication in the male reproductive system. *Biochim Biophys Acta* **1719**, 102-116.

Pradeepa, M. M., and Rao, M. R. (2007). Chromatin remodeling during mammalian spermatogenesis: role of testis specific histone variants and transition proteins. *Soc Reprod Fertil Suppl* **63**, 1-10.

Rago, V., Bilinska, B., Palma, A., Ando, S., and Carpino, A. (2003). Evidence of aromatase localization in cytoplasmic droplet of human immature ejaculated spermatozoa. *Folia Histochem Cytobiol* **41**, 23-27.

Rejraji, H., Sion, B., Prensier, G., Carreras, M., Motta, C., Frenoux, J. M., Vericel, E., Grizard, G., Vernet, P., and Drevet, J. R. (2006). Lipid remodeling of murine epididymosomes and spermatozoa during epididymal maturation. *Biol Reprod* **74**, 1104-1113.

Ren, X. Y., Zhou, Y., Zhang, J. P., Feng, W. H., and Jiao, B. H. (2003a). Metallothionein gene expression under different time in testicular Sertoli and spermatogenic cells of rats treated with cadmium. *Reprod Toxicol* **17**, 219-227.

Ren, X. Y., Zhou, Y., Zhang, J. P., Feng, W. H., and Jiao, B. H. (2003b). Expression of metallothionein gene at different time in testicular interstitial cells and liver of rats treated with cadmium. *World J Gastroenterol* **9**, 1554-1558.

Reynaert, I., Van Der Schueren, B., Degeest, G., Manin, M., Cuppens, H., Scholte, B., and Cassiman, J. J. (2000). Morphological changes in the vas deferens and expression of the cystic fibrosis transmembrane conductance regulator (CFTR) in control, deltaF508 and knock-out CFTR mice during postnatal life. *Mol Reprod Dev* **55**, 125-135.

Robaire, B., and Hermo, L. (1988). Efferent Ducts, Epididymis, and Vas Deferens: Structure, Functions and Their Regulation. In *The Physiology of Reproduction* (E. Knobil, and J. Neill, Eds.), pp. 999-1080. Raven Press, Ltd., New York.

Robaire, B., and Hinton, B. T. (2001). *The Epididymis: From Molecules To Clinical Practicea Comprehensive Survey Of The Efferent Ducts, The Epididymis And The Vas Deferens*. Springer Us.

Robaire, B., Hinton, B. T., and Orgebin-Crist, M.-C. (2006). The Epididymis. In *Knobil and Neill's Physiology of Reproduction* (J. D. Neill, Ed.), pp. 1071-1148. Elsevier, New York.

Robaire, B., and Viger, R. S. (1995). Regulation of epididymal epithelial cell functions. *Biol Reprod* **52**, 226-236.

Roberts, M. L., Scouten, W. H., and Nyquist, S. E. (1976). Isolation and characterization of the cytoplasmic droplet in the rat. *Biol Reprod* **14**, 421-424.

Roman, B. L., Pollenz, R. S., and Peterson, R. E. (1998). Responsiveness of the adult male rat reproductive tract to 2,3,7,8-tetrachlorodibenzo-p-dioxin exposure: Ah receptor and ARNT expression, CYP1A1 induction, and Ah receptor down-regulation. *Toxicol Appl Pharmacol* **150**, 228-239.

Ronfani, L., and Bianchi, M. E. (2004). Molecular mechanisms in male determination and germ cell differentiation. *Cell Mol Life Sci* **61**, 1907-1925.

Russel, L. D., Ettlin, R. A., Sinha Hikim, A. P., and Clegg, E. D. (1990). *Histological Histopathological Evaluation of the testis*. Cache River press, Clearwater. Fl.

Ruwanpura, S. M., McLachlan, R. I., and Meachem, S. J. (2010). Hormonal regulation of male germ cell development. *J Endocrinol* **205**, 117-131.

Sabik, H., Gagne, F., Blaise, C., Marcogliese, D. J., and Jeannot, R. (2003). Occurrence of alkylphenol polyethoxylates in the St. Lawrence River and their bioconcentration by mussels (Elliptio complanata). *Chemosphere* **51**, 349-356.

Saez, F., Frenette, G., and Sullivan, R. (2003). Epididymosomes and prostasomes: their roles in posttesticular maturation of the sperm cells. *J Androl* **24**, 149-154.

Salhanick, A. I., and Wiebe, J. P. (1980). FSH receptors in isolated Sertoli cells: changes in concentration of binding sites at the onset of sexual maturation. *Life sciences* **26**, 2281-2288.

Salian, S., Doshi, T., and Vanage, G. (2009). Neonatal exposure of male rats to Bisphenol A impairs fertility and expression of sertoli cell junctional proteins in the testis. *Toxicology* **265**, 56-67.

Santoni-Rugiu, E., and Silverman, J. A. (1997). Functional characterization of the rat mdr1b encoded P-glycoprotein: not all inducing agents are substrates. *Carcinogenesis* **18**, 2255-2263.

Sassone-Corsi, P. (2005). Transcription factors governing male fertility. *Andrologia* **37**, 228-229.

Saunders, P. T., Fisher, J. S., Sharpe, R. M., and Millar, M. R. (1998). Expression of oestrogen receptor beta (ER beta) occurs in multiple cell types, including some germ cells, in the rat testis. *J Endocrinol* **156**, R13-17.

Saunders, P. T., Sharpe, R. M., Williams, K., Macpherson, S., Urquart, H., Irvine, D. S., and Millar, M. R. (2001). Differential expression of oestrogen receptor alpha and beta proteins in the testes and male reproductive system of human and non-human primates. *Mol Hum Reprod* **7**, 227-236.

Scharenberg, C., Mannowetz, N., Robey, R. W., Brendel, C., Repges, P., Sahrhage, T., Jahn, T., and Wennemuth, G. (2009). ABCG2 is expressed in late spermatogenesis and is associated with the acrosome. *Biochem Biophys Res Commun* **378**, 302-307.

Scheffer, G. L., Pijnenborg, A. C., Smit, E. F., Muller, M., Postma, D. S., Timens, W., van der Valk, P., de Vries, E. G., and Scheper, R. J. (2002). Multidrug resistance related molecules in human and murine lung. *J Clin Pathol* **55**, 332-339.

Schinkel, A. H. (1997). The physiological function of drug-transporting P-glycoproteins. *Semin Cancer Biol* **8**, 161-170.

Schinkel, A. H., and Jonker, J. W. (2003). Mammalian drug efflux transporters of the ATP binding cassette (ABC) family: an overview. *Adv Drug Deliv Rev* **55**, 3-29.

Schinkel, A. H., Mol, C. A., Wagenaar, E., van Deemter, L., Smit, J. J., and Borst, P. (1995a). Multidrug resistance and the role of P-glycoprotein knockout mice. *Eur J Cancer* **31A**, 1295-1298.

Schinkel, A. H., Smit, J. J., van Tellingen, O., Beijnen, J. H., Wagenaar, E., van Deemter, L., Mol, C. A., van der Valk, M. A., Robanus-Maandag, E. C., te Riele, H. P., and et al. (1994). Disruption of the mouse mdr1a P-glycoprotein gene leads to a deficiency in the blood-brain barrier and to increased sensitivity to drugs. *Cell* **77**, 491-502.

Schinkel, A. H., Wagenaar, E., van Deemter, L., Mol, C. A., and Borst, P. (1995b). Absence of the mdr1a P-Glycoprotein in mice affects tissue distribution and pharmacokinetics of dexamethasone, digoxin, and cyclosporin A. *J Clin Invest* **96**, 1698-1705.

Schwab, D., Fischer, H., Tabatabaei, A., Poli, S., and Huwyler, J. (2003). Comparison of in vitro P-glycoprotein screening assays: recommendations for their use in drug discovery. *J Med Chem* **46**, 1716-1725.

Seeger, M. A., and van Veen, H. W. (2009). Molecular basis of multidrug transport by ABC transporters. *Biochim Biophys Acta* **1794**, 725-737.

Serre, V., and Robaire, B. (1999). Distribution of immune cells in the epididymis of the aging Brown Norway rat is segment-specific and related to the luminal content. *Biol Reprod* **61**, 705-714.

Setchell, B. P., and Breed, W. G. (2006). Anatomy, Vasculature, and Innervation of the Male Reproductive Tract. In *Knobil and Neill's Physiology of Reproduction* (J. Neill, Ed.), pp. 771-826. Elsevier Academic Press, San Diego.

Setchell, B. P., and Brooks, D. E. (1988). Anatomy, vasculature, innervation, and fluids of the male reproductive tract. In *The physiology of reproduction* (E. Knobil, and J. Neill, Eds.), pp. 753-836. Raven Press, Ltd., New York.

Shabbir, A., DiStasio, S., Zhao, J., Cardozo, C. P., Wolff, M. S., and Caplan, A. J. (2005). Differential effects of the organochlorine pesticide DDT and its metabolite p,p'-DDE on p-glycoprotein activity and expression. *Toxicol Appl Pharmacol* **203**, 91-98.

Shaha, C. (2007). Modulators of spermatogenic cell survival. *Soc Reprod Fertil Suppl* **63**, 173-186.

Sheweita, S. A., Tilmisany, A. M., and Al-Sawaf, H. (2005). Mechanisms of male infertility: role of antioxidants. *Curr Drug Metab* **6**, 495-501.

Show, M. D., Folmer, J. S., Anway, M. D., and Zirkin, B. R. (2004). Testicular expression and distribution of the rat bcl2 modifying factor in response to reduced intratesticular testosterone. *Biol Reprod* **70**, 1153-1161.

Shum, W. W., Da Silva, N., McKee, M., Smith, P. J., Brown, D., and Breton, S. (2008). Transepithelial projections from basal cells are luminal sensors in pseudostratified epithelia. *Cell* **135**, 1108-1117.

Simoni, M., Gromoll, J., and Nieschlag, E. (1997). The follicle-stimulating hormone receptor: biochemistry, molecular biology, physiology, and pathophysiology. *Endocr Rev* **18**, 739-773.

Singh, J., O'Neill, C., and Handelsman, D. J. (1995). Induction of spermatogenesis by androgens in gonadotropin-deficient (hpg) mice. *Endocrinology* **136**, 5311-5321.

Soares, A., Guieysse, B., Jefferson, B., Cartmell, E., and Lester, J. N. (2008). Nonylphenol in the environment: A critical review on occurrence, fate, toxicity and treatment in wastewaters. *Environ Int.*

Sreeramulu, K., Liu, R., and Sharom, F. J. (2007). Interaction of insecticides with mammalian P-glycoprotein and their effect on its transport function. *Biochim Biophys Acta* **1768**, 1750-1757.

St-Pierre, N., Dufresne, J., Rooney, A. A., and Cyr, D. G. (2003). Neonatal hypothyroidism alters the localization of gap junctional protein connexin 43 in the testis and messenger RNA levels in the epididymis of the rat. *Biol Reprod* **68**, 1232-1240.

Stachel, B., Dougherty, R. C., Lahl, U., Schlosser, M., and Zeschmar, B. (1989). Toxic environmental chemicals in human semen: analytical method and case studies. *Andrologia* **21**, 282-291.

Staud, F., Ceckova, M., Micuda, S., and Pavek, P. (2010). Expression and function of p-glycoprotein in normal tissues: effect on pharmacokinetics. *Methods Mol Biol* **596**, 199-222.

Stefanidou, M., Maravelias, C., and Spiliopoulou, C. (2009). Human exposure to endocrine disruptors and breast milk. *Endocr Metab Immune Disord Drug Targets* **9**, 269-276.

Steinberger, E., Root, A., Ficher, M., and Smith, K. D. (1973). The role of androgens in the initiation of spermatogenesis in man. *The Journal of clinical endocrinology and metabolism* **37**, 746-751.

Stoltenberg, M., Lund, L., Juhl, S., Danscher, G., and Ernst, E. (1997a). Histochemical demonstration of zinc ions in human epididymis using autometallography. *Histochem J* **29**, 721-726.

Stoltenberg, M., Sorensen, M. B., Danscher, G., Juhl, S., Andreasen, A., and Ernst, E. (1997b). Autometallographic demonstration of zinc ions in rat sperm cells. *Mol Hum Reprod* **3**, 763-767.

Sturm, A., Cravedi, J. P., and Segner, H. (2001). Prochloraz and nonylphenol diethoxylate inhibit an mdr1-like activity in vitro, but do not alter hepatic levels of P-glycoprotein in trout exposed in vivo. *Aquat Toxicol* **53**, 215-228.

Su, L., Cheng, C. Y., and Mruk, D. D. (2009). Drug transporter, P-glycoprotein (MDR1), is an integrated component of the mammalian blood-testis barrier. *Int J Biochem Cell Biol* **41**, 2578-2587.

Sullivan, R., Frenette, G., and Girouard, J. (2007). Epididymosomes are involved in the acquisition of new sperm proteins during epididymal transit. *Asian J Androl* **9**, 483-491.

Takano, H., and Abe, K. (2004). AFM study of surface structure changes in mouse spermatozoa associated with maturation. *Methods Mol Biol* **242**, 85-94.

Tapanainen, J. S., Aittomaki, K., Min, J., Vaskivuo, T., and Huhtaniemi, I. T. (1997). Men homozygous for an inactivating mutation of the follicle-stimulating hormone (FSH) receptor gene present variable suppression of spermatogenesis and fertility. *Nat Genet* **15**, 205-206.

Teerds, K. J., and Dorrington, J. H. (1993). Localization of transforming growth factor beta 1 and beta 2 during testicular development in the rat. *Biol Reprod* **48**, 40-45.

Tesarik, J., Greco, E., and Mendoza, C. (2001). Assisted reproduction with in-vitro-cultured testicular spermatozoa in cases of severe germ cell apoptosis: a pilot study. *Hum Reprod* **16**, 2640-2645.

Thiebaut, F., Tsuruo, T., Hamada, H., Gottesman, M. M., Pastan, I., and Willingham, M. C. (1987). Cellular localization of the multidrug-resistance gene product P-glycoprotein in normal human tissues. *Proc Natl Acad Sci U S A* **84**, 7735-7738.

Thimon, V., Frenette, G., Saez, F., Thabet, M., and Sullivan, R. (2008). Protein composition of human epididymosomes collected during surgical vasectomy reversal: a proteomic and genomic approach. *Hum Reprod* **23**, 1698-1707.

Tindall, D. J., Schrader, W. T., and Means, A. R. (1974). The production of androgen binding protein by Sertoli Cells. *Curr Top Mol Endocrinol* **1**, 167-175.

Turner, T. T. (1991). Spermatozoa are exposed to a complex microenvironment as they traverse the epididymis. *Annals of the New York Academy of Sciences* **637**, 364-383.

Vasquez-Duhalt, R., Marquez-Rocha, F. J., Ponce, E., Licea, A. F., and Viana, M. T. (2005). Nonylphnenol, an integrated vision of a pollutant. *Applied Ecology and Environmental Research* **4**, 125.

Verhoeven, G. (2005). A Sertoli cell-specific knock-out of the androgen receptor. *Andrologia* **37**, 207-208.

Verhoeven, G., Willems, A., Denolet, E., Swinnen, J. V., and De Gendt, K. (2010). Androgens and spermatogenesis: lessons from transgenic mouse models. *Philos Trans R Soc Lond B Biol Sci* **365**, 1537-1556.

Vierula, M. E., Rankin, T. L., and Orgebin-Crist, M. C. (1995). Electron microscopic immunolocalization of the 18 and 29 kilodalton secretory proteins in the mouse epididymis: evidence for differential uptake by clear cells. *Microsc Res Tech* **30**, 24-36.

Viger, R. S., and Robaire, B. (1994). Immunocytochemical localization of 4-ene steroid 5 alpha-reductase type 1 along the rat epididymis during postnatal development. *Endocrinology* **134**, 2298-2306.

Vigodner, M., and Morris, P. L. (2005). Testicular expression of small ubiquitin-related modifier-1 (SUMO-1) supports multiple roles in spermatogenesis: silencing of sex chromosomes in spermatocytes, spermatid microtubule nucleation, and nuclear reshaping. *Dev Biol* **282**, 480-492.

Visconti, P. E., Galantino-Homer, H., Ning, X., Moore, G. D., Valenzuela, J. P., Jorgez, C. J., Alvarez, J. G., and Kopf, G. S. (1999). Cholesterol efflux-mediated signal transduction in mammalian sperm. beta-cyclodextrins initiate transmembrane

signaling leading to an increase in protein tyrosine phosphorylation and capacitation. *J Biol Chem* **274**, 3235-3242.

Volk, H., Potschka, H., and Loscher, W. (2005). Immunohistochemical localization of P-glycoprotein in rat brain and detection of its increased expression by seizures are sensitive to fixation and staining variables. *J Histochem Cytochem* **53**, 517-531.

Wagner, M. S., Wajner, S. M., and Maia, A. L. (2008). The role of thyroid hormone in testicular development and function. *J Endocrinol* **199**, 351-365.

Wahlstrom, T., Huhtaniemi, I., Hovatta, O., and Seppala, M. (1983). Localization of luteinizing hormone, follicle-stimulating hormone, prolactin, and their receptors in human and rat testis using immunohistochemistry and radioreceptor assay. *The Journal of clinical endocrinology and metabolism* **57**, 825-830.

Walker, W. H. (2003). Nongenomic actions of androgen in Sertoli cells. *Curr Top Dev Biol* **56**, 25-53.

Walker, W. H., and Cheng, J. (2005). FSH and testosterone signaling in Sertoli cells. *Reproduction* **130**, 15-28.

Wang, Q., Yin, S., Ai, J. S., Liang, C. G., Hou, Y., Chen, D. Y., Schatten, H., and Sun, Q. Y. (2006a). Histone deacetylation is required for orderly meiosis. *Cell Cycle* **5**, 766-774.

Wang, R. S., Yeh, S., Chen, L. M., Lin, H. Y., Zhang, C., Ni, J., Wu, C. C., di Sant'Agnese, P. A., deMesy-Bentley, K. L., Tzeng, C. R., and Chang, C. (2006b). Androgen receptor in sertoli cell is essential for germ cell nursery and junctional complex formation in mouse testes. *Endocrinology* **147**, 5624-5633.

Wang, T., Chen, M., Yan, Y. E., Xiao, F. Q., Pan, X. L., and Wang, H. (2009). Growth retardation of fetal rats exposed to nicotine in utero: possible involvement of CYP1A1, CYP2E1, and P-glycoprotein. *Environ Toxicol* **24**, 33-42.

Wijnholds, J., Scheffer, G. L., van der Valk, M., van der Valk, P., Beijnen, J. H., Scheper, R. J., and Borst, P. (1998). Multidrug resistance protein 1 protects the oropharyngeal mucosal layer and the testicular tubules against drug-induced damage. *J Exp Med* **188**, 797-808.

Wilson, E. M., and Smith, A. A. (1975). Localization of androgen receptors in rat testis: biochemical studies. *Curr Top Mol Endocrinol* **2**, 281-286.

Woltje, M., Kraus, J., and Hollt, V. (1998). Identification of a functional 3',5'-cyclic adenosine monophosphate response element within the second promoter of the mouse somatostatin receptor type 2 gene. *FEBS Lett* **440**, 107-110.

Wong, E. W., Mruk, D. D., Lee, W. M., and Cheng, C. Y. (2010). Regulation of blood-testis barrier dynamics by TGF-beta3 is a Cdc42-dependent protein trafficking event. *Proc Natl Acad Sci U S A* **107**, 11399-11404.

Wong, P. Y. D., Gong, X.D., Leung, G.P.H., and Cheukj, B.I.Y. (2002). Formation of the epididymal fluid microenvironment. In B. Robaire and B. T. Hinton eds., The epididymis: From molecules to clinical practice. Plenum Publisers, New York. Pp119-130.

Woolveridge, I., de Boer-Brouwer, M., Taylor, M. F., Teerds, K. J., Wu, F. C., and Morris, I. D. (1999). Apoptosis in the rat spermatogenic epithelium following androgen withdrawal: changes in apoptosis-related genes. *Biol Reprod* **60**, 461-470.

Xia, W., Wong, E. W., Mruk, D. D., and Cheng, C. Y. (2009). TGF-beta3 and TNFalpha perturb blood-testis barrier (BTB) dynamics by accelerating the clathrin-mediated

endocytosis of integral membrane proteins: a new concept of BTB regulation during spermatogenesis. *Dev Biol* **327**, 48-61.

Xu, C., Li, C. Y., and Kong, A. N. (2005). Induction of phase I, II and III drug metabolism/transport by xenobiotics. *Arch Pharm Res* **28**, 249-268.

Yan, H. H., and Cheng, C. Y. (2005). Blood-testis barrier dynamics are regulated by an engagement/disengagement mechanism between tight and adherens junctions via peripheral adaptors. *Proc Natl Acad Sci U S A* **102**, 11722-11727.

Yang, F., De La Fuente, R., Leu, N., Baumann, C., McLaughlin, K., and Wang, P. (2006). Mouse SYCP2 is required for synaptonemal complex assembly and chromosomal synapsis during male meiosis. *J Cell Biol* **174**, 497-507.

Yeung, C. H., Barfield, J. P., and Cooper, T. G. (2006). Physiological volume regulation by spermatozoa. *Mol Cell Endocrinol* **250**, 98-105.

Yeung, C. H., Perez-Sanchez, F., Schroter, S., Kirchhoff, C., and Cooper, T. G. (2001). Changes of the major sperm maturation-associated epididymal protein HE5 (CD52) on human ejaculated spermatozoa during incubation. *Mol Hum Reprod* **7**, 617-624.

Ying, G. G., Williams, B., and Kookana, R. (2002). Environmental fate of alkylphenols and alkylphenol ethoxylates--a review. *Environ Int* **28**, 215-226.

Yuan, L., Liu, J., Brundell, E., Daneholt, B., and Hoog, C. (2000). The murine SCP3 gene is required for synaptonemal complex assembly, chromosome synapsis, and male fertility. *Mol Cell* **5**, 73-83.

Zhang, J., Yang, M., Zhang, Y., and Chen, M. (2008). Biotransformation of nonylphenol ethoxylates during sewage treatment under anaerobic and aerobic conditions. *J Environ Sci (China)* **20**, 135-141.

Zhao, M., Shirley, C. R., Hayashi, S., Marcon, L., Mohapatra, B., Suganuma, R., Behringer, R. R., Boissonneault, G., Yanagimachi, R., and Meistrich, M. L. (2004). Transition nuclear proteins are required for normal chromatin condensation and functional sperm development. *Genesis* **38**, 200-213.

Zini, A., Defreitas, G., Freeman, M., Hechter, S., and Jarvi, K. (2000). Varicocele is associated with abnormal retention of cytoplasmic droplets by human spermatozoa. *Fertil Steril* **74**, 461-464.