THE CONSEQUENCES ON THE RAT EPIDIDYMIS OF INHIBITING 5-ALPHA-REDUCTASE

Natali Anne Henderson

Department of Pharmacology and Therapeutics

McGill University

Montréal, Québec

May, 2005

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

Your file Votre référence
ISBN: 978-0-494-21654-5
Our file Notre référence
ISBN: 978-0-494-21654-5

Dedication

This thesis is dedicated with much love and thanks to my family.

To my parents, Carl and Lillian, for their unconditional love, guidance and support throughout all my endeavours in life.

To my sister and brother, Tobi and Ryan, for their encouragement and lasting friendship.

To Scott, who shared this journey with me, I thank you for your love, immeasurable patience and unwavering belief in my capabilities.

ABSTRACT

The epididymis functions in the transport, maturation, storage and protection of spermatozoa. The structure and functions of the epididymis are dependent on androgens, primarily dihydrotestosterone (DHT). 5α-Reductases (types 1 and 2) are thus key enzymes in this tissue because of their role in converting testosterone to DHT. The goal of this thesis was to examine the consequences on the rat epididymis and epididymal sperm of inhibiting DHT formation using novel dual 5α-reductase inhibitors (i.e. inhibitors specific to both isoforms of 5α-reductase). The first objective was to analyze the effects of 5α-reductase inhibitor treatment on gene expression in the four epididymal segments (initial segment, caput, corpus and cauda epididymidis) using cDNA arrays. Treatment had highly segment specific effects on epididymal gene expression. Affected genes included those involved in fatty acid and lipid metabolism, regulation of ion and fluid transport, luminal acidification, oxidative defense and protein processing and degradation; these are essential processes contributing to the formation of an optimal luminal microenvironment required for proper sperm maturation. The next objective was to determine whether the observed changes in gene expression actually translated into effects on epididymal sperm functions. Fertility and several key facets of epididymal sperm maturation were analyzed. The percentages of motile and progressively motile sperm from the cauda epididymidis decreased and characteristic sperm motion parameters were altered. An elevated proportion of sperm from this region also retained their

cytoplasmic droplet. Matings with treated males resulted in fewer successful pregnancies and a higher rate of preimplantation loss. Lastly, cDNA arrays and quantitative real-time RT-PCR were used to elucidate potential signaling mechanisms via which DHT mediates and/or regulates its differential effects on epididymal gene expression and functions. Analysis of the expression of the 5α-reductase isozymes, androgen receptor and members of the IGF, FGF, TGF and VEGF families revealed novel segment-specific expression profiles in the epididymis that were differentially affected by 5α-reductase inhibition. Collectively, these studies established dual 5α-reductase inhibition as a useful approach to study DHT action in the epididymis and revealed many novel aspects of epididymal physiology as well as avenues for future research.

RÉSUMÉ

L'épididyme est impliqué dans le transport, la maturation, le stockage et la protection des spermatozoïdes. La structure et les fonctions de l'épididyme dépendent des androgènes, principalement de la dihydrotestostérone (DHT). Les enzymes 5α-réductases de types 1 et 2 jouent un rôle clé au niveau de l'épididyme en convertissant la testostérone en DHT. L'objectif de cette thèse a été de déterminer les effets de l'inhibition de la formation de DHT sur l'épididyme et les spermatozoïdes chez le rat en utilisant de nouveaux inhibiteurs doubles de 5α-réductases (i.e. inhibiteurs spécifiques aux deux types d'isoformes). Le premier objectif a été d'analyser les effets de l'inhibition des enzymes 5α-réductases sur l'expression des gènes au niveau des quatre segments épididymaires (le segment initial, le caput, le corpus et le cauda) à l'aide de microdéploiements (microarray) d'ADNc. Ce traitement a induit des effets hautement spécifiques sur l'expression des gènes en fonction du segment épididymaire. Les gènes affectés incluent ceux impliqués dans le métabolisme des acides gras et des lipides, la régulation du transport des ions et des fluides, l'acidification de la lumière, les défenses oxydatives et le traitement ainsi que la dégradation des protéines; des processus essentiels qui contribuent à la formation d'un microenvironement optimal nécessaire à la maturation des spermatozoïdes. Le second objectif a été de déterminer si les changements observés au niveau de l'expression des gènes étaient répercutés sur les fonctions des spermatozoïdes épididymaires. La fertilité et différents aspects de la maturation des spermatozoïdes ont été analysés. Le pourcentage de

spermatozoïdes motiles et ceux ayant une mobilité progressive obtenus du cauda diminue et les paramètres caractéristiques du mouvement des spermatozoïdes sont altérés. Une proportion élevée de spermatozoïdes de ce segment épididymaire ont retenu leur gouttelette cytoplasmique. L'accouplement de rats mâles traités a résulté en une diminution du nombre de grossesses et une augmentation du taux des pertes de pré-implantation. Finalement, des microdéploiements d'ADNc et l'analyse quantitative par qRT-PCR en temps réel ont été employés afin d'élucider les mécanismes potentiels de signalisation par lesquels la DHT transduit et/ou régule ses différents effets sur les fonctions et l'expression des gènes épididymaires. L'analyse de l'expression des isozymes de la 5α-réductase, du récepteur à androgènes, et des membres des familles des IGF, FGF, TGF, et VEGF ont révélé de nouveaux profils d'expression génique spécifiques à des segments de l'épididyme qui sont affectés de façons différentielles par l'inhibition des 5α-réductases. L'ensemble de ces études établi que la double inhibition des 5α-réductases est une approche utile a l'étude de l'action de la DHT au niveau de l'épididyme et a révélé plusieurs nouveaux aspects de la physiologie épididymaire ainsi que de nouvelles possibilités pour de futures recherches.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

LIST OF TABLES

ACKNOWLEDGEMENTS

I would like to express my sincere thanks to my supervisor, Dr. Bernard Robaire, for his wisdom, guidance and encouragement. He possesses a rare ability to understand and appreciate the individuality of each of his graduate students and for this I will always be grateful.

I would also like to thank Dr. Hales and Dr. Trasler for their valuable feedback and suggestions regarding all aspects of my research project.

I express my gratitude to my advisor, Dr. Ribeiro-Da-Silva, for his kind words and encouragement.

I thank the Department of Pharmacology and Therapeutics for the privilege of pursuing my studies in the department.

I gratefully acknowledge the CIHR (formally MRC) for my studentship award and for financial support of these studies.

These studies would not have been possible without samples of the 5α-reductase inhibitors (PNU157706 and FK143) that were generously given to us by Pharmacia & Upjohn and Fujisawa Pharmaceutical Co.

I would also like to thank the following people who made the lab an enjoyable and often eventful place to be:

Patrick Syntin, my first real friend in the lab, who showed me the ropes and commiserated with me over many morning coffees and Friday night pints. I only wish you could have stuck around longer!

To the early Thompson House crew, especially Rob and Chris, who proved that graduate student life didn't always have to be about science.

To Shayesta, with whom it has been a privilege to collaborate with in the final stages of my studies.

To my conference roommates (Shayesta, Tara, Michelle, Alexis and Katia) who made those times memorable for so many reasons.

To Mani, Nadine, Sarah, Adriana, Wafa, Huge, Trang, Ludo and all other past and present lab and journal club members as well as my friends outside the lab, especially Scott's family, thank-you.

Last but not least, my thanks to Rufus, because he is so darn cool.

PREFACE

FORMAT OF THE THESIS

This is a manuscript-based thesis and conforms to section I.C. of the "Guidelines for Thesis Preparation" of the Faculty of Graduate Studies and Research of McGill University. This thesis is comprised of five chapters. Chapter one includes a general introduction to the male reproductive system and a comprehensive review of the literature regarding the structure, functions and regulation of the epididymis and the roles of 5α-reductase in the male reproductive system. Chapter one concludes with the rationale for the studies presented in this thesis. Chapters two to four are data chapters presented as the duplicated text of published papers or papers submitted for publication. Chapter two was published in Journal of Endocrinology: Henderson NA, Cooke GM, Robaire B. Effects of PNU157706, a dual 5alpha-reductase inhibitor, on gene expression in the rat epididymis. 2004 May;181:245-261. Chapter three was published in Biology of Reproduction: Henderson NA, Robaire B. Effects of PNU157706, a dual 5alpha-reductase inhibitor, on rat epididymal sperm maturation and fertility. Biol Reprod. 2005 Feb;72:436-443. The copyright agreements of the respective publishers, Journal of Endocrinology by the Society for Endocrinology (http://www.endocrinology-journals.org/) and Biology of Reproduction by the Society for the Study of Reproduction (http://www.biolreprod.org/), permit the inclusion of these manuscripts in this thesis provided they are for non-commercial use. Chapter four has been submitted for publication. Connecting texts are included between these chapters

to ensure continuity of the thesis. Chapter five contains a summary and discussion of the results of this thesis and the List of Original Contributions. References are included at the end of each chapter. The appendix contains the ethics certificates for work on animal subjects and for the use of radioactive material, the proprietary rights notices for the Journal of Endocrinology and Biology of Reproduction and the co-author permission waiver for inclusion of manuscripts as part of this thesis.

CONTRIBUTIONS OF AUTHORS

All of the experiments and analyses were completed by the candidate with the sole exception of the *in vitro* 5α-reductase activity assays included in chapters two and four. These assays were done by Dr. Gerry M. Cooke, who appears as a coauthor of these manuscripts; Dr. Cooke is affiliated with the Toxicology Research Division, Health Products and Foods Branch, Food Directorate, Health Canada, and the Reproductive Biology Unit, and Departments of Cellular and Molecular Medicine and Obstetrics and Gynecology of the University of Ottawa.

CHAPTER 1

INTRODUCTION

A. General Introduction: Male Reproductive System

The main focus of this thesis is the epididymis, however, in order to fully appreciate this unique tissue, it is necessary to understand the biological context in which it exists. Hence, a general overview of the structure, functions and regulation of the male reproductive system will be presented. Since androgens are crucial regulators of reproductive function, special focus is paid to the mechanisms of androgen action. The general background presented will also provide a means of comparing the regulation and functions of the epididymis to those of the other reproductive tissues. Consideration of the unique spermatozoa-related functions and regulatory mechanisms pertaining to the epididymis is imperative for the development of therapies that target the epididymis, for example, as a means of contraception. Later sections of the introduction will deal exclusively with the epididymis and the key role of 5α-reduced androgens in this tissue.

In the simplest terms, the predominant role of the male reproductive system is to create and maintain functional spermatozoa and deliver them to the female reproductive tract. Achieving these tasks actually requires complex and coordinated interactions between the different cells, tissues and secretions of the male reproductive system.

1. The Testis

The testes are the primary organs of the male reproductive system. The testes occur in pairs and are located within the scrotum where a temperature 2-3

degrees lower than body temperature is maintained, a necessity for proper testicular function (1). The testis is protected by the tunica albuginea, a thick fibrous membrane composed of connective tissue and muscle fibers that encapsulates the testis and has contractile ability (2, 3). The testis is anatomically and functionally divided into two major compartments: the seminiferous epithelium and the interstitium. These compartments carry out the testicular functions of spermatogenesis and steroidogenesis, respectively. The complexity and significance of these processes warrant their own sections and are presented below.

The structural units of the seminiferous epithelium are the long, convoluted seminiferous tubules. Layers of peritubular myoid cells encase the seminiferous tubules; the tubules consist of both somatic cells (Sertoli cells) and developing germ cells. Sertoli cells were named after Enrico Sertoli who first described these very unique cells in the mid 1800s (4); since then, much has been learned about these tall, columnar cells that extend from the basement membrane to the lumen of the seminiferous tubule. Intercellular tight junctions between adjacent Sertoli cells form a major component of the blood-testis barrier, thus providing a protected and intimate environment for spermatogenesis to occur (5). Furthermore, the Sertoli cells are sustentacular or "nursing" cells with very unique shelf-like cytoplasmic processes that support and nourish the developing germ cells (6). As they differentiate, the germ cells progressively migrate across the seminiferous epithelium while maintaining essential contact with the Sertoli cells. This intimate physical association was first established by

Russell (6); since then the intricate communications between the Sertoli and germ cells have been extensively studied (reviewed in 7, 8). The cellular communication network is bi-directional and very elaborate involving paracrine factors, signaling molecules, proteases and antiproteases, transport/binding proteins, extracellular matrix components, energy metabolites and membrane components (7, 8).

The interstitium surrounds the seminiferous tubules and is comprised of blood vessels, lymphatics, nerves and several different cell types (9). Most notably, the steroid-producing Leydig cells are found in the interstitial space. Over 150 years ago, Franz von Leydig discovered these cells, noting their abundance and location in the testicular interstitium clustered around blood vessels and the seminiferous tubules (10). The significance of their anatomical location became apparent many years later upon the elucidation of the endocrine nature of these cells as the primary producer and supplier of testosterone to the blood stream and directly to the seminiferous epithelium (11). Leydig cell characteristics indicative of their steroidogenic role are the presence of abundant smooth endoplasmic reticulum, mitochondria with tubular cristae, and lipid droplets (12). Less common cell types in the interstitium include macrophages, monocytes, mast cells and fibroblasts (9, 13).

1.1. Spermatogenesis

In the early 1950s, landmark studies by Leblond and Clermont first defined the well-organized and highly regulated process of spermatogenesis (14). Each

spermatogenic cycle essentially consists of three phases. In the initial proliferative phase, diploid spermatogonial cells, from a self-renewing stem cell pool, undergo a period of mitotic divisions giving rise to spermatocytes, which then enter a meiotic phase (DNA synthesis and two meiotic divisions) that gives rise to haploid spermatids. In the last phase, termed spermiogenesis, the round spermatids mature morphologically into elongated spermatids. This is a critical process that not only involves the differentiation of sperm into the familiar head, midpiece and tail structures, but also the shedding of all but a small remnant of cytoplasm (cytoplasmic droplet), and nuclear events such as extensive chromatin remodelling (chromatin condensation) facilitated by the replacement of histones first by transition proteins and then by protamines (15, 16).

Spermatogenesis is a unique process that begins during fetal life, is arrested in infancy and restarts at puberty to continue throughout life in the male. The duration of the spermatogenic cycle differs among species (e.g., 64 days in humans, 49-52 days in rats (depending on the strain)), and new cycles are initiated prior to the completion of previous cycles such that older germ cells are continuously pushed toward the lumen (14, 17). These spermatogenic "waves" give rise to the distinctive stratification of the seminiferous epithelium, wherein groups of germ cells in different stages of development reside longitudinally within the same tubule. Upon reaching the luminal border, elongated spermatids disconnect from the Sertoli cells, cast off their excess cytoplasm (residual body) and are released as spermatozoa into the tubular lumen in a process called

spermiation (17). The residual bodies are engulfed and digested by the Sertoli cells (18).

1.2. Steroidogenesis

Testosterone is the primary male sex steroid. Greater than 95% of circulating testosterone is produced by the testicular Leydig cells (11). Within Leydig cells, cholesterol, the precursor of all steroids, is transferred from the outer to the inner mitochondrial membrane by steroidogenic acute regulatory protein (StAR), where the conversion of cholesterol to pregnenolone is catalyzed by the cholesterol sidechain cleavage cytochrome P-450 enzyme complex (P450ssc) (19). This is the rate-limiting step in the androgen biosynthetic pathway. The subsequent enzymatic steps occur in the smooth endoplasmic reticulum: 17α-hydroxylase/C17-20-lyase converts pregnenolone to dehydroepiandrosterone, 17β-hydroxysteroid dehydrogenase converts dehydroepiandrosterone to androstenediol, and finally 3β-hydroxysteroid dehydrogenase converts androstenediol to testosterone (20).

While testosterone acts locally within the testis itself, the extent of its functions go well beyond the tissue where it is produced Testosterone, bound to carrier proteins (i.e., SHBG and albumin), travels via the blood stream to carry out specific functions throughout the body. In addition to extratesticular components of the reproductive system, androgens also target many tissues including fat, muscle, bone, the central nervous system, the cardiovascular system and the immune system (21-25). Importantly, within specific target

tissues, testosterone can be reduced by 5α-reductase to the more potent androgen dihydrotestosterone (DHT), or be converted to estradiol by the enzyme P450-aromatase (26, 27). These conversions allow testosterone to achieve an even greater range of complex physiological functions.

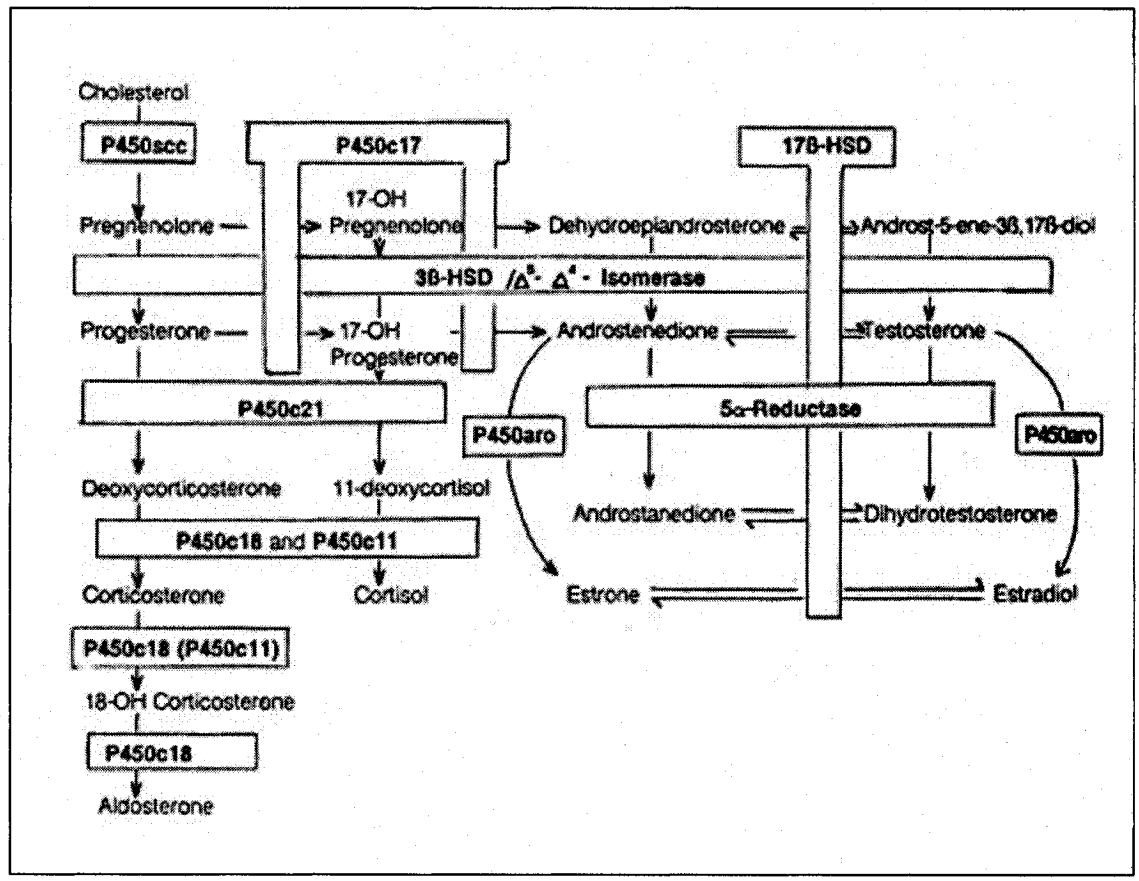


Figure 1. The steroidogenic pathway.

2. The Extratesticular Components

In addition to the testes, there are several other important components of the male reproductive system; these tissues include a series of sperm-conducting ducts, the sex accessory glands and the external genitalia (penis and scrotum).

2.1. The Excurrent Ducts

The sperm conducting tissues, referred to as the excurrent duct system, provide a pathway for sperm from the seminiferous epithelium in the testis to the site of ejaculation; sequentially, this pathway is comprised of the efferent ducts, epididymis, and vas deferens (28).

The rete testis, not technically part of the excurrent duct system as it is located within the testis, is a network of anastomising channels lined by a cuboidal epithelium that collects sperm from the terminal ends of the seminiferous tubules. Connected to the rete testis are the efferent ducts (vasa efferentia) that carry sperm out of the testis proper. The efferent ducts are a series of straight or slightly convoluted tubules with a columnar epithelium that eventually anastomose to form one tubule that connects to the head of the epididymis. The efferent ducts are unique in that they are the only region in the excurrent duct system lined by cilia (29); initially thought to propel sperm towards the epididymis, it is now believed the cilia actually serve to mix the fluid contents of the lumen since these cilia do not beat in the same direction (30). Upon leaving the efferent ducts, sperm pass through the epididymis. The epididymis will be presented in depth in a subsequent part of this introductory chapter. The vas deferens (ductus deferens) is a straight muscular tube that transports sperm out of the epididymis upon ejaculation. Composed mainly of principal cells (columnar cells), the epithelium lining the proximal, distal and terminal regions of the vas deferens is morphologically distinct.

Several forces are involved in moving the sperm through the excurrent duct system, including hydrostatic pressure created by constant secretion of testicular fluid, contractions of the tunica albuginea of the testis, the peritubular myoid cells surrounding the seminiferous tubules, the smooth muscle surrounding the excurrent ducts and a vacuum force created by the ejaculation of stored sperm (28).

Originally viewed as a simple conduit system for sperm, it is now known that the excurrent duct system has more specialized roles. For example, it was postulated as early as 1920 that the epithelium of the efferent ducts had fluid reabsorption capabilities (31); this was confirmed in 1959 by tracer re-uptake studies (32). It is now known that, depending on the species, the efferent ducts are responsible for the reabsorption of up to 95% of the fluid produced by the testis (reviewed in 33). Furthermore, the reabsorption of fluid and the absorption and secretion of various molecules by the specialized epithelium lining the excurrent ducts actively modulates the composition of the luminal fluid bathing the sperm. Lastly, the efferent ducts and the vas deferens are also capable of spermiophagy (phagocytosis of spermatozoa) that usually occurs in response to abnormal conditions (e.g., tubule obstruction) but potentially under normal conditions as well (34-36).

2.2. The Sex Accessory Tissues

The sex accessory glands include the seminal vesicles and bulbourethral (Cowper's) glands, which occur in pairs, and the prostate (37). All species have

a prostate gland, while some species lack the seminal vesicles and/or bulbourethral glands. Located near the vas deferens-urethra junction, the predominant role of these glands is the secretion of seminal fluids that provide a vehicle for sperm at the time of ejaculation (37-39). The seminal vesicles contribute the largest amount of seminal fluid (~65%) and all together the sex accessory gland secretions make up greater than 95% of the ejaculate volume (37). The composition of the seminal fluid includes rich energy sources (sorbitol, fructose), protective factors such as buffering ions and proteins that shield the sperm from the foreign female reproductive tract environment, proteolytic enzymes that facilitate sperm penetration of the cervical mucous, prostaglandins believed to stimulate the female tract and some molecules of unknown function (e.g., spermine) (38, 40-42). Additionally, in rodent species, the seminal fluid coagulates to form a hard copulatory plug that ensures the retention and transport of sperm in the female reproductive tract (43).

B. Regulation of the Male Reproductive System

The regulation of the male reproductive system is extremely complex, involving numerous external and internal factors and the interplay between its different components. Many reviews have been published that extensively cover the numerous factors and mechanisms involved in the regulation of male reproductive function (28, 37, 44, 45). Therefore, the following section will only serve to present major regulatory mechanisms and some factors particularly

relevant to this thesis. Special attention is focussed on the roles of androgens and subsequently the mechanisms of androgen action.

1. The Hypothalamus-Pituitary-Testis Axis

Very early studies using the classic endocrinology technique of endocrine gland removal followed by replacement with glandular extracts first revealed the important neuroendocrine regulation of reproductive function mediated by the hypothalamus and pituitary (46, 47). The pulsatile release of gonadotropin-releasing hormone (GnRH) from the hypothalamus controls the synthesis and secretion of two circulating gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH), from the anterior pituitary. Both LH and FSH target the testis; LH, secreted in a pulsatile fashion, stimulates Leydig cells to produce testosterone while FSH acts on the Sertoli cells and is required for the initiation of normal spermatogenesis. FSH also stimulates Sertoli cells to secrete androgen-binding protein (ABP), a carrier molecule for extracellular androgens.

The integrated hypothalamus-pituitary-testicular axis is controlled by complex negative feedback mechanisms. Testosterone feeds back to the hypothalamus and pituitary where it negatively controls the release of LH, and to a lesser extent FSH (48-50). The active metabolites of testosterone, estradiol and DHT, have different effects on the hypothalamic-pituitary-testicular axis; like testosterone, estradiol can also diminish LH release while DHT appears to have little or no effect on gonadotropin release (48, 50-53). In addition to steroidal feedback control, FSH secretion from the pituitary is also negatively regulated by

the Sertoli cell-produced peptide hormone inhibin, thus forming a separate testis-pituitary feedback loop (54, 55).

2. Steroid Hormones

Androgens are crucial regulators of sexual differentiation and development of the male phenotype and sexual maturation at puberty. Androgen insensitivity syndromes (AIS), caused by various androgen receptor gene mutations that impair androgen receptor function, unequivocally demonstrate the fundamental roles of androgens in male reproductive physiology (reviewed in 56). First described over 50 years ago, AIS range from the total abolishment of androgen receptor (AR) function causing complete feminization at birth, to partial disruptions of AR function resulting in different degrees of feminization or ambiguous genitalia, to subtle AR mutations that depress spermatogenesis while causing no overt sexual phenotype (56-58).

Testosterone and its active metabolite, DHT, both act via the single androgen receptor. DHT is more potent than testosterone due to a higher binding affinity for the AR leading to the formation of a more stable receptor-androgen complex (59-61). The relative potency of DHT is tissue dependent; in the prostate for example, DHT is approximately 4-5 times more potent than testosterone (62). Initially thought to simply amplify the action of testosterone, it is now also believed that DHT has biologically separate roles during development and sexual maturation, as well as in adulthood. Supporting this is the occurrence of mutations in the gene encoding 5α-reductase, the enzyme responsible for

converting testosterone to DHT, that result in a unique form of male pseudohermaphroditism despite normal or elevated plasma testosterone levels (63). Though genetically male (46, XY) and possessing bilateral testes and normal internal Wolffian-duct structures (epididymides, vasa deferentia, seminal vesicles, ejaculatory ducts), individuals deficient in 5α-reductase have highly underdeveloped prostates and ambiguous external genitalia at birth with subsequent virilization (including phallic growth) at the time of puberty (63, 64).

It is now commonly accepted that during embryogenesis, testosterone is responsible for the normal development of the Wolffian-duct structures and DHT is responsible for the development of the prostate, urethra, penis and scrotum that derive from the urogenital sinus and tubercle. Additionally, DHT appears to be responsible for sexual maturation at puberty characterized by altered facial and body hair growth patterns and maturation of the external genitalia while testosterone mediates the pubertal and post pubertal increase in muscle mass and the development of male sex drive (libido) (65, 66). Androgens are also fundamental for the control and maintenance of sexual function and behaviour during adulthood (65).

Testosterone, once secreted from the Leydig cell, binds to ABP and is maintained at very high concentrations within the testis where it is the primary hormonal regulator of spermatogenesis. Interestingly, the effect of testosterone on spermatogenesis is biphasic; depending on the dose, testosterone can both promote or inhibit the process *in vivo* (67-69). The testosterone-dependent regulation of spermatogenesis in different species has recently been reviewed

(70). In particular, several studies have demonstrated that testosterone is critical for later stages of spermatogenesis and spermiogenesis (68, 71-73) as well as spermiation (74).

O'Donnell et al. reported a peak in 5α-reductase isoenzymes and 5α-reduced metabolite production in the testis during puberty that coincided with the first wave of spermatogenesis in the rat, suggesting a role for 5α-reduced metabolites in the initiation, but not the maintenance, of spermatogenesis (75). Earlier studies by this group showed that 5α-reduced androgens are important for maintaining androgen action on rat spermatogenesis (particularly mid-spermiogenesis) only when testicular testosterone concentrations are experimentally reduced (76, 77). Under normal physiological conditions, the need to amplify testosterone signaling in the testis by conversion to DHT seems unlikely given that local testosterone concentrations are already extremely high. For example, within the human testis, testosterone concentrations are greater than 100-fold serum levels (78). Indeed, the very low levels of testicular 5α-reductases present in the adult do not appear to play an important role in spermatogenesis.

While the testis is regarded as a primarily testosterone-dependent tissue, DHT is known to be the primary androgen acting in other reproductive tissues. The prostate is perhaps the most well characterized DHT-dependent tissue in the male reproductive system. This is largely due to the prevalence of two clinically important diseases (benign prostatic hyperplasia and prostate cancer) that has engendered numerous studies aimed at understanding the physiology of this

tissue. There are numerous reviews that describe the important role of DHT in the prostate (79, 80). The seminal vesicles are also highly dependent on androgens, and DHT is the primary androgen in this tissue (37, 81). Furthermore, evidence indicates a role for DHT in seminal vesicle development, which brings into question the generally accepted role of testosterone in the development of this particular Wolffian-duct derived tissue (82). The roles for DHT in epididymal physiology will be discussed in later sections.

Clearly, androgens are the predominant hormonal regulators of reproductive function; however, roles for other hormones are quickly emerging. The ability of exogenous estrogen exposure to adversely affect spermatogenesis and male fertility was the first indication that estrogens may play a role in male reproduction (83, 84). A clear confirmation of the role of estrogen in the male is the perturbed reproductive phenotypes of recently generated transgenic mice lacking estrogen receptors or p450-aromatase (85-87). Interestingly, detailed histological studies of the estrogen receptor α knockout mice (ERαKO) carried out by Hess et al. have shown that the efferent ducts are primary targets for estrogen regulation (87, 88). The inability of the efferent ducts to carry out their primary function of fluid reabsorption is largely responsible for the impairment of spermatogenesis and fertility in the ERαKO mice. These observations highlight the important contributions of the extratesticular components of the male reproductive system to normal fertility.

3. Mechanisms of Androgen Action

The traditional mode of androgen action is via the androgen receptor, a classical nuclear receptor comprising an amino-terminal domain, a DNA-binding domain, a hinge region and a ligand-binding domain (89). Basically, within an androgen target cell, testosterone (or DHT depending on the target tissue) binds to the ligand-binding domain of the androgen receptor causing the dissociation of accessory proteins (e.g., heat shock proteins), receptor conformation changes that reveal the DNA-binding domain and subsequent translocation of the receptor into the nucleus where the DNA-binding domain interacts with specific DNA sequence elements (androgen response elements or AREs) on target genes to modulate transcriptional activity. The first link between steroid hormones and gene activation was established more than 40 years ago by Clever and Karlson (90) who injected the steroid ecdysone into insect larvae and observed changes in chromosome structure (so-called "puff reactions") within a certain time interval. Since then the genomic actions of steroid hormones have been firmly established and extensively reviewed (91-94). The discovery of coregulators has added a new layer of complexity to androgen receptor-dependent transcriptional regulation (reviewed in 95); essentially, coregulators (coactivators and corepressors) comprise a novel group of proteins that modulates the nuclear receptor transactivation function in a cell- and tissue-specific manner.

Interestingly, while the expression of many genes has been described as androgen-regulated, a biologically functional androgen response element (ARE) within the promoter and/or intronic regions of only a handful of genes has been

demonstrated thus far. The human prostate-specific genes PSA and hKLK2 and the rat prostate-specific genes probasin and the C subunit of prostatein are among these genes (96-99). Advances in genome sequencing should facilitate the identification of putative ARE sequences among known androgen-regulated genes as well as the discovery of candidate androgen-regulated genes.

In contrast to the well-characterized genomic events mediated by androgens, research accumulated over the past 20 years has revealed unequivocal non-genomic or "extranuclear" effects of these same hormones. These effects are rapid (seconds to minutes) and therefore incompatible with the classic genomic model of hormone action characterized by latent effects due to the time-course of transcriptional and translational events. Indeed, unlike the genomic effects, the non-genomic effects are insensitive to inhibitors of transcription and translation. These alternative steroid effects have been described for all classes of steroids and in a number of different tissues (reviewed in 100, 101).

In regards to the non-genomic effects of androgens in particular, testosterone has been shown to induce increases in Ca^{2+} levels by both influx of Ca^{2+} into target cells and Ca^{2+} release from intracellular stores, and to induce Ca^{2+}-mediated diacylglycerol and inositol 1,4,5-triphosphate formation (102-104). DHT has also been shown to rapidly and reversibly activate mitogen-activated protein kinases (MAPKs) in prostate cancer cell lines (105) and to interact with the Src/Raf-1/Erk-2 pathway (106, 107). As with other steroid hormones, the non-genomic actions of androgens are widely presumed to be mediated by cell-

surface membrane receptors as evidenced by studies using plasma-membrane impermeable testosterone-BSA conjugates (104). Treatment with antiandrogens either attenuates or does not affect the non-genomic actions of testosterone, indicating that the androgen receptor itself is likely only involved in some cases of non-genomic androgen action (100, 101). While the current knowledge of non-genomic steroid action is by no means complete, the diversity of the mechanisms of non-genomic actions is very evident and presents a fascinating and challenging new area of research.

4. Growth Factors

As with all tissues, growth factors have obvious roles in regulating the growth and development of the male reproductive system. Furthermore, growth factors are also known to figure prominently in reproductive tissue pathologies, for example, during tumour growth and benign prostatic enlargement (108-111). It is therefore not surprising that growth factors are thought to be involved in regulating the functions of normal adult reproductive tissues.

In the case of the testis, the expression, localization and production of components of several different growth factor systems (IGF, TGF, EGF, FGF, PDGF, NGF) have been characterized (reviewed in 112). IGF-1, for example, has well-documented effects on the gonad and data indicate that this particular growth factor is essential for reproductive maturation (reviewed in 113). Furthermore, IGF-1 is a potential regulator of adult neuro-endocrine functions via actions at the level of the hypothalamus where it is co-expressed with the IGF-1

receptor (113, 114). Other studies indicate that both IGF-1 and TGF-β1 regulate Leydig cell function in an autocrine manner; IGF-1 is a positive regulator while TGF-β1 inhibits Leydig cell function (115). In fact, the TGF-β family is one of the best characterized growth factor families acting in the testis; in addition to roles in testis development, various TGF-β isoforms are known to regulate spermatogenesis, steroidogenesis, extracellular matrix synthesis and Sertoli cell tight junction dynamics in this tissue (reviewed in 116).

In the case of the prostate, it is likely that the androgen sensitivity of this tissue is mediated by the production of peptide growth factors from stromal cells which act in a paracrine manner as direct intermediates of androgen action on epithelial cells (117). Different growth factors have stimulatory (e.g. FGFs, IGFs) and inhibitory (e.g. TGF-β1) effects that maintain an equilibrium of prostate epithelial cell numbers (117). Interestingly, studies using prostate cell lines have provided evidence to support androgen receptor cross talk with growth factor signaling pathways, resulting in both enhanced androgen responses and receptor ligand-independent activation and modulation of androgen receptor transactivation (118-121).

Though the local expression and production of growth factors and growth factor receptors in healthy adult reproductive tissues certainly implies important functions for these molecules, for the most part, definitive local roles for most growth factor systems in these tissues remain unresolved. This is especially true for reproductive tissues other than the testis and prostate where data concerning growth factor expression, localization and production is even sparser.

C. The Epididymis

Early researchers in reproduction generally thought that sperm from the testis were already functionally mature, in other words, they were fully motile and possessed all the features required for fertilization of the egg. When it was observed as early as 1897 that fluid and sperm from the proximal and distal parts of the epididymis had different characteristics, a controversy on the role of the epididymis in sperm maturation was born (122, 123). Some researchers maintained that sperm were already fully mature before reaching the epididymis, others believed that sperm maturation was intrinsic to the germ cells themselves and was fulfilled by the time it took sperm to traverse the epididymis, while still others speculated that the epididymis, via its secretions, was actively involved in sperm maturation (124-127). This controversy lasted throughout much of the first half of the 20th century until renewed interest and advances in methodology allowed it to be resolved. An excellent historical perspective on the epididymis, from its earliest mention in the 4th century BC up to the present time, was documented by Orgebin-Crist and provides useful insight and accounts of the breakthroughs and advances in epididymal research over the years (128). In the late 1960s, pioneering studies by Orgebin-Crist (129) herself as well as Bedford (130) were the first to demonstrate that sperm maturation was not simply due to the passage of time, rather, the acquisition of motility and fertilizing ability required the exposure of sperm to the changing luminal environment of the epididymis. These findings finally revealed an important role for the epididymis in sperm maturation, sparking a renewed interest in this tissue. Since then,

research efforts devoted to increasing our understanding of epididymal physiology have dramatically increased. The following part of the introduction will focus on the structure, functions and regulation of the epididymis, highlighting the unique attributes of this tissue in comparison to other components of the male reproductive system.

1. Structure

Epididymides occur in pairs, one attached to the back of each testis. They are the major components of the excurrent duct system, receiving testicular inputs of sperm and fluid via the efferent ducts and emptying into the vas deferens (28, 131). The epididymis is a single, long and highly convoluted tubule comprised of two main compartments: the epididymal epithelium and the interior lumen. The lumen contains fluid with a complex and evolving composition and the maturing germ cells. The tubule is surrounded by connective tissue occupied by layers of smooth muscle, nerves, blood vessels and lymphatics (28, 131). Depending on the species, the tubule measures between 3 and 80 meters when unwound (132, 133) and it takes sperm approximately 8-11 days to travel from one end of the duct to the other (28, 134). The epididymis is structurally divided into four major segments: the initial segment closest to the testis, followed by the caput, corpus and cauda epididymides (28, 131, 135). These divisions are not only based on gross anatomical and morphological differences along the duct, but biochemical and functional differences as well. There is also evidence to suggest that the proximal part of the epididymis stems from a different

embryological origin (mesonephric tubules) than the rest of the duct (mesonephric or Wolffian duct) (136).

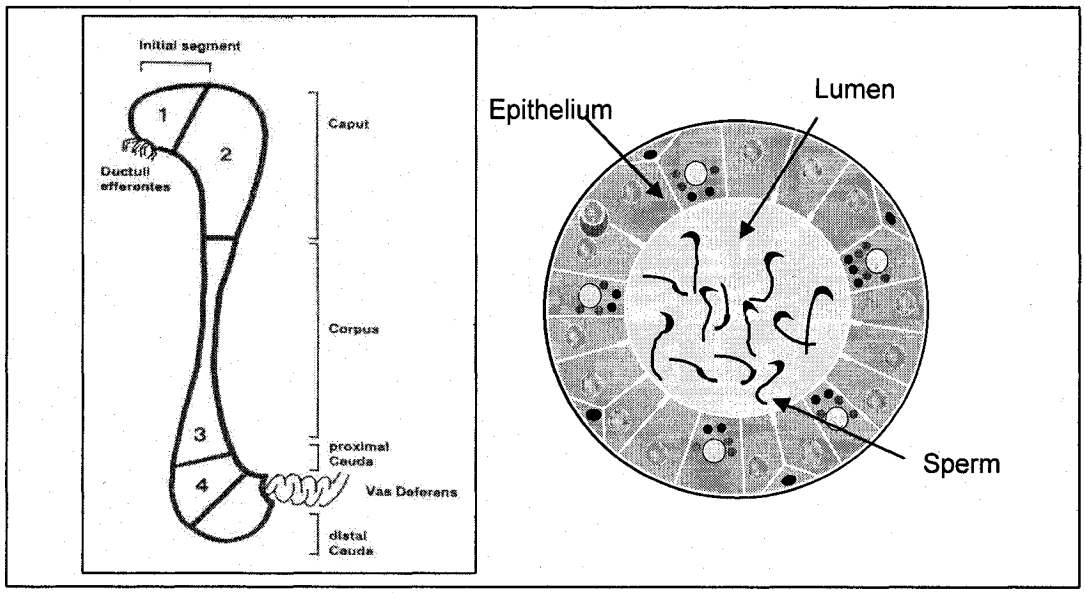


Figure 2. Epididymal segments and tubule compartments.

2. Histology

The epididymal epithelium is comprised of several different cell types; the presence and proportion of the different cell types is region-specific. A brief review of the different cell types will be presented. A more thorough description of the structural features of these cells can be found in comprehensive reviews (28, 137).

The principal cell is the predominant cell type within the epididymis. Present throughout the length of the duct, principal cells represent nearly 80% of the epithelial cell content in the initial segment and approximately 65% in the cauda epididymidis. The principal cells are columnar in shape and extend from the basement membrane to the lumen. They show regional differences in cell height; principal cells are very tall in the initial segment and become much

shorter in the cauda epididymidis. This is consistent with the increased luminal diameter of the more distal regions of the duct. Regional differences also exist in the appearance and organization of the secretory and endocytic organelles (28, 137). These differences likely reflect their different functions along the epididymis. Indeed, the coordinated secretory and endocytic activities of the principal cells and other epithelial cell types within the different epididymal segments directly modulate the composition of the luminal fluid (28, 137, 138-142). Exposure of the germ cells to the continuously changing luminal environment is crucial for proper sperm maturation, as will be discussed later on.

Basal cells are also found throughout the epididymis and constitute the second most abundant cell type in the epithelium (28, 137). Unlike principal cells, basal cells do not come in contact with the lumen; rather, they reside in between principal cells at the base of the epithelium in close contact with the basement membrane. Basal cells are flat, elongated cells with processes that extend along the basement membrane and sometimes up towards the lumen (143). Specific functions have yet to be ascribed to this cell type. A role in local immune defense has been proposed by some investigators who found that basal cells express macrophage antigens (144, 145); however, this feature appears to be species-specific (146, 147).

The clear cells are large cells that span the epithelium; they are most abundant in the cauda epididymidis but are also present in the caput and corpus epididymides. Clear cells have endocytic properties and are thought to actively remove material from the luminal compartment (28, 137). Part of this material

appears to be the contents of the cytoplasmic droplet that is shed from sperm as they transit through the epididymis (148).

The halo, narrow and apical cells are the least abundant cell types in the epididymis (28, 137). Halo cells are small cells located throughout the epididymis. Data exists to suggest that the halo cells are the primary immune cells in the epididymis; this cell type consists of helper T lymphocytes, cytotoxic T lymphocytes and monocytes (147). Narrow and apical cells are confined to the initial segment of the epididymis. Aside from endocytic capability, little is known regarding the function of either of these cell types.

2.1. The Blood-Epididymis Barrier

The blood-epididymis barrier prevents the transfer of materials from the general circulation into the epididymal lumen. Structurally, apical tight junctions between adjacent principal cells are responsible for the formation of the blood-epididymis barrier. Functionally, these cellular contacts are crucial for creating a protective and selective microenvironment for sperm maturation to occur (reviewed in 149).

The existence of a blood-epididymis barrier was first established in the early 1970s (150). In 1995, Cyr et al. observed that there were regional differences in the ultrastructural features of tight junctional complexes between epididymal principal cells (151). Ongoing work from Cyr's lab has focused on the characterization of the integral membrane proteins involved in epididymal tight-junction formation (e.g., cadherin, occludin, claudin-1); these studies, as well as

others, have contributed to our knowledge of the development, localization and regulation of tight-junctional complexes in the epididymis (149, 152-154).

3. Functions

In addition to sharing sperm transport functions with the other components of the excurrent duct system, the epididymis carries out other crucial sperm-related functions including sperm maturation, storage and protection.

3.1. Sperm Maturation

Sperm released from the testis are not yet functionally mature; in other words, they cannot swim or fertilize eggs. The acquisition of forward motility and fertilizing ability is referred to as maturation; sperm mature as they pass through the epididymis (129). While sperm passage through the epididymis is an absolute requirement for maturation, the exact region in the epididymis where sperm first acquire fertilizing potential varies among species. Generally, sperm become fertile by the time they reach the distal corpus/proximal cauda epididymidis (155-158). Similarly, sperm gain the potential for progressive motility by the time they reach the cauda epididymidis (155, 159). The gradual acquisition of potential for forward motility is reflected by the characteristic motion patterns of sperm isolated from the caput and cauda epididymides; caput epididymal sperm display a curved or circular swimming pattern, while cauda epididymal sperm are more vigorous and swim in a straight path (159).

3.1.1. Sperm Modifications During Epididymal Transit

In addition to the obvious changes in motility and fertilizing potential, several morphological and biochemical changes in spermatozoa also occur during epididymal transit (160). Morphologically, the most apparent change is the migration of the cytoplasmic droplet along the midpiece of the sperm and its eventual shedding in the distal regions of the epididymis (160, 161). Intracellular sperm organelles are also modified during maturation. For example, structural changes in several organelles, including the nucleus and mitochondria, arise due the formation of stabilizing disulfide bonds (162, 163). The extensive remodelling of the lipid, protein and glycoprotein composition of the sperm plasma membrane is another feature of sperm maturation (164-167). The ratios of cholesterol:phospholipid and saturated:unsaturated fatty acids are altered in the maturing sperm membrane leading to changes in membrane fluidity (164, 168). The acquisition of new proteins and/or the modification of existing sperm membrane proteins is important for the capacitation of sperm in the female reproductive tract and sperm interaction with the female gametes (164, 165).

Since spermatozoa are generally considered to be biosynthetically inert once they leave the testis, maturational changes are necessarily mediated by proteins that were already present in sperm within the testis that become modified and activated in the epididymis (e.g., by proteolysis), or by the permanent or transient interaction of sperm directly with enzymes, binding molecules and other components present in the epididymal luminal fluid (165). Either way, sperm maturation ultimately relies on the proper functioning of the

epididymal epithelium, which controls the regional composition of the luminal fluid (169).

3.1.2. Creation of the Epididymal Luminal Environment

Micropuncture studies have been instrumental in determining the composition of the luminal fluid in the different epididymal regions (170). It is now known that the fluid microenvironment is very dynamic and dependent on the selective absorption and secretion of components by the epididymal epithelium (28, 137, 171). Major constituents include small organic molecules, ions, amino acids, peptides, proteins and lipids (172-177).

Many individual processes are involved in the formation of the luminal environment; these processes include fluid and ion transport, luminal acidification as well as protein and lipid secretion, processing and degradation.

3.1.2.1. Fluid and Ions

The major role of fluid transport across the epididymal epithelium is to concentrate sperm as they travel through the tubule (177); by the time spermatozoa reach the cauda epididymidis, they have been concentrated twenty-fold (171). In addition, this process regulates the concentration of constituents in the luminal fluid to which sperm are exposed.

Much of the fluid reabsorption occurs in the efferent ducts and the proximal portion of the epididymis (171). The expression of aquaporins (water channels), a local renin-angiotensin system and active ion transport are factors

involved in fluid transport (177, 178). In fact, the ionic composition of epididymal luminal fluid is markedly different in the different epididymal regions (177). Ions involved in mediating the absorption of water include chloride (caput) and sodium (cauda) (179, 180).

The ionic composition of the luminal fluid also determines its pH. In the rat, intraluminal pH decreases between the initial segment (pH 7.2) and the cauda epididymidis (pH 6.8) (181, 182). The distribution of luminal bicarbonate ions along the epididymis mirrors the pattern of pH decline (181, 183). In 1980, Au and Wong showed that the epithelial reabsorption of both bicarbonate and sodium ions were responsible for the decline in pH (184). Anion exchangers, ion channels, specific ATPases and carbonic anhydrase are implicated in the acidification process (176, 185, 186). Still, a specific role for luminal acidification in sperm maturation remains unclear. Several lines of evidence do however suggest a role for luminal acidification in sperm storage (176).

3.1.2.2. Proteins and Lipids

In earlier studies, SDS polyacrylamide gel electrophoresis was used to characterize the protein content of the luminal fluid from the different regions of the epididymis and compare it to that of blood or rete testis fluid (187, 188). These studies revealed very few similarities between the protein content of epididymal luminal fluid and blood or rete testis fluid; in addition, the protein profiles from the different epididymal regions differed markedly. The conclusion was that the epididymis must be able to synthesize and secrete new proteins into

the lumen. This was confirmed in 1980 by Brooks and Higgins who showed that epididymal tissues *in vitro* incorporated the radioactive amino acid precursor ^{35}S-methionine into new proteins and secreted them into the incubation medium (189). Since then, the characterization and identification of proteins secreted by the epididymal epithelium have been the focus of intense investigation (138, 190-192). Advances in proteomic analysis techniques have greatly aided in these tasks, allowing the elucidation of various secreted proteins including different types of binding proteins, glycosidases, antioxidant enzymes, clusterin, proteases and protease inhibitors (193, 194).

The sequential exposure of sperm to the various types of proteins synthesized and secreted by the epididymal epithelium is imperative for proper sperm maturation (139, 193, 194). Moreover, while fluid regulation assures the right quantity of proteins present in the epididymal lumen, the qualitative evolution of proteins along the tubule involves post-secretory events such as protein processing and degradation and reabsorption of proteins by the epithelium. Indeed, the ability of the epididymal epithelium to endocytose specific proteins has been demonstrated; for example, the endocytosis of androgen binding protein, clusterin, alpha-2 macroglobulin and transferrin has been characterized in the rat (140, 142, 195, 196).

The epididymal lumen contains many lipids and lipid substrates (176). The lipid-rich luminal environment is thought to be important for the maturation of the sperm plasma membrane as well as the maintenance of the apical membrane of epididymal epithelial cells. However, lipid-dependent mechanisms

that may mediate these events remain unresolved (164). Nevertheless, it is well established that the modification and exchange of sperm membrane lipids is an important feature of sperm maturation (166-168).

It has been known for many decades that some of the most highly concentrated constituents in the lumen are the phospholipid glycerylphosphorylcholine (GPC) and the organic compound carnitine (197, 198). Epididymal principal cells synthesize GPC and the concentration of GPC in the lumen increases towards the cauda epididymidis (197, 199). In addition to a presumed role in sperm membrane maturation, GPC and other phospholipids may act as oxygen radical scavengers in the lumen. Still, future studies are required to confirm the exact roles for lipids in epididymal function.

Carnitine is involved in the β-oxidation of fatty acids in tissues such as skeletal and cardiac muscle (200), however the reason for high carnitine concentrations in epididymal luminal fluid remains unresolved. The absorption of carnitine in the proximal epididymis may have a role in sperm motility (201, 202). Also, the infertility observed in male mice with a primary carnitine deficiency implicates a necessary role for carnitine in epididymal function (203).

3.2. Sperm Transport and Storage

It was initially thought that the only role of the epididymis was the transport of sperm from the testis to the site of ejaculation. As with other components of the excurrent duct system, the transport of sperm through the epididymis is mediated by hydrostatic pressure and contractions of smooth muscle

surrounding the tubule (28). Innervation of the epididymis is functionally important for epididymal contractility (204). The cauda epididymidis in particular has prominent innervation coinciding with the prevalence of smooth muscle in this region (205, 206). Neuronal input controls sperm movement through the tubule and expulsion of sperm into the vas deferens during ejaculation (204, 207).

The cauda epididymidis is the primary site for sperm storage. In mammals, between 50 to 80% of spermatozoa within the excurrent ducts are located in this region (208). Sperm have acquired full forward motility by the time they reach the cauda epididymidis, however they are kept in an immotile state until ejaculation (159, 209, 210). In the rat, immobilin, a large mucus-like protein secreted by the proximal epididymal segments, closely associates with sperm in the cauda epididymis rendering them immotile (141, 211). This interaction is thought to protect sperm from mechanical shearing forces during ejaculation. Clear cells in the distal cauda epididymidis selectively endocytose immobilin to maintain steady concentrations within the lumen (141). The acid pH of cauda luminal fluid may also play a role in the immobilization of sperm during storage, but this has yet to be confirmed *in vivo* (209, 212, 213).

3.3. Sperm Protection

The blood-epididymis barrier can protect sperm from most external factors by preventing their access to the luminal environment (149); however, sperm remain susceptible to damage from factors that arise internally. Spermatozoa

generate reactive oxygen species required for capacitation and chromatin condensation, however, spermatozoa are also highly susceptible to the damaging effects of reactive oxygen species (214). Antioxidant defense mechanisms are thus of great importance in the epididymis to protect maturing spermatozoa as well as the epididymal epithelium from oxidative damage.

The epididymis has many antioxidant defense mechanisms. Epididymal plasma contains vitamin E, zinc and taurine which can act as antioxidants (172, 215, 216). Important antioxidant and glutathione-cycle enzymes are also present. For example, indoleamine dioxygenase, superoxide dismutase and several glutathione peroxidases have been characterized in the epididymis (215 217-221). Moreover, high glutathione-S-transferase (GST) activity and the expression of different GSTs have been established in the epididymis (143, 222-224). Lastly, multiple gamma-glutamyl-transpeptidases (GGTs II-IV), enzymes responsible for glutathione degradation, are differentially expressed along the epididymis (225).

4. Gene Expression in the Epididymis

Underlying the many integrated functions of the epididymal epithelium is the controlled expression of a multitude of genes. A large amount of research has focused on examining gene expression in the epididymis (reviewed in 226-229). From these numerous studies, one major feature of epididymal gene expression has emerged, namely, epididymal gene expression profiles are highly segment specific. This is not surprising since segment-specific morphology,

biochemistry and function are hallmark characteristics of the tissue. Moreover, epididymal gene expression responds in a segment-specific manner to the experimental circumstances of androgen withdrawal, stress and aging (230-232).

Much like the diverse nature of proteins secreted by the epididymal epithelium, genes expressed in the epididymis encode a vast array of gene products (226-229). While the expression patterns of several epididymis-specific and/or epididymis-relevant genes have been analyzed along the epididymis, understanding the structure and function of this complex tissue requires more than analyzing the properties of isolated genes. Recently, gene array technology has provided a powerful method for large-scale, parallel gene expression analysis thereby generating greater insight into the link between epididymal gene expression and epididymal function (233). In conjunction with advances in genomic technology, proteomic studies are now possible due to the technique of matrix-assisted laser desorption/ionization (MALDI) imaging mass spectrometry (IMS). Using this technique to analyze mouse epididymal tissue sections and laser-captured microdissected epididymal cells, Chaurand et al. have monitored over 400 different protein profiles in the epididymis (234). The identity of only a few of these proteins is known, however, it was noted that more than 50 of these proteins show a regionalized epididymal expression or secretion pattern which is consistent with what is observed at the level of gene expression. Further elucidation of these proteins should contribute greatly to our understanding of epididymal physiology and allow specific links to be made between epididymal gene transcription, translation and function.

D. Regulation of the Epididymis

Many factors are involved in the regulation of epididymal functions, including estrogens, retinoids, growth factors and unidentified testicular factors (235-238). Androgens, however, are recognized as the primary hormonal regulators of epididymal structure and functions (239-241). As early as 1926, Benoit demonstrated that orchidectomy (removal of the testes) had consequences on the epididymis (242). It was determined a few years later that the testicular substance that maintained the epididymis was testosterone (243). When deprived of androgens, the epididymis atrophies and sperm do not survive (239, 242, 244). The structural, morphological, cellular and ultrastructural changes in the epididymis that occur in response to androgen withdrawal have been well documented (239, 245, 246). The following section focuses primarily on the androgen regulation of epididymal functions, though other modes of regulation are also addressed.

1. Steroid Hormones

The epididymis is a highly androgen-dependent tissue. Androgen sources include androgens directly input by the adjacent testis via the efferent ducts and circulating androgens supplied by the vasculature. Androgen levels in the testicular fluid entering the epididymis are approximately ten-fold higher than circulating androgen levels (247). Androgen action is mediated by binding of androgens to the androgen receptor; androgen receptor levels are relatively constant throughout the epididymis (248, 249). Additionally, androgens are also

expected to exert non-genomic effects in the epididymis, as they have been shown to do in other tissues (100, 101).

Several in vivo and in vitro studies have confirmed that it is not testosterone, but rather dihydrotestosterone (DHT), that is the main androgen acting in the epididymis (247, 250-254). Despite these important findings, little effort has focused on distinguishing between the effects of total androgens and DHT in the epididymis. Indeed, the main methodologies used to study the androgen-dependence of epididymal functions are based on the removal of total androgens by orchidectomy (often accompanied by testosterone replacement studies), suppression of androgen synthesis (GnRH antagonists, hypophysectomy) or blocking androgen action (AR receptor antagonists). Nevertheless, using these various methods, most of the previously described epididymal functions have been shown to be regulated by androgens (reviewed in 246). The role of 5α-reduced androgens in the epididymis will be further discussed in a later section. The drastic effect of androgen withdrawal on epididymal sperm clearly reflects the crucial role for androgens in regulating all facets of epididymal function. Some of the epididymal epithelial processes that have been shown to be dependent on androgens include the transport of specific ions and small organic molecules across the epididymal epithelium and regulation of intraluminal pH. For example, androgens influence fluid balance in the epididymis by regulating sodium and anion transport across the epithelium (179, 255, 256). Additionally, the transport of carnitine and inositol across the epithelium is regulated by androgens (257, 258). The anti-androgen flutamide

has been shown to elevate epididymal luminal pH in rats, thus indicating that acidification is under the control of androgens (259).

Studies where androgens have been shown to regulate proteins and enzymes involved in the numerous epididymal functions at the mRNA, protein and/or activity levels are plentiful (246); the following cases constitute only a few representative examples. The regulation of protein processing enzymes such as cathepsin B (260, 261) and lipid metabolizing enzymes such as phopholipase A (262) is androgen-dependent. In regard to fluid transport, androgens modulate the epididymal expression of aquaporins (263) as well as specific components of the renin-angiotensin system, namely angiotensinogen and angiotensin II receptor type 1 (256, 264). The mRNA for the acidification enzyme carbonic anhydrase IV, predominantly expressed in the corpus epididymidis, decreases following androgen withdrawal by castration (186). The expression of another enzyme implicated in acidification, H^+K^+ATPase, depends on testosterone in all regions of the epididymis except the initial segment (265). Relating to the protective functions of the epididymis, orchidectomy and androgen replacement studies demonstrate that the epididymal glutathione S-transferases are under separate control and are differentially regulated by androgens (143, 266). Similarly, multiple gamma-glutamyl transpeptidases are differentially regulated by androgens in different segments of the epididymis (225). Lastly, androgens are known to regulate components of tight junctions in the epididymis that are critical for the formation of the protective blood-epididymis barrier (154, 267).

Less well characterized are the roles for other hormones in the regulation of epididymal functions. An important role for estrogens in regulating the uptake of fluid in the efferent ducts has already been clearly established. Whether this finding is indicative of a similar regulatory role for estrogens in the epididymis remains unclear, though the abnormal epididymal phenotype in ERαKO mice suggests that it is (88).

As early as 1975, Danzo speculated that estrogen could bind to receptors in the epididymis (268). Autoradiographical techniques confirmed this binding nearly ten years later (269). Unfortunately, attempts to differentiate between the estrogen receptor subtypes (ERα and ERβ) in the epididymis using multiple immunocytochemical techniques have yielded conflicting results, particularly for ERα, necessitating further studies to clarify their localization within the epididymis (reviewed in 235). Notably, estrogen (estradiol) is an active metabolite of testosterone. There exists no evidence to date that estradiol can be synthesized by the epididymal epithelium. This is unlike the case for the other active metabolite of testosterone, DHT, which is well known to be made by the epididymis. Alternatively, germ cells can synthesize estrogen (270) and p450-aromatase has been localized to the cytoplasmic droplet attached to epididymal sperm (271). This suggests that the germ cells act as "endocrine" cells providing estrogen to the epididymis where it may be involved in regulating epididymal functions.

2. Testicular factors

It has been well established that the epididymis, in particular the initial segment, is dependent on the testicular input of factors other than androgens directly to the epididymal lumen (245, 272-275); depriving the epididymis of these testicular factors has well-documented effects on morphology and gene expression in the initial segment (28, 229, 238, 276). This type of regulation has been called "lumicrine" by some and refers specifically to "the regulation of cells via factors secreted or produced by another group of cells through a luminal or ductal system" (238, 274). Such regulation has been demonstrated by two different methods: efferent duct ligation (EDL) and orchidectomy followed by androgen replacement. EDL results in the blockage of direct testicular input to the epididymis but leaves androgen production (though not spermatogenesis) by the testes intact and thus capable of synthesizing and supplying androgens to the epididymis via the bloodstream; consequently, EDL allows a method of differentiating between the testicular delivery of androgens and nonandrogenic factors to the epididymis. Effects of EDL on the histology of the initial segment of the epididymis were reported over 25 years ago (272, 273). Orchidectomy generally induces a loss of function that can often be recovered by the reintroduction of exogenous androgens; a lack of recovery following androgen replacement indicates regulation by a non-androgenic testicular factor.

Many examples of lumicrine regulation of the epididymis exist. For instance, the accumulation of carnitine in the cauda epididymidis decreases with orchidectomy and carnitine uptake is only partially restored by subsequent

androgen replacement (277). Accordingly, carnitine accumulation in the cauda epididymidis also decreases after EDL (277). More often, lumicrine regulation is observed within the proximal regions of the epididymis, particularly the initial segment. For example, the expression of 5α-reductase, gamma-glutamyl transpeptidase IV, claudin-1, proenkephalin and cystatin-related epididymal specific (CRES) in the initial segment is dependent on luminal factors from the testis (154, 225, 278-280).

3. Growth Factors

Investigation of growth factor systems in the epididymis has been fairly limited; however, several clues suggest they may play a key role in this tissue. Vascular endothelial growth factor (VEGF) protein has been shown to be localized in the rat epididymal epithelium in a region- and cell-specific pattern (281). Interestingly, the overexpression of VEGF in the testis and epididymis of transgenic mice causes infertility (282). This finding suggests a role for VEGF in the male reproductive system that is likely different from its classical role in regulating endothelial (blood vessel) growth and permeability. Future studies are needed to determine whether the infertility in these mice stems from a testicular or epididymal effect, or a combination of both. In a different animal model, the GH-deficient dwarf (dw/dw) rat, the administration of insulin-like growth factor 1 increases motility and improves morphology of immature spermatozoa (283). Again, whether an epididymal mechanism contributes to this effect is not yet clear.

Several different fibroblast growth factor (FGF) receptors are expressed in the initial segment of the rat epididymis (284). This is interesting since FGF is a candidate testicular factor that regulates the epididymis in a lumicrine fashion (237). It has also been suggested that certain growth factors secreted by the proximal epididymis may act as paracrine regulators of the more distal epididymal segments. Candidates for this paracrine mechanism of regulation include platelet-derived growth factor, transforming growth factor β and nerve growth factor (285-288). It thus seems plausible that several different growth factor systems are involved in epididymal regulation. Further investigation is needed to elucidate mechanisms of growth factor regulation in the epididymis and to determine their reliance on androgens.

E. 5α-Reductase

5α-Reductase (EC 1.3.1.22) is the enzyme that catalyzes the conversion of testosterone into DHT. In species including humans, monkeys, dogs, rats and mice, two isoforms of 5α-reductase have been identified and are termed type 1 and type 2 (289, 290). The presence of two isozymes that carry out the same reaction likely underscores the need for 5α-reduced androgens in male physiology. Research efforts focused on the characterization of both isozymes of 5α-reductase have revealed differences in sequence and chromosomal location as well as differences in tissue, cellular and subcellular distributions, enzyme kinetics, and regulation.

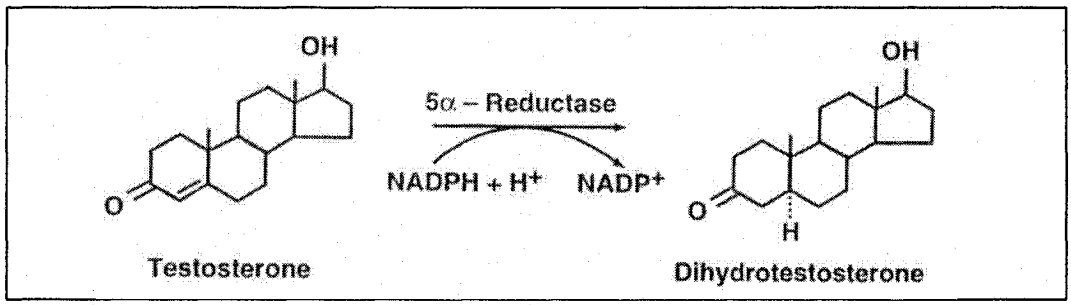


Figure 3. Reaction catalyzed by 5α-reductase.

In humans, the 5α-reductase isozymes share similar gene structures and are approximately 46% homologous in sequence; type 1 is located on chromosome 5 and type 2 is located on chromosome 2 (291-293). Mutations in the 5α-reductase type 2 gene are responsible for 5α-reductase deficiency in humans that results in male pseudohermaphroditism; more than 20 different mutations of this gene have been reported (63, 64, 291). Rat 5α-reductase type 1 and type 2 are approximately 60% and 77% identical in sequence to their respective human homologs, and approximately 44% homologous to each other (289, 290). The 5α-reductase genes encode a highly hydrophobic membrane-bound protein; because of these properties, attempts to purify this protein have been unsuccessful to date.

Type 2 5α-reductase is the predominant isoform in male reproductive tissues such as the prostate (290). The type 1 isoform predominates in peripheral tissues such as the liver and skin, however, it is also present in male reproductive tissues (290). Liver 5α-reductase, along with 3α-hydroxysteroid dehydrogenase, is presumably involved in the catabolism or breakdown of steroid hormones in preparation for their elimination (294). In other tissues,

rather than inactivating steroid hormones, 5α-reductase potentiates hormone action via the formation of DHT (26). While tissue localization may predict catabolic or anabolic roles for 5α-reductase, it remains unknown whether there are independent physiological roles for each isozyme.

1. 5α-Reductase in the Male Reproductive System

A comparison of 5α-reductase within two different androgen-dependent reproductive tissues, the testis and the prostate, serves to illustrate the differences in cellular and subcellular distribution and regulation of this enzyme.

1.1. Testis

Within the testis, 5α-reductase activity has been predominantly localized to cells in the interstitial compartment, especially during development (295-297). Supporting this is the demonstration that purified immature Leydig cells have substantial 5α-reductase activity (295). Indeed, it was already mentioned that peaks in 5α-reductase isoenzyme expression and 5α-reductase activity in the testis occur at puberty, reflecting a likely role for 5α-reduced androgens in the initiation of spermatogenesis at this time (75). It would seem that androgens do not have a prominent role in the regulation of testicular 5α-reductase activity. This is evidenced by studies in hypophysectomized rats where LH administration increases 5α-reductase activity independent of testosterone production (298, 299). More recently, however, it has been shown that the two isozymes of 5α-reductase in the testis are differentially regulated by testosterone at the mRNA

level: testosterone regulates 5α-reductase type 1 mRNA and activity in a negative manner but does not affect 5α-reductase type 2. Conversely, FSH regulates 5α-reductase type 1 mRNA in a positive manner and also appears to regulate type 2 (300). The activity of both 5α-reductase isozymes decreases dramatically after puberty, consistent with a limited role for DHT in the adult testis (301). Testicular 5α-reductase activity has been primarily localized to the microsomal subcellular fraction (302). The role of each 5α-reductase isozyme in the testis remains unclear; however, it appears that 5α-reductase type 1 is predominant in this tissue (75).

1.2. Prostate

5α-reductase has been extensively studied in the prostate because of the well-established role for DHT in the development of prostate diseases. Within the prostate, 5α-reductase activity increases post-pubertally and reaches a stable plateau in adulthood (303). This is in marked contrast to testicular 5α-reductase activity. In the prostate, 5α-reductase activity has been localized to both microsomal and nuclear subcellular fractions (304, 305). The prostate is composed of stroma and epithelium and studies have localized 5α-reductase activity to both compartments (306). 5α-reductase activity in the prostate has been shown to be regulated by androgens. For example, testosterone administration rescues the loss of 5α-reductase activity that occurs following orchidectomy (307). Additionally, androgens control the expression of both 5α-reductase isozymes in the prostate (308). Unlike the testis, LH does not appear

to regulate 5α-reductase in the prostate (309). While some studies suggest that type 2 5α-reductase predominates in the prostate, both isozymes have been localized in the tissue (305, 310). Furthermore, specific 5α-reductase type 1 enzyme activity has been demonstrated in the prostate, supporting a significant role for both isozymes in this tissue (311).

2. 5α-Reductase in the Epididymis

A number of key studies have unequivocally demonstrated that DHT is the main androgen acting in the epididymis. Several different groups have demonstrated the ability of epididymal cells to synthesize 5α-reduced metabolites from testosterone *in vitro* (250-252). In 1972, Tindall et al. showed that the active androgen present in epididymal cell nuclei after injection of radiolabelled testosterone is DHT (253). Subsequently, in 1974, Orgebin-Crist demonstrated that 5α-reduced androgens are more potent than testosterone in maintaining epididymal functions *in vivo* (254). Finally, micropuncture experiments confirmed that, beyond the efferent ducts, the predominant androgens in epididymal luminal fluid are the 5α-reduced metabolites of testosterone (247). More recently, epididymal 5α-reductases have been characterized at the activity, mRNA and protein levels.

2.1. Enzyme Activity

In the epididymis, 5α-reductase activity is present in both the microsomal and nuclear fractions (312). Microsomal 5α-reductase activity is expressed at

low levels throughout the epididymis (313). In contrast, nuclear 5α-reductase activity exists in a striking positional gradient along the epididymis; it is highest in the initial segment and drops significantly towards the end of the tubule (314). Following bilateral orchidectomy, nuclear 5α-reductase activity declines dramatically, especially in the proximal regions, and is only partially rescued by testosterone replacement, even at supraphysiological levels (314). Thus it is apparent that nuclear 5α-reductase activity is regulated in a lumicrine manner by a factor coming from the testis. The decreased activity observed following efferent duct ligation and unilateral castration is also consistent with this mode of regulation (314, 315).

Attempts have been made to identify the lumicrine factor regulating 5α-reductase activity. Experimentally removing sperm, by administering testosterone at a dose that suppresses spermatogenesis, has no effect on nuclear 5α-reductase activity, thereby eliminating the possibility that sperm (or a sperm-associated substance) is the testicular factor in question (316). Alternatively, hypophysectomy (removal of the pituitary) followed by administration of testosterone at a dose that maintains spermatogenesis and Sertoli-cell secretion, also maintains nuclear 5α-reductase activity (317). A Sertoli-cell product secreted under the control of androgens was thus proposed as the testicular factor regulating nuclear 5α-reductase activity. Androgen-binding protein (ABP) is a likely candidate given that its synthesis by Sertoli cells is androgen dependent (318). Moreover, ABP is actively endocytosed by the

epithelium in the initial segment of the epididymis, the region where nuclear 5α-reductase activity is highest (319, 320).

2.2. mRNA Levels

Analysis of the localization and regulation of the mRNAs for 5α-reductase type 1 and type 2 in the rat epididymis was done by Viger in the early 1990s (278, 321). The expression of 5α-reductase type 1 mRNA is highest in the initial segment and decreases longitudinally. As with nuclear 5α-reductase activity, the expression of 5α-reductase type 1 mRNA decreases significantly following orchidectomy and efferent duct ligation. Following orchidectomy, exogenous testosterone can recover the expression of type 1 mRNA in all regions of the epididymis except the initial segment (321). These findings indicate that a lumicrine factor originating in the testis is required for the regulation of 5α-reductase type 1 expression uniquely in the initial segment while the expression in the rest of the tissue is regulated by circulating androgens.

5α-Reductase type 2 mRNA is expressed at higher levels than 5α-reductase type 1 in all segments of the epididymis. Longitudinally, type 2 expression is high in the initial segment, peaks in the caput epididymidis and then decreases in the corpus and cauda epididymides, though to a lesser extent than type 1 expression (278). Surprisingly, levels of 5α-reductase type 2 mRNA increase nearly 2-fold in the initial segment in response to efferent duct ligation, while expression in the rest of the tissue is unaffected (278). This was in marked contrast to the effects of efferent duct ligation on 5α-reductase type 1 mRNA

expression, and was the first instance where the differential regulation of both isozymes of 5α-reductase in the same tissue was observed. Interestingly, the developmental expression profiles for type 1 and type 2 5α-reductase are also different in the epididymis; the segment-specific expression of 5α-reductase type 1 mRNA varies dramatically at different postnatal ages while 5α-reductase type 2 expression is not altered post-natally (278, 321).

2.3. Protein Levels

The development of antibodies specific to each 5α-reductase isozyme has proved challenging. Consequently, there has yet to be a clear differentiation of the localization of type 1 and type 2 5α-reductase proteins along the epididymis. Nevertheless, immunohistochemical studies using an antibody raised against the rat type 1 isoform have shown that the pattern of protein expression in the epididymis is similar to the expression profiles for 5α-reductase type 1 mRNA and nuclear 5α-reductase activity; the localization was most intense in a discrete lobule of the initial segment and decreased longitudinally (322). Efferent duct ligation abolished 5α-reductase type 1 protein staining in the initial segment exclusively (249).

3. 5α-Reductase Inhibitors

Several characteristics of the 5α-reductase deficiency phenotype, including underdeveloped prostates, altered facial and body hair growth patterns, decreased incidences of temporal hairline regression and decreased incidences

of acne, were the first indicators that the inhibition of 5α-reductase may be of therapeutic potential in normal, non-deficient males. Biomedical research and drug discovery efforts have since led to the development of 5α-reductase inhibitors, primarily for the treatment of benign prostatic hyperplasia (BPH), which is extremely prevalent in older males (>60% of men over 55 years old) (323). More recently, 5α-reductase inhibitors have also been marketed for the treatment of male pattern baldness (324), and basic and clinical research indicates potential roles for 5α-reductase inhibitors in the treatment of acne (325) and the early treatment or prevention of prostate cancer (326, 327).

It has been shown that inhibitors have different selectivity for the two isoforms of 5α-reductase. Finasteride, a type 2 selective 5α-reductase inhibitor, was first marketed for the treatment of benign prostatic hyperplasia in 1992 (328). Consequently, the long-term efficacy and safety of finasteride have been extensively studied for this indication (reviewed in 79, 80). Finasteride treatment decreases serum and prostate DHT levels by approximately 65-70% and 85-90% respectively (80). The lack of specificity of finasteride for the type 1 isoform has been proposed as the reason why this drug is only moderately effective in the treatment of benign prostatic hyperplasia (79, 80). Efforts to discover more therapeutically effective compounds, i.e., that decrease DHT levels to a greater extent, have led to the development of a novel class of dual 5α-reductase inhibitors (329-332). For example, dutasteride (GI198745) is a dual 5α-reductase inhibitor that was selected for clinical development due to its remarkable potency. It is perhaps the most potent 5α-reductase inhibitor

reported thus far, reducing serum DHT levels to >95% (331). In early 2003, dutasteride (GI198745) became the first dual 5α-reductase inhibitor available for the treatment of BPH (333). In comparison to finasteride, dutasteride and other dual compounds achieve almost total suppression of DHT levels (329, 330, 333).

Compared to the multitude of studies investigating the effects of 5α-reductase inhibition in the prostate, there are a limited number of studies that examine the effects of finasteride on other reproductive tissues or fertility (334-337) and fewer still that examine the extraprostatic effects of the novel class of dual inhibitors (329, 333).

As early as 1981, Cohen et al. presented data that a 5α-reductase inhibitor could affect fertility (338). However, interpretation of these early studies needs to be made with caution since many of the earlier inhibitory compounds employed in these studies also have known effects on other steroid metabolizing enzymes (e.g., 3α-hydroxysteroid dehydrogenase) or on the androgen receptor (339-342). Therefore any effect cannot solely or conclusively be attributed to the inhibition of 5α-reductase.

Later inhibitor studies employed the type 2 selective inhibitor finasteride. In 1991, Cukierski *et al.* (334) reported a partial decrease in fertility following chronic finasteride treatment (80 mg/kg/day for 24 weeks) of adult male rats. Wise *et al.* reported a similar effect in a related study (335). In these earlier studies, testis weight and histology were unaffected by treatment and the subfertility was attributed solely to a deficit in the formation of copulatory plugs due to the effects of treatment on seminal vesicle and prostate weights.

However, any data pertaining to the epididymis were lacking, therefore it is possible that the compromised fertility could have been due to an effect on this tissue as well.

Interestingly, with respect to other 5α-reductase inhibitor studies, no adverse effects of 5α-reductase inhibitor treatment on testis weight, histology and/or sperm counts, and hence spermatogenesis, have been reported following treatment with either finasteride or a novel dual inhibitor (79, 337, 343, 344). To date, however, still little or no information exists pertaining to the effects of 5α-reductase inhibition in the epididymis and no studies have focused on the epididymal consequences of inhibiting both isozymes of 5α-reductase with a dual inhibitor.

F. Formulation of the Project

Scientific research into the physiology of male reproduction has obvious clinical implications for the development of novel treatments for reproductive tissue cancers and age-related pathologies such as BPH, for discerning the treatable causes of male infertility or subfertility, as well as for the design of effective male contraceptives. For these reasons, both the prostate and the testis have historically been the focus of major research efforts; however, over the past few decades it has become increasingly apparent that epididymal research is also of great importance. Of particular relevance are the multiple sperm-related functions of the epididymis that have made this tissue an appealing post-testicular target for the development of safe, rapid and reversible

male contraceptives. Additionally, the epididymis is clearly a potential site of origin of male infertility and therefore, elucidating epididymal causes of male infertility has therapeutic potential for the management of this prevalent pathology. Despite these therapeutic incentives, much remains to be learned about the specific factors and mechanisms involved in mediating and regulating epididymal functions. Therefore, novel approaches are required to further our understanding of epididymal physiology.

The maintenance of epididymal structure and functions is known to be highly dependent on the presence of androgens. Furthermore, several in vivo and in vitro studies have confirmed that it is not testosterone, but rather dihydrotestosterone (DHT), that is the main androgen acting in this tissue. Despite the central role for DHT in epididymal physiology, very few studies have examined the specific consequences on the epididymis of inhibiting 5α-reductase, the enzyme that catalyzes DHT formation. The availability of suitable inhibitors may have precluded these studies; however, with the advent of specific dual inhibitors that concurrently inhibit both isozymes of 5α-reductase, this is no longer the case. Indeed, studying the consequences of inhibiting DHT formation is likely to provide important information regarding the androgen-regulation of epididymal functions. Additionally, given that all current evidence precludes an effect of 5α-reductase inhibition on the testis, the inhibition of DHT production is an interesting experimental approach for studying the regulation of sperm maturation in the epididymis while presumably having little or no effect on sperm production in the testis. The goal of the present thesis is to use novel 5α-

reductase inhibitors in combination with advanced gene expression and sperm analysis techniques in order to increase our current understanding of the roles of DHT in epididymal physiology.

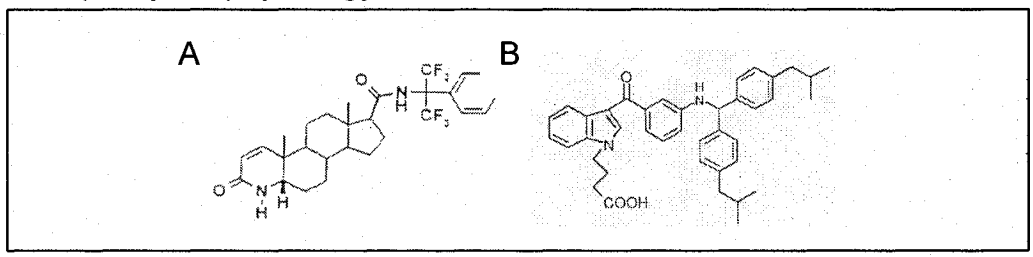


Figure 4. Structures of dual 5α-reductase inhibitors. A) PNU157706, Pharmacia & Upjohn. B) FK143, Fujisawa Pharmaceutical Co.

Specifically, the studies comprised in chapter 2 of the present thesis employ the novel dual 5α-reductase inhibitor PNU157706 to examine the effects of inhibiting DHT formation on epididymal gene expression. This is accomplished on a large-scale with cDNA array technology. Subsequently, the studies in chapter 3 examine the effects of PNU157706 treatment on epididymal sperm maturation, enabling a correlation between changes in epididymal gene expression and epididymal function. For the first time, techniques are used to assess sperm morphology, motility and fertilizing ability following treatment with a dual 5α-reductase inhibitor. Lastly, in chapter 4, previous effects of inhibiting DHT formation on epididymal gene expression are confirmed by using another dual 5α-reductase inhibitor, FK143. Additionally, gene expression analysis following treatment with both inhibitor compounds is expanded using real-time quantitative RT-PCR techniques to include genes involved in mediating androgen action and in multiple growth factor signaling pathways. Conclusively, these studies give insight into the role of DHT in regulating epididymal gene expression

and functions, establish 5α-reductase inhibition as a new approach to study epididymal physiology and identify novel directions and targets for continued epididymal research.

References

1. Rock J, Robinson D. 1965 Effect of induced intrascrotal hyperthermia on testicular function in man. Am J Obstet Gynecol. 93:793-801.
2. Davis JR, Langford G, Kirby PJ. 1970 The testicular capsule. In: Johnson AD, Gomes WR, Vandemark NL (eds), The testis: development, anatomy and physiology. New York: Academic Press. pp 281–337.
3. Middendorff R, Muller D, Mewe M, Mukhopadhyay AK, Holstein AF, Davidoff MS. 2002 The tunica albuginea of the human testis is characterized by complex contraction and relaxation activities regulated by cyclic GMP. J Clin Endocrinol Metab. 87:3486-3499.
4. Sertoli E. 1865 Dell'esistenza di particolari cellule ramificate nei canalicoli seminiferi del testicolo umano. Morgagni. 7: 31-40.
5. Setchell BP 1980 The functional significance of the blood-testis barrier. J Androl. 1:3-10.
6. Russell LD, Griswold MD. 1993 (eds) The Sertoli Cell. Clearwater, FL: Cache River Press.
7. Enders GC. 1993 Sertoli-Sertoli and Sertoli-germ cell communications. In: Russell LD, Griswold MD (eds.), The Sertoli Cell. Clearwater, FL: Cache River Press. pp 448-460.
8. Jegou B. 1993 The Sertoli-germ cell communication network in mammals. Int Rev Cytol. 147: 25-96.

9. Holstein AF, Davidoff MS. 1997 Organization of the intertubular tissue of the human testis. In: Motta PM (ed), Recent Advances in Microscopy of Cells, Tissues and Organs. Rome: Antonio Delfino. pp 569-577.
10. von Leydig F. 1850 Zur Anatomie der männlichen Geschlechtsorgane und Analdrüsen der Säugethiere. Zeitschrift für wissenschaftliche Zoologie. 2: 1-57.
11. Bardin CW. 1996 Androgens: Early Attempts to Evaluate Leydig Cell Function in Man. In: Payne A, Hardy M, Russell LD (eds), The Leydig Cell. Clearwater, FL: Cache River Press. pp 31-42.
12. Pudney J. 1996 Comparitive cytology of the Leydig cell. In: Payne A, Hardy M, Russell LD (eds), The Leydig Cell. Clearwater, FL: Cache River Press. pp 97-142.
13. Niemi M, Sharpe RM, Brown WRA 1986 Macrophages in the interstitial tissue of the rat testis. Cell Tissue Res. 243:337-433.
14. Leblond CP, Clermont Y. 1952 Definition of stages of the cycle of the seminiferous epithelium in the rat. Ann NY Acad Sci 55:548–573.
15. Lalli M, Clermont Y. 1981 Structural changes of the head components of the rat spermatid during late spermiogenesis. Am J Anat. 160:419-434.
16. Courtens JL, Loir M. 1981 A cytochemical study of nuclear changes in boar, bull, goat, mouse, rat, and stallion spermatids. J Ultrastruct Res. 74:327-340.
17. Russell LD, Ettlin R, Sinha Hikim AP, Clegg ED. 1990 Histological and histopathological evaluation of the testis. Clearwater, FL: Cache River Press.

18. Kerr JB, de Kretser DM. 1974 Proceedings: The role of the Sertoli cell in phagocytosis of the residual bodies of spermatids. J Reprod Fertil. 136:439-440.
19. Lin D, Sugawara T, Strauss III JF, Clark BJ, Stocco DM, Saenger P, Rogol A, Miller WL. 1995 Role of steroidogenic acute regulatory protein in adrenal and gonadal steroidogenesis. Science 267:1828-1831.
20. Hall PF. 1994 Testicular steroid synthesis: Organization and regulation. In: Knobil E, Neill JD (eds), The Physiology of Reproduction, Second Edition. New York: Raven Press, Ltd. pp 1335-1362.
21. Bhasin S, Woodhouse L, Storer TW. 2003 Androgen effects on body composition. Growth Horm IGF Res. 13 Suppl A:S63-71.
22. Vanderschueren D, Vandenput L, Boonen S, Lindberg MK, Bouillon R, Ohlsson C. 2004 Androgens and bone. Endocr Rev. 25:389-425.
23. Wolf OT. 2003 Cognitive functions and sex steroids. Ann Endocrinol (Paris). 64:158-161.
24. Liu PY, Death AK, Handelsman DJ. 2003 Androgens and cardiovascular disease. Endocr Rev. 24:313-340.
25. Grossman CJ. 1984 Regulation of the immune system by sex steroids. Endocr Rev. 5:435-455.
26. Bruchovsky N, Wilson JD. 1968 The conversion of testosterone to 5-alpha-androstan-17-beta-ol-3-one by rat prostate in vivo and in vitro. J Biol Chem. 243:2012-2021.

27. Brodie AM. 1979 Recent advances in studies on estrogen biosynthesis. J Endocrinol Invest. 2:445-460.
28. Robaire B, Hermo L. 1988 Efferent ducts, epididymis, and vas deferens: structure, functions, and their regulation. In: Knobil E , Neill JD, (eds), The Physiology of Reproduction. New York: Raven Press Ltd. pp 999-1080.
29. Becker O. 1856 Uber Flimmerepithelium im Nebenhoden des Menschen. Wien ned Wchnschr. 6:184.
30. Talo A. 1981 In-vitro spontaneous electrical activity of rat efferent ductules. J Reprod Fertil. 63:17.
31. Mollendorf WV. 1920 Vitale Farbungen an tierischen Zellen. Egr Physiol. 18:141.
32. Burgos MH, Montes de Oca M, Montorzi NM. 1959 La incorporacion de Fe59 en los conductos efferents del hamster. Rev Soc Argent Biol. 35:26.
33. Hess RA, Bunick D, Lee KH, Bahr J, Taylor JA, Korach KS, Lubahn DB. 1997 A role for oestrogens in the male reproductive system. Nature. 390:509–512.
34. Hoffer AP, Hamilton DW. 1974 Phagocytosis of sperm by the epithelial cells in ductuli efferentes of experimental rats. Anat Rec. 178:376.
35. Hoffer AP, Hamilton DW, Fawcett DW. 1975 Phagocytosis of spermatozoa by the epithelial cells of the ductuli efferentes after epididymal obstruction in the rat.
J Reprod Fertil. 44:1-9.

36. Aire TA. 2000 Active spermiophagy in the initial part of the proximal efferent duct of the epididymis of normal domestic fowl (Gallus domesticus). Res Vet Sci. 68:135-140.
37. Luke MC, Coffey DS. 1994 Male Sex Accessory Tissues Structure, Androgen Action, and Physiology. In: Knobil E, Neill JD (eds), The Physiology of Reproduction, 2nd edition. New York: Raven Press. pp 1478
38. Mann T. 1974 Secretory function of the prostate, seminal vesicle and other male accessory organs of reproduction. J Reprod Fertil. 37:179-188.
39. Ventura WP, Freund M. 1972 The role of semen and accessory gland secretions in sperm transport in the rat. Acta Eur Fertil. 3:87-103.
40. Curry PT, Atherton RW. 1990 Seminal vesicles: development, secretory products, and fertility. Arch Androl. 25:107-113.
41. Kavanagh JP. 1985 Sodium, potassium, calcium, magnesium, zinc, citrate and chloride content of human prostatic and seminal fluid. J Reprod Fertil. 75:35-41.
42. Chow PH, Chan CW, Cheng YL. 1993 Contents of fructose, citric acid, acid phosphatase, proteins and electrolytes in secretions of the accessory sex glands of the male golden hamster. Int J Androl. 16:41-45.
43. Matthews M, Adler NT. 1977 Facilitative and inhibitory influences of reproductive behavior on sperm transport in rats. J Comp Physiol Psychol. 91:727-741.

44. Sharpe RM. 1994 Regulation of spermatogenesis. In: Knobil E, Neill JD (eds), The Physiology of Reproduction, Second Edition. New York: Raven Press, Inc. pp 1363-1434.
45. Saez JM. 1994 Leydig cells: endocrine, paracrine, and autocrine regulation. Endocr Rev. 15:574-626.
46. Smith PE. 1927 The disabilities caused by hypophysectomy and their repair JAMA 88:158-161.
47. Greep RO, Febold HL, Hisaw FL. 1932 Effects of two hypophyseal gonadotrophic hormones on reproductive system of male rat. Anat Rec. 65:261-269.
48. Winters SJ, Janick JJ, Loriaux DL, Sherins RJ. 1979 Studies on the role of sex steroids in the feedback control of gonadotropin concentrations in men. II. Use of the estrogen antagonist, clomiphene citrate. J Clin Endocrinol Metab. 48:222-227.
49. Matsumoto AM, Bremner WJ. 1984 Modulation of pulsatile gonadotropin secretion by testosterone in man. J Clin Endocrinol Metab. 58:609-614.
50. Sherins RJ, Loriaux DL. 1973 Studies of the role of sex steroids in the feedback control of FSH concentrations in men. J Clin Endocrinol Metab. 36:886-893.
51. Bagatell CJ, Dahl KD, Bremner WJ. 1994 The direct pituitary effect of testosterone to inhibit gonadotropin secretion in men is partially mediated by aromatization to estradiol. J Androl. 15:15-21.

52. Wang C, Iranmanesh A, Berman N, McDonald V, Steiner B, Ziel F, Faulkner SM, Dudley RE, Veldhuis JD, Swerdloff RS. 1998 Comparative pharmacokinetics of three doses of percutaneous dihydrotestosterone gel in healthy elderly men--a clinical research center study. J Clin Endocrinol Metab. 83:2749-2757.
53. Rittmaster RS, Lemay A, Zwicker H, Capizzi TP, Winch S, Moore E, Gormley GJ. 1992 Effect of finasteride, a 5 alpha-reductase inhibitor, on serum gonadotropins in normal men. J Clin Endocrinol Metab. 75:484-488.
54. McCullagh DR. 1932 Dual endocrine activity of the testis. Science. 76:19-20.
55. Robertson DM, Foulds LM, Leversha L, Morgan FJ, Hearn MT, Burger HG, Wettenhall RE, de Kretser DM. 1985 Isolation of inhibin from bovine follicular fluid. Biochem Biophys Res Commun. 126:220-226.
56. Quigley CA, De Bellis A, Marschke KB, el-Awady MK, Wilson EM, French FS. 1995 Androgen receptor defects: historical, clinical, and molecular perspectives. Endocr Rev. 16:271-321.
57. Morris JM. 1953 The syndrome of testicular feminization in male pseudohermaphrodites. Am J Obstet Gynecol. 65:1192-1211.
58. Yong EL, Loy CJ, Sim KS. 2003 Androgen receptor gene and male infertility. Hum Reprod Update. 9:1-7.
59. Traish AM, Muller RE, Wotiz HH. 1984 Differences in the physicochemical characteristics of androgen-receptor complexes formed in vivo and in vitro. Endocrinology. 114:1761-1769.

60. Toth M, Zakar T. 1982 Relative binding affinities of testosterone, 19-nortestosterone and their 5 alpha-reduced derivatives to the androgen receptor and to other androgen-binding proteins: a suggested role of 5 alpha-reductive steroid metabolism in the dissociation of "myotropic" and "androgenic" activities of 19-nortestosterone. J Steroid Biochem. 17:653-660.
61. Zhou ZX, Lane MV, Kemppainen JA, French FS, Wilson EM. 1995 Specificity of ligand-dependent androgen receptor stabilization: receptor domain interactions influence ligand dissociation and receptor stability. Mol Endocrinol. 9:208-218.
62. Wilbert DM, Griffin JE, Wilson JD. 1983 Characterization of the cytosol androgen receptor of the human prostate. J Clin Endocrinol Metab. 56:113-120.
63. Imperato-McGinley J, Zhu YS. 2002 Androgens and male physiology the syndrome of 5alpha-reductase-2 deficiency. Mol Cell Endocrinol. 198:51-59.
64. Wilson JD, Griffin JE, Russell DW. 1993 Steroid 5 alpha-reductase 2 deficiency. Endocr Rev. 14:577-593.
65. George FW, Wilson JD. 1994 Sex determination and differentiation. In: Knobil E, Neill JD (eds), The Physiology of Reproduction, Second Edition. New York: Raven Press, Inc. pp 13-28.
66. Siiteri PK, Wilson JD. 1973 Testosterone formation and metabolism during sexual differentiation in the human embryo. J Clin Endocrinol Metab. 38:113-125.

67. Walsh PC, Swerdloff RS.1973 Biphasic effect of testosterone on spermatogenesis in the rat. Invest Urol. 11:190-193.

68. Sun YT, Irby DC, Robertson DM, de Kretser DM. 1989 The effects of exogenously administered testosterone on spermatogenesis in intact and hypophysectomized rats. Endocrinology. 125:1000-1010.

69. Zirkin BR, Santulli R, Awoniyi CA, Ewing LL. 1989 Maintenance of advanced spermatogenic cells in the adult rat testis: quantitative relationship to testosterone concentration within the testis. Endocrinology. 124:3043-3049.

70. McLachlan RI, O'Donnell L, Meachem SJ, Stanton PG, de Kretser DM, Pratis K, Robertson DM. 2002 Identification of specific sites of hormonal regulation in spermatogenesis in rats, monkeys, and man. Recent Prog Horm Res. 57:149-179.

71. Awoniyi CA, Santulli R, Sprando RL, Ewing LL, Zirkin BR. 1989 Restoration of advanced spermatogenic cells in the experimentally regressed rat testis: quantitative relationship to testosterone concentration within the testis. Endocrinology. 124:1217-1223.

72. McLachlan RI, Wreford NG, Meachem SJ, De Kretser DM, Robertson DM. 1994 Effects of testosterone on spermatogenic cell populations in the adult rat. Biol Reprod. 51:945-955.

73. O'Donnell L, McLachlan RI, Wreford NG, Robertson DM. 1994 Testosterone promotes the conversion of round spermatids between stages VII and VIII of the rat spermatogenic cycle. Endocrinology. 135:2608-2614.

74. Saito K, O'Donnell L, McLachlan RI, Robertson DM. 2000 Spermiation failure is a major contributor to early spermatogenic suppression caused by hormone withdrawal in adult rats. Endocrinology. 141:2779-2785.

75. Killian J, Pratis K, Clifton RJ, Stanton PG, Robertson DM, O'Donnell L. 2003 5alpha-reductase isoenzymes 1 and 2 in the rat testis during postnatal development. Biol Reprod. 68:1711-1718.

76. O'Donnell L, Stanton PG, Wreford NG, Robertson DM, McLachlan RI. **1996** Inhibition of 5 alpha-reductase activity impairs the testosterone-dependent restoration of spermiogenesis in adult rats. Endocrinology. 137:2703-2710.

77. O'Donnell L, Pratis K, Stanton PG, Robertson DM, McLachlan RI. 1999 Testosterone-dependent restoration of spermatogenesis in adult rats is impaired by a 5alpha-reductase inhibitor. J Androl. 20:109-117.

78. Jarow JP, Chen H, Rosner TW, Trentacoste S, Zirkin BR. 2001 Assessment of the androgen environment within the human testis: minimally invasive method to obtain intratesticular fluid. J Androl. 22:640-645.

79. Steers WD. 2001 5alpha-reductase activity in the prostate. Urology. 58(6 Suppl 1):17-24.

80. Bartsch G, Rittmaster RS, Klocker H. 2002 Dihydrotestosterone and the concept of 5alpha-reductase inhibition in human benign prostatic hyperplasia. World J Urol. 19:413-425.

81. Rastogi RK, Milone M, Caliendo MF, Di Meglio M. 1979 A study of the enzyme activity in the seminal vesicles of castrated and hormone-replaced castrated mice. Experientia. 35:419-420.

82. Shima H, Tsuji M, Young P, Cunha GR. 1990 Postnatal growth of mouse seminal vesicle is dependent on 5 alpha-dihydrotestosterone. Endocrinology. 127:3222-3233.

83. McLachlan JA, Newbold RR, Bullock B. 1975 Reproductive tract lesions in male mice exposed prenatally to diethylstilbestrol. Science. 190:991-992.

84. Gill WB, Schumacher GF, Bibbo M, Straus FH 2nd, Schoenberg HW. 1979 Association of diethylstilbestrol exposure in utero with cryptorchidism, testicular hypoplasia and semen abnormalities. J Urol. 122:36-39.

85. Lubahn DB, Moyer JS, Golding TS, Couse JF, Korach KS, Smithies O. 1993 Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. PNAS. 90:11162-11166.

86. Eddy EM, Washburn TF, Bunch DO, Goulding EH, Gladen BC, Lubahn DB, Korach KS. 1996 Targeted disruption of the estrogen receptor gene in male mice causes alteration of spermatogenesis and infertility. Endocrinology. 137:4796-4805.

87. Hess RA, Bunick D, Lee KH, Bahr J, Taylor JA, Korach KS, Lubahn DB. 1997 A role for oestrogens in the male reproductive system. Nature. 390:509-512.

88. Hess RA, Bunick D, Lubahn DB, Zhou Q, Bouma J. 2000 Morphologic changes in efferent ductules and epididymis in estrogen receptor-alpha knockout mice. J Androl. 21:107-121.

89. Evans RM. 1988 The steroid and thyroid hormone receptor superfamily. Science **240:**889-895.

90. Clever U, Karlson P. 1960 Induktion von Puff-Veränderungen in Speicheldrüsen-chromosomen von Chironomus tentans durch Ecdyson. Exp Cell Res. **20:**623-626.

91. Beato M, Chavez S, Truss M. 1996 Transcriptional regulation by steroid hormones. Steroids. 61:240-251.

92. Beato M, Klug J. 2000 Steroid hormone receptors: An update. Hum Reprod Update. 6:225-236.

93. Lee HJ, Chang C. 2003 Recent advances in androgen receptor action. Cell Mol Life Sci. 60:1613-1622.

94. Gobinet J, Poujol N, Sultan Ch. 2002 Molecular action of androgens. Mol Cell Endocrinol. 198:15-24.

95. Heinlein CA, Chang C. 2002 Androgen Receptor (AR) Coregulators: An Overview. Endocr Rev. 23:175-200.

96. Riegman PHJ, Vlietstra RJ, Korput JAGM van der, Brinkmann AO, Trapman J 1991 The promoter of the prostate-specific antigen gene contains a functional androgen responsive element. Mol Endocrinol. 5:1921–1930.

97. Murtha P, Tindall DJ, Young CY. 1993 Androgen induction of a human prostate-specific kallikrein, hKLK2: characterization of an androgen response element in the 5' promoter region of the gene. Biochemistry. 32:6459-6464.

98. Kasper S, Rennie PS, Bruchovsky N, Sheppard PC, Cheng H, Lin L, Shiu RP, Snoek R, Matusik RJ. 1994 Cooperative binding of androgen receptors to two DNA sequences is required for androgen induction of the probasin gene. J Biol Chem. 269:31763-31769.

99. Claessens F, Celis L, Peeters B, Heyns W, Verhoeven G, Rombauts W. 1989 Functional characterization of an androgen response element in the first intron of the C3(1) gene of prostatic binding protein. Biochem Biophys Res Commun. 164:833-840.

100. Falkenstein E, Tillmann HC, Christ M, Feuring M, Wehling M. **2000** Multiple actions of steroid hormones--a focus on rapid, nongenomic effects. Pharmacol Rev. 52:513-556.

101. Revelli A, Massobrio M, Tesarik J. 1998 Nongenomic Actions of Steroids in Reproductive Tissues. Endocr Rev. 19:3-17.

102. Benten WP, Lieberherr M, Sekeris CE, Wunderlich F. 1997 Testosterone induces Ca^{2+} influx via non-genomic surface receptors in activated T cells. FEBS Lett. 407:211-214.

103. Benten WP, Lieberherr M, Stamm O, Wrehlke C, Guo Z and Wunderlich F. 1999b Testosterone signaling through internalizable surface receptors in androgen receptor-free macrophages. Mol Biol Cell. 10:3113-3123.

104. Lieberherr M, Grosse B. 1994 Androgens increase intracellular calcium concentration and inositol 1,4,5-trisphosphate and diacylglycerol formation via a pertussis toxin-sensitive G-protein. J Biol Chem. 269:7217-7223.

105. Peterziel H, Mink S, Schonert A, Becker M, Klocker H, Cato AC. 1999 Rapid signalling by androgen receptor in prostate cancer cells. Oncogene. 18:6322-6329.

106. Migliaccio A, Castoria G, Di Domenico M, de Falco A, Bilancio A, Lombardi M, Barone MV, Ametrano D, Zannini MS, Abbondanza C, Auricchio F. 2000 Steroid-induced androgen receptor-oestradiol receptor beta-Src complex triggers prostate cancer cell proliferation. EMBO J. 19:5406-5417.

107. Kousteni S, Bellido T, Plotkin LI, O'Brien CA, Bodenner DL, Han L, Han K, DiGregorio GB, Katzenellenbogen JA, Katzenellenbogen BS, Roberson PK, Weinstein RS, Jilka RL, Manolagas SC. 2001 Nongenotropic, sex-nonspecific signaling through the estrogen or androgen receptors: dissociation from transcriptional activity. 104:719-730.

108. Turner HE, Harris AL, Melmed S, Wass JA. 2003 Angiogenesis in endocrine tumors. Endocr Rev. 24:600-632.

109. Khandwala HM, McCutcheon IE, Flyvbjerg A, Friend KE. 2000 The effects of insulin-like growth factors on tumorigenesis and neoplastic growth. Endocr Rev. 21:215-244.

110. Polnaszek N, Kwabi-Addo B, Wang J, Ittmann M. 2004 FGF17 is an autocrine prostatic epithelial growth factor and is upregulated in benign prostatic hyperplasia. Prostate. 60:18-24.

111. Aksoy Y, Aksoy H, Bakan E, Atmaca AF, Akcay F. 2004 Serum insulin-like growth factor-I and insulin-like growth factor-binding protein-3 in localized,

metastasized prostate cancer and benign prostatic hyperplasia. Urol Int. 72:62-65.

112. Gnessi L, Fabbri A, Spera G. 1997 Gonadal peptides as mediators of development and functional control of the testis: an integrated system with hormones and local environment. Endocr Rev. 18:541-609.

113. Wilson ME. 2001 The impact of the GH-IGF-I axis on gonadotropin secretion: inferences from animal models. J Pediatr Endocrinol Metab. 14:115-40.

114. Daftary SS, Gore AC. 2005 IGF-1 in the brain as a regulator of reproductive neuroendocrine function. Exp Biol Med. 230:292-306.

115. Le Roy C, Lejeune H, Chuzel F, Saez JM, Langlois D. 1999 Autocrine regulation of Leydig cell differentiated functions by insulin-like growth factor I and transforming growth factor beta. J Steroid Biochem Mol Biol. 69:379-384.

116. Lui WY, Lee WM, Cheng CY. 2003 TGF-betas: their role in testicular function and Sertoli cell tight junction dynamics. Int J Androl. 26:147-160.

117. Byrne RL, Leung H, Neal DE. 1996 Peptide growth factors in the prostate as mediators of stromal epithelial interaction. Br J Urol. 77:627-633.

118. Culig Z, Hobisch A, Cronauer MV, Radmayr C, Trapman J, Hittmair A, Bartsch G, Klocker H. 1994 Androgen receptor activation in prostatic tumor cell lines by insulin-like growth factor-I, keratinocyte growth factor, and epidermal growth factor. Cancer Res 54:5474-5478.

119. Culig Z, Hobisch A, Cronauer MV, Hittmair A, Radmayr C, Bartsch G, Klocker H. 1995 Activation of the androgen receptor by polypeptide growth factors and cellular regulators. World J Urol 13:285-289.

120. Orio F Jr, Terouanne B, Georget V, Lumbroso S, Avances C, Siatka C, Sultan C. 2002 Potential action of IGF-1 and EGF on androgen receptor nuclear transfer and transactivation in normal and cancer human prostate cell lines. Mol Cell Endocrinol 198:105-114.

121. Huang D, Liu X, Plymate SR, Idowu M, Grimes M, Best AM, McKinney JL, Ware JL. 2004 Proteomic identification of 14-3-3 sigma as a common component of the androgen receptor and the epidermal growth factor receptor signaling pathways of the human prostate epithelial cell line M12. Oncogene. 23:6881-6889.

122. Hammar JA. 1897. Űber secretionserscheinungen in Nebenhoden des Hundes. Zugleich ein Beitrag zur Physiologie des Zellenkerns. Arch Anat Entwicklungsgeschichte Suppl. 1-42.

123. Tournade A. 1913 Différence de motilité des spermatozoids prélevés dans diverse segments de l'épididyme. Compte Rend Soc Biol. 74:738-739.

124. von Haller A. 1765 Partes Genitales Masculae. In: Haak LB (ed), Elementa Physiologiae Corporis Humani, vol 7:410

125. Myers-Ward CF. 1897 Preliminary note on the structure and function of the epididymis and the vas deferens in the higher mammalia. J Anat. 135-140

126. van der Stricht O. 1893 La signification des cellules epithéliales de l'épididyme de Lacerta vivipara. Soc Biol. 45:799.

127. Young A. 1929 A study of the function of the epididymis I: Is the attainment of full spermatozoan motility attributable to some specific actions of the epididymal secretion? J Morphol. 48:475-491.

128. Orgebin-Crist MC. 1998 The epididymis across 24 centuries. J Reprod Fertil Suppl. 53:285-292.

129. Orgebin-Crist MC. 1967 Sperm maturation in rabbit epididymis. Nature. 216:816-818.

130. Bedford JM. 1967 Effects of duct ligation on the fertilizing ability of spermatozoa from different regions of the rabbit epididymis. J Exp Zool. 166:271-281.

131. Reid D, Cleland K. 1957 The structure and function of the epididymis. 1. The histology of the rat epididymis. Aust J Zool. 5:223-246.

132. Maneely R. 1959 A histological and critical review. Acta Zool. 40:1-21.

133. Turner TT. 1979 On the epididymis and its function. Invest Urol. 16:311-321.

134. Amann RP, Johnson L, Thompson DL Jr, Pickett BW. 1976 Daily spermatozoal production, epididymal spermatozoal reserves and transit time of spermatozoa through the epididymis of the rhesus monkey. Biol Reprod. 15.:586-592.

135. Hamilton DW. 1975 Structure and function of the epithelium lining the ductuli efferents ductus epididymidis and ductus deferens in the rat. In: Hamilton DW, Greep RO (eds), Handbook of Physiology, Section 7. Washington DC: American Physiological Society. vol 5 pp 259-301.

136. Marshall FF, Reiner WG, Goldberg BS. 1979 The embryologic origin of the caput epididymidis in the rat. Invest Urol. 17:78-82.

137. Hermo L, Robaire B. 2002 Epididymal cell types and their functions. In: Robaire B, Hinton BT (eds), The Epididymis: From Molecules to Clinical Practice. New York: Kluwer Academic/Plenum Press Publishers. pp 81-102.

138. Turner TT, Avery EA, Sawchuk TJ. 1994 Assessment of protein synthesis and secretion by rat seminiferous and epididymal tubules in vivo. Int J Androl. 17:205-213.

139. Turner TT. 1991 Spermatozoa are exposed to a complex microenvironment as they traverse the epididymis. Ann N Y Acad Sci. 637:364-383.

140. Hermo L, Wright J, Oko R, Morales CR. 1991 Role of epithelial cells of the male excurrent duct system of the rat in the endocytosis or secretion of sulfated glycoprotein-2 (clusterin). Biol Reprod. 44:1113-1131.

141. Hermo L, Oko R, Robaire B. 1992 Epithelial cells of the epididymis show regional variations with respect to the secretion of endocytosis of immobilin as revealed by light and electron microscope immunocytochemistry. Anat Rec. 232:202-220.

142. Hermo L, Barin K, Oko R. 1998 Androgen binding protein secretion and endocytosis by principal cells in the adult rat epididymis and during postnatal development. J Androl. 19:527-541.

143. Veri JP, Hermo L, Robaire B. 1993 Immunocytochemical localization of the Yf subunit of glutathione S-transferase P shows regional variation in the

staining of epithelial cells of the testis, efferent ducts, and epididymis of the male rat. J Androl. 14:23-44.

144. Seiler P, Wenzel I, Wagenfeld A, Yeung CH, Nieschlag E, Cooper TG. 1998 The appearance of basal cells in the developing murine epididymis and their temporal expression of macrophage antigens. Int J Androl. 21:217-226.

145. Yeung CH, Nashan D, Sorg C, Oberpenning F, Schulze H, Nieschlag E, Cooper TG. 1994 Basal cells of the human epididymis--antigenic and ultrastructural similarities to tissue-fixed macrophages. Biol Reprod. 50:917-926.

146. Flickinger CJ, Bush LA, Howards SS, Herr JC. 1997 Distribution of leukocytes in the epithelium and interstitium of four regions of the Lewis rat epididymis. Anat Rec. 248:380-390.

147. Serre V, 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.

148. Hermo L, Dworkin J, 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.

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

150. Friend DS, Gilula NB. 1972 Variations in tight and gap junctions in mammalian tissues. J Cell Biol. 53:758-776.

151. Cyr DG, Robaire B, Hermo L. 1995 Structure and turnover of junctional complexes between principal cells of the rat epididymis. Microsc Res Tech. 30:54-66.

152. Cyr DG, Hermo L, Egenberger N, Mertineit C, Trasler JM, Laird DW. 1999 Cellular immunolocalization of occludin during embryonic and postnatal development of the mouse testis and epididymis. Endocrinology. 140:3815-3825.

153. Cyr DG, Hermo L, Robaire B. 1993 Developmental changes in epithelial cadherin messenger ribonucleic acid and immunocytochemical localization of epithelial cadherin during postnatal epididymal development in the rat. Endocrinology. 132:1115-1124.

154. Gregory M, Dufresne J, Hermo L, Cyr D. 2001 Claudin-1 is not restricted to tight junctions in the rat epididymis. Endocrinology. 142:854-863.

155. Blandau RJ, Rumery R. 1964 The relationship of swimming movements of epididymal spermatozoa to their fertilizing capacity. Fertil Steril. 15:571-579.

156. Horan AH, Bedford JM. 1972 Development of the fertilizing ability of spermatozoa in the epididymis of the Syrian hamster. J Reprod Fertil. 30:417-423

157. Dyson AL, Orgebin-Crist MC. 1973 Effect of hypophysectomy, castration and androgen replacement upon the fertilizing ability of rat epididymal spermatozoa. Endocrinology. 93:391-402.

158. Moore HD, Hartman TD, Pryor JP. 1983 Development of the oocyte-penetrating capacity of spermatozoa in the human epididymis. Int J Androl. 6:310-318.

159. Yeung CH, Cooper TG. 2002 Acquisition and Development of Sperm Motility upon Maturation in the Epididymis. In: Robaire B, Hinton BT (eds), The Epididymis: From Molecules to Clinical Practice. New York: Kluwer Academic/Plenum Press Publishers. pp 417-434.

160. Olsen GE, Nagdas SK, Winfrey VP. 2002 Structural Differentiation of Spermatozoa During Post-Testicular Maturation. In: Robaire B, Hinton BT (eds), The Epididymis: From Molecules to Clinical Practice. New York: Kluwer Academic/Plenum Press Publishers. pp 371-388.

161. Jones RC. 1971 Studies of the structure of the head of boar spermatozoa from the epididymis. J Reprod Fertil Suppl. 13:Suppl 13:51-64.

162. Calvin HI, Bedford JM. 1971 Formation of disulphide bonds in the nucleus and accessory structures of mammalian spermatozoa during maturation in the epididymis. J Reprod Fertil Suppl. 13:Suppl 13:65-75.

163. Bedford JM, Calvin HI. 1974 Changes in -S-S- linked structures of the sperm tail during epididymal maturation, with comparative observations in sub-mammalian species. J Exp Zool. 187:181-204.

164. Jones R. 2002 Plasma Membrane Composition and Organisation During Maturation of Spermatozoa in the Epididymis. In: Robaire B, Hinton BT (eds), The Epididymis: From Molecules to Clinical Practice. New York: Kluwer Academic/Plenum Press Publishers. pp 405-416.

165. Cuasnicu PS, Cohen DJ, Ellerman DA, Busso D, Da Ros VG, Morgenfeld MM. 2002 Changes in Specific Sperm Proteins During Epididymal Maturation. In: Robaire B, Hinton BT (eds), The Epididymis: From Molecules to Clinical Practice. New York: Kluwer Academic/Plenum Press Publishers. pp 389-403.

166. Hall JC, Hadley J, Doman T. 1991 Correlation between changes in rat sperm membrane lipids, protein, and the membrane physical state during epididymal maturation. J Androl. 12:76-87.

167. Jones R. 1998 Plasma membrane structure and remodelling during sperm maturation in the epididymis. J Reprod Fertil Suppl. 53:73-84.

168. Nolan JP, Hammerstedt RH. 1997 Regulation of membrane stability and the acrosome reaction in mammalian sperm. FASEB J. 11:670-682.

169. Hinton BT, Palladino MA. 1995 Epididymal epithelium: its contribution to the formation of a luminal fluid microenvironment. Microsc Res Tech. 30:67-81.

170. Hinton BT. 1980 The epididymal microenvironment: a site of attack for a male contraceptive? Invest Urol. 18:1-10.

171. Turner TT. 1984 Resorption versus secretion in the rat epididymis. J Reprod Fertil. 72:509-514.

172. Hinton BT. 1990 The testicular and epididymal luminal amino acid microenvironment in the rat. J Androl. 111:498-505.

173. Veeramachaneni DN, Amann RP, Palmer JS, Hinton BT. 1990 Proteins in luminal fluid of the ram excurrent ducts: changes in composition and evidence for differential endocytosis. J Androl. 1990 11:140-154.

174. Olson GE, Hinton BT. 1985 Regional differences in luminal fluid polypeptides of the rat testis and epididymis revealed by two-dimensional gel electrophoresis. J Androl. 6:20-34.

175. Hinton BT, Setchell BP. 1980 Concentrations of glycerophosphocholine, phosphocholine and free inorganic phosphate in the luminal fluid of the rat testis and epididymis. J Reprod Fertil. 58:401-406.

176. Turner TT. 2002 Necessity's Potion: Inorganic Ions and Small Organic Molecules in the Epididymal Lumen. In: Robaire B, Hinton BT (eds), The Epididymis: From Molecules to Clinical Practice. New York: Kluwer Academic/Plenum Press Publishers. pp 131-150.

177. Wong PYD, Gong XD, Leung GPH, Cheuk BLY. 2002 Formation of the Epididymal Fluid Microenvironment. In: Robaire B, Hinton BT (eds), The Epididymis: From Molecules to Clinical Practice. New York: Kluwer Academic/Plenum Press Publishers. pp 119-130.

178. Leung PS, Sernia C. 2003 The renin-angiotensin system and male reproduction: new functions for old hormones. J Mol Endocrinol. 30:263-270.

179. Wong PY, Yeung CH. 1977 Hormonal regulation of fluid reabsorption in isolated rat cauda epididymidis. Endocrinology. 101:1391-1397.

180. Wong PY, Yeung CH. 1978 Absorptive and secretory functions of the perfused rat cauda epididymidis. J Physiol. 275:13-26.

181. Caflisch CR. 1992 Acidification of testicular and epididymal fluids in the rat after surgically-induced varicocele. Int J Androl. 15:238-245.

182. Levine N, Kelly H. 1978 Measurement of pH in the rat epididymis in vivo. J Reprod Fertil. 52:333-335.

183. Levine N, Marsh DJ. 1971 Micropuncture studies of the electrochemical aspects of fluid and electrolyte transport in individual seminiferous tubules, the epididymis and the vas deferens in rats. J Physiol. 213:557-570.

184. Au CL, Wong PY. 1980 Luminal acidification by the perfused rat cauda epididymidis. J Physiol. 309:419-427.

185. Jensen LJ, Stuart-Tilley AK, Peters LL, Lux SE, Alper SL, Breton S. 1999 Immunolocalization of AE2 anion exchanger in rat and mouse epididymis. Biol Reprod. 61:973-980.

186. Kaunisto K, Fleming RE, Kneer J, Sly WS, Rajaniemi H. 1999 Regional expression and androgen regulation of carbonic anhydrase IV and II in the adult rat epididymis. Biol Reprod. 61:1521-1526.

187. Amann RP, Killian GJ, Benton AW. 1973 Differences in the electrophoretic characteristics of bovine rete testis fluid and plasma from the cauda epididymidis. J Reprod Fertil. 1973 35:321-330.

188. Koskimies AI, Kormano M. 1975 Proteins in fluids from different segments of the rat epididymis. J Reprod Fertil. 43:345-348.

189. Brooks DE, Higgins SJ. 1980 Characterization and androgen-dependence of proteins associated with luminal fluid and spermatozoa in the rat epididymis. J Reprod Fertil. 59:363-375.

190. Vreeburg JT, Holland MK, Cornwall GA, Orgebin-Crist MC. 1990 Secretion and transport of mouse epididymal proteins after injection of 35S-methionine. Biol Reprod. 43:113-120.

191. Vreeburg JT, Holland MK, Orgebin-Crist MC. 1992 Binding of epididymal proteins to rat spermatozoa in vivo. Biol Reprod. 47:588-597.

192. Syntin P, Dacheux F, Druart X, Gatti JL, Okamura N, Dacheux JL. 1996 Characterization and identification of proteins secreted in the various regions of the adult boar epididymis. Biol Reprod. 55:956-974.

193. Dacheux JL, Dacheux F. 2002 Protein Secretion in the Epididymis. In: Robaire B, Hinton BT (eds), The Epididymis: From Molecules to Clinical Practice. New York: Kluwer Academic/Plenum Press Publishers. pp 151-168.

194. Gatti JL, Castella S, Dacheux F, Ecroyd H, Metayer S, Thimon V, Dacheux JL. 2004 Post-testicular sperm environment and fertility. Anim Reprod Sci. 82-83:321-339.

195. Djakiew D, Byers SW, Lewis DM, Dym M. 1985 Receptor-mediated endocytosis of alpha 2-macroglobulin by principal cells in the proximal caput epididymidis in vivo. J Androl. 6:190-196.

196. Djakiew D, Griswold MD, Lewis DM, Dym M. 1986 Micropuncture studies of receptor-mediated endocytosis of transferrin in the rat epididymis. Biol Reprod. 34:691-699.

197. Dawson RMC, Rowlands IW. 1958 Glycerylphosphorylcholine in the male reproductive tract of rats and guinea pigs. Quart J Exp Physiol. 44: 26-34.

198. Marquis NR, Fritz IB. 1964 Enzymological Determination of Free Carnitine Concentrations in Rat Tissues. J Lipid Res. 41:184-187.

199. Killian GJ, Chapman DA. 1980 Glycerylphosphorylcholine, sialic acid and protein in epithelial cells isolated from the rat caput epididymidis by elutriation. Biol Reprod. 22:846-850.

200. Atar D, Spiess M, Mandinova A, Cierpka H, Noll G, Luscher TF. 1997 Carnitine--from cellular mechanisms to potential clinical applications in heart disease. Eur J Clin Invest. 27:973-976.

201. Hinton BT, Brooks DE, Dott HM, Setchell BP. 1981 Effects of carnitine and some related compounds on the motility of rat spermatozoa from the caput epididymidis. J Reprod Fertil. 61:59-64.

202. Hinton BT, Hernandez H. 1985 Selective luminal absorption of L-carnitine from the proximal regions of the rat epididymis. Possible relationships to development of sperm motility. J Androl. 6:300-305.

203. Toshimori K, Kuwajima M, Yoshinaga K, Wakayama T, Shima K. 1999 Dysfunctions of the epididymis as a result of primary carnitine deficiency in juvenile visceral steatosis mice. FEBS Lett. 446:323-326.

204. Setchell BP. 2002 Innervation and Vasculature of the Excurrent Duct System. In: Robaire B, Hinton BT (eds) The Epididymis: From Molecules to Clinical Practice. New York: Kluwer Academic/Plenum Press Publishers. pp 35-48.

205. El-Badawi A, Schenk EA. 1967 The distribution of cholinergic and adrenergic nerves in the mammalian epididymis: a comparative histochemical study. Am J Anat. 121:1-14.

206. Baumgarten HG, Holstein AF, Rosengren E. 1971 Arrangement, ultrastructure, and adrenergic innervation of smooth musculature of the ductuli efferentes, ductus epididymidis and ductus deferens of man. Z Zellforsch Mikrosk Anat. 120:37-79.

207. Kaleczyc J. 1998 Origin and neurochemical characteristics of nerve fibres supplying the mammalian vas deferens. Microsc Res Tech. 42:409-422.

208. Amann RP, Howards SS. 1980 Daily spermatozoal production and epididymal spermatozoal reserves of the human male. J Urol. 124:211-215.

209. Turner TT, Reich GW. 1985 Cauda epididymal sperm motility: a comparison among five species. Biol Reprod. 32:120-128.

210. Jones RC, Clulow J. 1994 Interactions of sperm and the reproductive ducts of the male tammar wallaby, Macropus eugenii (Macropodidae: Marsupialia). Reprod Fertil Dev. 6:437-444.

211. Usselman MC, Cone RA. 1983 Rat sperm are mechanically immobilized in the caudal epididymis by "immobilin," a high molecular weight glycoprotein. Biol Reprod. 29:1241-1253.

212. Acott TS, Carr DW. 1984 Inhibition of bovine spermatozoa by caudal epididymal fluid: II. Interaction of pH and a quiescence factor. Biol Reprod. 30:926-935.

213. Clulow J, Jones RC, Murdoch RN. 1992 Maturation and regulation of the motility of spermatozoa in the epididymis of the tammar wallaby (Macropus eugenii). J Reprod Fertil. 94:295-303.

214. Aitken RJ. 2002 Active Oxygen in Spermatozoa During Epididymal Transit. In: Robaire B, Hinton BT (eds), The Epididymis: From Molecules to Clinical Practice. New York: Kluwer Academic/Plenum Press Publishers. pp 435-448.

215. Tramer F, Rocco F, Micali F, Sandri G, Panfili E. 1998 Antioxidant systems in rat epididymal spermatozoa. Biol Reprod. 59:753-758.

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

217. Ozaki Y, Nichol CA, Duch DS. 1987 Utilization of dihydroflavin mononucleotide and superoxide anion for the decyclization of L-tryptophan by murine epididymal indoleamine 2,3-dioxygenase. Arch Biochem Biophys. 257:207-216.

218. Perry AC, Jones R, Hall L. 1993 Isolation and characterization of a rat cDNA clone encoding a secreted superoxide dismutase reveals the epididymis to be a major site of its expression. Biochem J. 293 (Pt 1):21-25.

219. Schwaab V, Faure J, Dufaure JP, Drevet JR. 1998 GPx3: the plasma-type glutathione peroxidase is expressed under androgenic control in the mouse epididymis and vas deferens. Mol Reprod Dev. 51:362-372.

220. Vernet P, Rigaudiere N, Ghyselinck N, Dufaure JP, Drevet JR. 1996 In vitro expression of a mouse tissue specific glutathione-peroxidase-like protein lacking the selenocysteine can protect stably transfected mammalian cells against oxidative damage. Biochem Cell Biol. 74:125-131.

221. Vernet P, Faure J, Dufaure JP, Drevet JR. 1997 Tissue and developmental distribution, dependence upon testicular factors and attachment to spermatozoa of GPX5, a murine epididymis-specific glutathione peroxidase. Mol Reprod Dev. 47:87-98.

222. Hales BF, Hachey C, Robaire B. 1980 The presence and longitudinal distribution of the glutathione S-transferases in rat epididymis and vas deferens. Biochem J. 189:135-142.

223. Veri JP, Hermo L, Robaire B. 1994 Immunocytochemical localization of glutathione S-transferase Yo subunit in the rat testis and epididymis. J Androl. 15:415-434.

224. Papp S, Robaire B, Hermo L. 1995 Immunocytochemical localization of the Ya, Yc, Yb1, and Yb2 subunits of glutathione S-transferases in the testis and epididymis of adult rats. Microsc Res Tech. 30:1-23.

225. Palladino MA, Hinton BT. 1994 Expression of multiple gamma-glutamyl transpeptidase messenger ribonucleic acid transcripts in the adult rat epididymis is differentially regulated by androgens and testicular factors in a region-specific manner. Endocrinology. 135:1146-1156.

226. Kirchhoff C. Gene expression in the epididymis. 1999 Int Rev Cytol. 188:133-202.

227. Kirchhoff C. 2002 Specific Gene Expression in the Human and Non-Human Primate Epididymis. In: Robaire B, Hinton BT (eds), The Epididymis: From Molecules to Clinical Practice. New York: Kluwer Academic/Plenum Press Publishers. pp 201-218.

228. Cornwall GA, Hann SR. 1995 Specialized gene expression in the epididymis. J Androl. 16:379-383.

229. Cornwall GA, Lareyre JJ, Matusik RJ, Hinton BT, Orgebin-Crist MC. 2002 Gene Expression and Epididymal Function. In: Robaire B, Hinton BT (eds) The Epididymis: From Molecules to Clinical Practice. New York: Kluwer Academic/Plenum Press Publishers. pp 169-200.

230. Jervis KM, Robaire B. 2002 Changes in gene expression during aging in the Brown Norway rat epididymis. Exp Gerontol. 37:897-906.

231. Jervis KM, Robaire B. 2003 Effects of caloric restriction on gene expression along the epididymis of the Brown Norway rat during aging. Exp Gerontol. 38:549-560.

232. Ezer N, Robaire B. 2003 Gene expression is differentially regulated in the epididymis after orchidectomy. Endocrinology. 144:975-988.

233. Jervis KM, Robaire B. 2001 Dynamic changes in gene expression along the rat epididymis. Biol Reprod. 65:696-703.

234. Chaurand P, Fouchecourt S, DaGue BB, Xu BJ, Reyzer ML, Orgebin-Crist MC, Caprioli RM. 2003 Profiling and imaging proteins in the mouse epididymis by imaging mass spectrometry. Proteomics. 3:2221-2239.

235. Hess RA, Zhou Q, Nie R. 2002 The Role of Estrogens in the Endocrine and Paracrine Regulation of the Efferent Ductules, Epididymis and Vas Deferens. In: Robaire B, Hinton BT (eds), The Epididymis: From Molecules to Clinical Practice. New York: Kluwer Academic/Plenum Press Publishers. pp 317-338.

236. Orgebin-Crist MC, Lareyre JJ, Suzuki K, Araki Y, Fouchécourt S, Matusik RJ, Ong DE. 2002 Retinoids and Epididymal Function. In: Robaire B, Hinton BT (eds) The Epididymis: From Molecules to Clinical Practice. New York: Kluwer Academic/Plenum Press Publishers. pp 339-352.

237. Lan ZJ, Labus JC, Hinton BT. 1998 Regulation of gamma-glutamyl transpeptidase catalytic activity and protein level in the initial segment of the rat epididymis by testicular factors: role of basic fibroblast growth factor. Biol Reprod. 58:197-206.

238. Hinton BT, Lan ZJ, Rudolph DB, Labus JC, Lye RJ. 1998 Testicular regulation of epididymal gene expression. J Reprod Fertil Suppl. 53:47-57.

239. Orgebin-Crist MC, Tichenor PL. 1973 Effect of testosterone on sperm maturation in vitro. Nature. 245:328-329.

240. Blaquier JA, Cameo MS, Burgos MH. 1972 The role of androgens in the maturation of epididymal spermatozoa in the guinea pig. Endocrinology. 90:839-842.

241. Cameo MS, Blaquier JA, Burgos MH. 1971 The androgen dependency of the process of sperm maturation in the guinea pig. Acta Physiol Lat Am. 21:254-255.

242. Benoit MJ. 1926 Recherches anatomiques, cytologiques et histophysiologiques sur les voies excrétrices du testicule, chez les mammifères. Arch d'Anat d'histo et d'embryo. 5:173-412.

243. Butenandt A .1931 Uber die chemish untersuchungder der sexualhormone. Z Angnew Chem. 44:905-908.

244. White WE. 1932 The effect of hypophysectomy on the survival of spermatozoa in the male rat. Anat Rec. 54:253-273.

245. Fan X, Robaire B. 1998 Orchidectomy induces a wave of apoptotic cell death in the epididymis. Endocrinology. 139:2128-2136.

246. Ezer N, Roabire B. 2002 Androgenic Regulation of the Structure and Functions of the Epididymis. In: Robaire B, Hinton BT (eds), The Epididymis: From Molecules to Clinical Practice. New York: Kluwer Academic/Plenum Press Publishers. pp 297-316.

247. Turner TT, Jones CE, Howards SS, Ewing LL, Zegeye B, Gunsalus GL. 1984 On the androgen microenvironment of maturing spermatozoa. Endocrinology. 115:1925-1932.

248. Tindall DJ, Hansson V, McLean WS, Ritzen EM, Nayfeh SN, French FS. 1975 Androgen-binding proteins in rat epididymis: properties of a cytoplasmic receptor for androgen similar to the androgen receptor in ventral prostate and different from androgen-binding protein (ABP). Mol Cell Endocrinol. 3:83-101.

249. Robaire B, Viger RS. 1995 Regulation of epididymal epithelial cell functions. Biol Reprod. 52:226-236.

250. Gloyna RE, Wilson JD. 1969 A comparative study of the conversion of testosterone to 17-beta-hydroxy-5-alpha-androstan-3-one (Dihydrotestosterone) by prostate and epididymis. J Clin Endocrinol Metab. 29:970-977.

251. Robaire B, Ewing LL, Zirkin BR, Irby DC. 1977 Steroid delta4-5alpha-reductase and 3alpha-hydroxysteroid dehydrogenase in the rat epididymis. Endocrinology. 101:1379-1390.

252. Monsalve A, Blaquier JA. 1977 Partial characterization of epididymal 5 alpha reductase in the rat. Steroids. 30:41-51.

253. Tindall DJ, French FS, Nayfeh SN. 1972 Androgen uptake and binding in rat epididymal nuclei, in vivo. Biochem Biophys Res Commun. 49:1391-1397.

254. Orgebin-Crist MC, Davies J. 1974 Functional and morphological effects of hypophysectomy and androgen replacement in the rabbit epididymis. Cell Tissue Res. 148:183-201.

255. Au CL, Ngai HK, Yeung CH, Wong PY. 1978 Effect of adrenalectomy and hormone replacement on sodium and water transport in the perfused rat cauda epididymidis. J Endocrinol. 77:265-266.

256. Leung PS, Wong TP, Chung YW, Chan HC. 2002 Androgen dependent expression of AT1 receptor and its regulation of anion secretion in rat epididymis. Cell Biol Int. 26:117-122.

257. Brooks DE, Hamilton DW, Mallek AH. 1973 The uptake of L-(methyl- 3 H) carnitine by the rat epididymis. Biochem Biophys Res Commun. 52:1354-1360.

258. Pholpramool C, White RW, Setchell BP. 1982 Influence of androgens on inositol secretion and sperm transport in the epididymis of rats. J Reprod Fertil. 66:547-553.

259. Caflisch CR. 1993 Effect of A Nonsteroidal Antiandrogen, Flutamide on Intraluminal Acidification in Rat Testis and Epididymis. Andrologia. 25:363-367.

260. Igdoura SA, Morales CR, Hermo L. 1995 Differential expression of cathepsins B and D in testis and epididymis of adult rats. J Histochem Cytochem. 43:545-557.

261. Tomomasa H, Waguri S, Umeda T, Koiso K, Kominami E, Uchiyama Y. 1994 Lysosomal cysteine proteinases in rat epididymis. J Histochem Cytochem. 42:417-425.

262. Beck B. 1980 Tissue specificity of the epididymal androgen dependent phospholipase A. Int J Androl. 3:349-362.

263. Pastor-Soler N, Isnard-Bagnis C, Herak-Kramberger C, Sabolic I, Van Hoek A, Brown D, Breton S. 2002 Expression of aquaporin 9 in the adult rat epididymal epithelium is modulated by androgens. Biol Reprod. 66:1716-1722.

264. Leung PS, Wong TP, Lam SY, Chan HC, Wong PY. 2000 Testicular hormonal regulation of the renin-angiotensin system in the rat epididymis. Life Sci. 66:1317-1324.

265. Andonian S, Adamali H, Hermo L. 2001 Expression and regulation of H+K+ATPase in lysosomes of epithelial cells of the adult rat epididymis. Mol Reprod Dev. 58:398-410.

266. Robaire B, Hales BF. 1982 Regulation of epididymal glutathione S-transferases: effects of orchidectomy and androgen replacement. Biol Reprod. 26:559-565.

267. Cyr DG, Hermo L, Blaschuk OW, Robaire B. 1992 Distribution and regulation of epithelial cadherin messenger ribonucleic acid and immunocytochemical localization of epithelial cadherin in the rat epididymis. Endocrinology. 130:353-363.

268. Danzo BJ, Eller BC, Judy LA, Trautman JR, Orgebin-Crist MC. 1975 Estradiol binding in cytosol from epididymides of immature rabbits. Mol Cell Endocrinol. 2:91-105.

269. Schleicher G, Drews U, Stumpf WE, Sar M. 1984 Differential distribution of dihydrotestosterone and estradiol binding sites in the epididymis of the mouse. An autoradiographic study. Histochemistry. 81:139-147.

270. Nitta H, Bunick D, Hess RA, Janulis L, Newton SC, Millette CF, Osawa Y, Shizuta Y, Toda K, Bahr JM. 1993 Germ cells of the mouse testis express P450 aromatase. Endocrinology. 132:1396-1401.

271. Janulis L, Hess RA, Bunick D, Nitta H, Janssen S, Asawa Y, Bahr JM. 1996 Mouse epididymal sperm contain active P450 aromatase which decreases as sperm traverse the epididymis. J Androl. 17:111-116.

272. Danzo BJ, Cooper TG, Orgebin-Crist MC. 1977 Androgen binding protein (ABP) in fluids collected from the rete testis and cauda epididymidis of sexually mature and immature rabbits and observations on morphological changes in the epididymis following ligation of the ductuli efferentes. Biol Reprod. 17:64-77.

273. Fawcett DW, Hoffer AP. 1979 Failure of exogenous androgen to prevent regression of the initial segments of the rat epididymis after efferent duct ligation or orchidectomy. Biol Reprod. 20:162-181.

274. Nicander L, Osman DI, Ploen L, Bugge HP, Kvisgaard KN. 1983 Early effects of efferent ductile ligation on the proximal segment of the rat epididymis. Int J Androl. 6: 91-102.

275. Turner TT, Riley TA. 1999 p53 independent, region-specific epithelial apoptosis is induced in the rat epididymis by deprivation of luminal factors. Mol Reprod Dev. 53:188-197.

276. Hinton, BT, Lan, ZJ, Lye RJ, Labus JC. 2000 Regulation of epididymal function by testicular factors: The lumicrine hypothesis. In: Goldberg E (ed), The Testis. From Stem Cell to Sperm Function. New York: Serono Symposia USA, Springer. pp 163-173.

277. Bohmer T, Hansson V. 1975 Androgen-dependent accumulation of carnitine by rat epididymis after injection of [3H]butyrobetaine in vivo. Mol Cell Endocrinol. 3:103-115.

278. Viger RS, Robaire B. 1996 The mRNAs for the steroid 5 alpha-reductase isozymes, types 1 and 2, are differentially regulated in the rat epididymis. J Androl. 17:27-34.

279. Garrett JE, Garrett SH, Douglass J. 1990 A spermatozoa-associated factor regulates proenkephalin gene expression in the rat epididymis. Mol Endocrinol. 4:108-118.

280. Cornwall GA, Orgebin-Crist MC, Hann SR. 1992 The CRES gene: a unique testis-regulated gene related to the cystatin family is highly restricted in its expression to the proximal region of the mouse epididymis. Mol Endocrinol. 6:1653-1664.

281. Zhang QY, Qiu SD, Ge L. 2004 Studies on expression and location of VEGF protein in rat testis and epididymis Shi Yan Sheng Wu Xue Bao. 37:1-8.

282. Korpelainen EI, Karkkainen MJ, Tenhunen A, Lakso M, Rauvala H, Vierula M, Parvinen M, Alitalo K. 1998 Overexpression of VEGF in testis and epididymis causes infertility in transgenic mice: evidence for nonendothelial targets for VEGF. J Cell Biol. 143:1705-1712.

283. Vickers MH, Casey PJ, Champion ZJ, Gravance CG, Breier BH. 1999 IGF-I treatment increases motility and improves morphology of immature spermatozoa in the GH-deficient dwarf (dw/dw) rat. Growth Horm IGF Res. 9:236-240.

284. Kirby JL, Yang L, Labus JC, Hinton BT. 2003 Characterization of fibroblast growth factor receptors expressed in principal cells in the initial segment of the rat epididymis. Biol Reprod. 68:2314-2321.

285. Okamura N, Tamba M, Uchiyama Y, Sugita Y, Dacheux F, Syntin P, Dacheux JL. 1995 Direct evidence for the elevated synthesis and secretion of procathepsin L in the distal caput epididymis of boar. Biochim Biophys Acta. 1245:221-226.

286. Desai KV, Flanders KC, Kondaiah P. 1998 Expression of transforming growth factor-beta isoforms in the rat male accessory sex organs and epididymis. Cell Tissue Res. 294:271-277.

287. Bomgardner D, Wehrenberg U, Rune GM. 1999 TGF-beta could be involved in paracrine actions in the epididymis of the marmoset monkey (Callithrix jacchus). J Androl. 20:375-383.

288. Ayer-LeLievre C, Olson L, Ebendal T, Hallbook F, Persson H. 1988 Nerve growth factor mRNA and protein in the testis and epididymis of mouse and rat. PNAS. 85:2628-2632.

289. Andersson S, Russell DW. 1990 Structural and biochemical properties of cloned and expressed human and rat steroid 5 alpha-reductases. PNAS. 87:3640-3644.

290. Normington K, Russell DW. 1992 Tissue distribution and kinetic characteristics of rat steroid 5 alpha-reductase isozymes. Evidence for distinct physiological functions. J Biol Chem. 267:19548-19554.

291. Andersson S, Berman DM, Jenkins EP, Russell DW. 1991 Deletion of steroid 5 alpha-reductase 2 gene in male pseudohermaphroditism. Nature. 354(6349):159-161.

292. Jenkins EP, Andersson S, Imperato-McGinley J, Wilson JD, Russell DW. 1992 Genetic and pharmacological evidence for more than one human steroid 5 alpha-reductase. J Clin Invest. 89:293-300.

293. Thigpen AE, Davis DL, Milatovich A, Mendonca BB, Imperato-McGinley J, Griffin JE, Francke U, Wilson JD, Russell DW. 1992 Molecular genetics of steroid 5 alpha-reductase 2 deficiency. J Clin Invest. 90:799-809.

294. Schneider JJ. 1952 Conversion of desoxycorticosterone to four allopregnane metabolites by rat liver in vitro. J Biol Chem. 199:235-244.

295. Murono EP. 1989 Maturational changes in steroidogenic enzyme activities metabolizing testosterone and dihydrotestosterone in two populations of testicular interstitial cells. Acta Endocrinol (Copenh). 121:477-483.

296. Yoshizaki K, Matsumoto K, Samuels LT. 1978 Localization of delta4-5alpha-reductase in immature rat testes. Endocrinology. 102:918-925.

297. Viger RS, Robaire B. 1995 Steady state steroid 5 alpha-reductase messenger ribonucleic acid levels and immunocytochemical localization of the type 1 protein in the rat testis during postnatal development. Endocrinology. 136:5409-5415.

298. Nayfeh SN, Coffey JC, Kotite NJ, French FS. 1975 Gonadotropic regulation of 5 alpha-reductase activity in the interstitial cells and whole testis homogenate of the immature rat. Curr Top Mol Endocrinol. 2:53-64.

299. Chase DJ, Payne AH. 1985 Prolactin involvement in regulation of testicular 5 alpha-reductase activity in the immature rat. Biol Reprod. 33:637-643.

300. Pratis K, O'Donnell L, Ooi GT, Stanton PG, McLachlan RI, Robertson DM. 2003 Differential regulation of rat testicular 5alpha-reductase type 1 and 2 isoforms by testosterone and FSH. J Endocrinol. 176:393-403.

301. Pratis K, O'Donnell L, Ooi GT, McLachlan RI, Robertson DM. 2000 Enzyme assay for 5alpha-reductase type 2 activity in the presence of 5alpha-reductase type 1 activity in rat testis. J Steroid Biochem Mol Biol. 75:75-82.

302. Watkins WJ, Goldring CE, Gower DB. 1988 Properties of 4-ene-5 alpha-reductase and studies on its solubilization from porcine testicular microsomes. J Steroid Biochem. 29:325-331.

303. Orlowski J, Bird CE, Clark AF. 1988 Androgen metabolism and regulation of rat ventral prostate growth and acid phosphatase during sexual maturation. J Endocrinol. 1988 116:81-90.

304. Nozu K, Tamaoki BI. 1974 Intranuclear and intermicrosomal distributions of 3-oxo-5alpha-steroid:NADP+ delta4-oxidoreductase in rat ventral prostate. Biochim Biophys Acta. 348:321-333.

305. Span PN, Sweep CG, Benraad TJ, Smals A. 1996 Differential subcellular distribution of rat prostatic steroid 5alpha-reductase isozyme activities. Eur J Endocrinol. 134:386-392.

306. Djoseland O, Bruchovsky N, Rennie PS, Otal N, Hoglo S. 1983 5 alpha-reductase activity in stroma and epithelium of rat prostate and epididymis. A contribution to elucidation of the mechanism for development of hyperplastic growth of prostatic tissue. Acta Endocrinol (Copenh). 103:273-281.

307. Kitahara S, Higashi Y, Takeuchi S, Oshima H. 1989 Relationship of changing delta 4-steroid 5 alpha-reductase activity to [125I]iododeoxyuridine uptake during regeneration of involuted rat prostates. Biol Reprod. 40:793-799.

308. Torres JM, Ruiz E, Ortega E. 2003 Development of a quantitative RT-PCR method to study 5alpha-reductase mRNA isozymes in rat prostate in different androgen status. Prostate. 56:74-79.

309. Moore RJ, Wilson JD. 1973 The effect of androgenic hormones on the reduced nicotinamide adenine dinucleotide phosphate: Δ^4-3-ketosteroid 5α-oxidoreductase of rat ventral prostate. Endocrinology 93:581-592.

310. Pelletier G, Luu-The V, Li S, Ouellet J, Labrie F. 2004 Cellular localization of mRNA expression of enzymes involved in the formation and inactivation of hormonal steroids in the mouse prostate. J Histochem Cytochem. 52:1351-1356.

311. Span PN, Benraad ThJ, Sweep CG, Smals AG. 1996 Kinetic analysis of rat steroid 5alpha-reductase activity in prostate and epididymis homogenates at neutral pH: evidence for type I activity in epididymis. J Steroid Biochem Mol Biol. 57:95-101.

312. Scheer H, Robaire B. 1983 Subcellular distribution of steroid delta 4-5 alpha-reductase and 3 alpha-hydroxysteroid dehydrogenase in the rat epididymis during sexual maturation. Biol Reprod. 29:1-10.

313. Scheer H, Robaire B. 1983 Solubilization and partial characterization of rat epididymal delta 4-steroid 5 alpha-reductase (cholestenone 5 alpha-reductase). Biochem J. 211:65-74.

314. Robaire B, Ewing LL, Zirkin BR, Irby DC. 1977 Steroid delta4-5alpha-reductase and 3alpha-hydroxysteroid dehydrogenase in the rat epididymis. Endocrinology. 101:1379-1390.

315. Robaire B. 1979 Effects of unilateral orchidectomy on rat epididymal delta 4-5 alpha-reductase and 3 alpha-hydroxysteroid dehydrogenase. Can J Physiol Pharmacol. 57:998-1003.

316. Robaire B, Viger RS. 1993 Regulation of Epididymal Epithelial Functions. In: Zirkin BR, Whitcomb R (eds), Understanding Male Infertility: Basic and Clinical Approaches. New York: Raven Press, Ltd. pp 183-210

317. Robaire B, Zirkin BR. 1981 Hypophysectomy and simultaneous testosterone replacement: effects on male rat reproductive tract and epididymal delta 4-5 alpha-reductase and 3 alpha-hydroxysteroid dehydrogenase. Endocrinology. 109:1225-1233.

318. Tindall DJ, Means AR. 1976 Concerning the hormonal regulation of androgen binding protein in rat testis. Endocrinology. 99:809-818.

319. French FS, Ritzen EM. 1973 A high-affinity androgen-binding protein (ABP) in rat testis: evidence for secretion into efferent duct fluid and absorption by epididymis. Endocrinology. 93:88-95.

320. Felden F, Leheup B, Fremont S, Bouguerne R, Egloff M, Nicolas JP, Grignon G, Gueant JL. 1992 The plasma membrane of epididymal epithelial

cells has a specific receptor which binds to androgen-binding protein and sex steroid-binding protein. J Steroid Biochem Mol Biol. 42:279-285.

321. Viger RS, Robaire B. 1992 Expression of 4-ene steroid 5 alpha-reductase messenger ribonucleic acid in the rat epididymis during postnatal development. Endocrinology. 131:1534-1540.

322. Viger RS, 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.

323. Rosen R, Altwein J, Boyle P, Kirby RS, Lukacs B, Meuleman E, O'Leary MP, Puppo P, Chris R, Giuliano F. 2004 Lower urinary tract symptoms and male sexual dysfunction: the multinational survey of the aging male (MSAM-7). Prog Urol. 14:332-344.

324. Libecco JF, Bergfeld WF. 2004 Finasteride in the treatment of alopecia. Expert Opin Pharmacother. 5:933-940.

325. Chen W, Zouboulis CC, Orfanos CE. 1996 The 5 alpha-reductase system and its inhibitors. Recent development and its perspective in treating androgen-dependent skin disorders. Dermatology. 193:177-184.

326. Zaccheo T, Giudici D, di Salle E. 1998 Effect of the dual 5alpha-reductase inhibitor PNU 157706 on the growth of dunning R3327 prostatic carcinoma in the rat. J Steroid Biochem Mol Biol. 64:193-198.

327. Andriole GL, Humphrey P, Ray P, Gleave ME, Trachtenberg J, Thomas LN, Lazier CB, Rittmaster RS. 2004 Effect of the dual 5alpha-reductase inhibitor

dutasteride on markers of tumor regression in prostate cancer. J Urol. 172:915-919.

328. Gormley GJ, Stoner E, Bruskewitz RC, Imperato-McGinley J, Walsh PC, McConnell JD, Andriole GL, Geller J, Bracken BR, Tenover JS, Vaughan ED, Pappas F, Taylor A, Binkowitz B, Ng J. 1992 The effect of finasteride in men with benign prostatic hyperplasia. The Finasteride Study Group. N Engl J Med. 327:1185-1191.

329. Foley CL, Kirby RS. 2003 5 alpha-reductase inhibitors: what's new? Curr Opin Urol. 13:31-37.

330. di Salle E, Giudici D, Radice A, Zaccheo T, Ornati G, Nesi M, Panzeri A, Delos S, Martin PM. 1998 PNU 157706, a novel dual type I and II 5alpha-reductase inhibitor. J Steroid Biochem Mol Biol. 64:179-186.

331. Frye SV, Bramson HN, Hermann DJ, Lee FW, Sinhababu AK, Tian G. 1998 Discovery and development of GG745, a potent inhibitor of both isozymes of 5 alpha-reductase. Pharm Biotechnol. 11:393-422.

332. Hirosumi J, Nakayama O, Chida N, Inami M, Fagan T, Sawada K, Shigematsu S, Kojo H, Notsu Y, Okuhara M. 1995 FK143, a novel nonsteroidal inhibitor of steroid 5 alpha-reductase: (2) In vivo effects on rat and dog prostates. J Steroid Biochem Mol Biol. 52:365-373.

333. Roehrborn CG, Boyle P, Nickel JC, Hoefner K, Andriole G; ARIA3001 ARIA3002 and ARIA3003 Study Investigators. 2002 Efficacy and safety of a dual inhibitor of 5-alpha-reductase types 1 and 2 (dutasteride) in men with benign prostatic hyperplasia. Urology. 60:434-441.

334. Cukierski MA, Sina JL, Prahalada S, Wise LD, Antonello JM, MacDonald JS, Robertson RT. 1991 Decreased fertility in male rats administered the 5 alpha-reductase inhibitor, finasteride, is due to deficits in copulatory plug formation. Reprod Toxicol. 5:353-362.

335. Wise LD, Minsker DH, Cukierski MA, Clark RL, Prahalada S, Antonello JM, MacDonald JS, Robertson RT. 1991 Reversible decreases of fertility in male Sprague-Dawley rats treated orally with finasteride, a 5 alpha-reductase inhibitor. Reprod Toxicol. 5:337-346.

336. Iguer-Ouada M, Verstegen JP. 1997 Effect of finasteride (Proscar MSD) on seminal composition, prostate function and fertility in male dogs. J Reprod Fertil Suppl. 51:139-149.

337. George FW, Johnson L, Wilson JD. 1989 The effect of a 5 alpha-reductase inhibitor on androgen physiology in the immature male rat. Endocrinology. 125:2434-2438.

338. Cohen J, Ooms MP, Vreeburg JT. 1981 Reduction of fertilizing capacity of epididymal spermatozoa by 5 alpha-steroid reductase inhibitors. Experientia. 37:1031-1032.

339. Robaire B, Covey DF, Robinson CH, Ewing LL. 1977 Selective inhibition of rat epididymal steroid delta4-5alpha-reductase by conjugated allenic 3-oxo-5, 10-secosteroids. J Steroid Biochem. 8:307-310.

340. de Larminat MA, Blaquier JA. 1979 Effect of in vivo administration of 5 alpha reductase inhibitors on epididymal function. Acta Physiol Lat Am. 29:1-6.

341. Vazquez MH, Tezon JG, Blaquier JA. 1987 Studies on the mechanism of the antiandrogenic effect of a putative 5 alpha-reductase inhibitor. J Steroid Biochem. 28:227-231.

342. Cooke GM, Robaire B. 1986 The effects of diethyl-4-methyl-3-oxo-4-aza-5 alpha-androstane-17 beta-carboxamide (4-MA) and (4R)-5,10-SECO-19-norpregna-4,5-diene-3,10,20-trione (SECO) on androgen biosynthesis in the rat testis and epididymis. J Steroid Biochem. 24:877-886.

343. Overstreet JW, Fuh VL, Gould J, Howards SS, Lieber MM, Hellstrom W, Shapiro S, Carroll P, Corfman RS, Petrou S, Lewis R, Toth P, Shown T, Roy J, Jarow JP, Bonilla J, Jacobsen CA, Wang DZ, Kaufman KD. 1999 Chronic treatment with finasteride daily does not affect spermatogenesis or semen production in young men. J Urol. 162:1295-1300.

344. Rhoden EL, Gobbi D, Menti E, Rhoden C, Teloken C. 2002 Effects of the chronic use of finasteride on testicular weight and spermatogenesis in Wistar rats. BJU Int. 89:961-963.

CHAPTER 2

Effects of PNU157706, a Dual 5α-Reductase Inhibitor, on Gene Expression in the Rat Epididymis

Natali Anne Henderson, Gerard M. Cooke[1] and Bernard Robaire

Published in Journal of Endocrinology

J Endocrinol. 2004 May;181(2):245-261.

[1] G.M. Cooke did the *in vitro* 5α-reductase enzyme assays described in this chapter.

Abstract

The epididymis is the site of sperm maturation and storage. 5α-Reductases (types 1 and 2) are key enzymes in this tissue because they convert testosterone to dihydrotestosterone (DHT), the main androgen regulating epididymal functions. Examining the consequences of inhibiting DHT formation is likely to provide important information regarding the regulation of epididymal functions, yet few inhibitor studies have focused on this tissue. To understand better DHT-mediated regulation of epididymal gene expression, we employed a dual 5α-reductase inhibitor and cDNA microarrays to examine the effects of 5α-reductase inhibition on gene expression in the initial segment, caput, corpus, and cauda epididymidis. Inhibition of epididymal 5α-reductase activity by PNU157706 was confirmed by *in vitro* enzyme assays. Rats were treated with 0, 0.1, 1.0 or 10 mg/kg/day of PNU157706 for 28 days. The weights of DHT-dependent tissues, including the epididymis, were decreased following treatment. The effect of treatment on gene expression was dose-dependent and highly segment-specific. The initial segment responded uniquely in that a similar number of genes increased and decreased in expression compared with the other segments where the majority of affected genes decreased in expression. Some of the more dramatically affected genes were involved in signal transduction as well as fatty acid and lipid metabolism, regulation of ion and fluid transport, luminal acidification, oxidative defense and protein processing and degradation. These are essential processes contributing to the formation of an optimal luminal microenvironment required for proper sperm maturation. These

results provide novel insight into the DHT-dependent mechanisms that control epididymal functions.

Introduction

The epididymis is a single, highly convoluted tubule that receives the input of sperm and fluid from the testis and that empties into the vas deferens (Robaire & Hinton 2002). It is structurally divided into four main segments (initial segment, caput, corpus and cauda epididymidis) and functions in the transport, protection, maturation and storage of spermatozoa (Robaire & Hinton 2002, Orgebin-Crist 1967). The multifaceted sperm-related functions of the epididymis have made this tissue an appealing target for the control of male contraception and fertility. Achieving these therapeutic goals, however, necessitates a greater understanding of the mechanisms regulating epididymal structure and functions.

Not surprisingly, given the complexity of this tissue, many factors are involved in the regulation of epididymal functions, including estrogens, retinoids, growth factors and unidentified testicular factors (Hess *et al.* 2002, Orgebin-Crist *et al.* 2002, Lan *et al.* 1998, Hinton *et al.* 1998). Androgens, however, are recognized as the primary hormonal regulators of epididymal structure and functions (Orgebin-Crist & Tichenor 1973, Blaquier *et al.* 1972). Furthermore, several in vivo and in vitro studies have confirmed that it is not testosterone, but rather dihydrotestosterone (DHT), that is the main androgen acting in this tissue (Orgebin-Crist *et al.* 1976, Tindall *et al.* 1972, Turner *et al.* 1984, Gloyna & Wilson 1969). 5α-Reductase (EC 1.3.1.22) is thus a key enzyme in the

epididymis because of its role in locally converting testosterone to DHT. Two isoforms of 5α-reductase have been identified and are termed type 1 and type 2 (Andersson & Russell 1990, Normington & Russell 1992). Both isoforms are present in the epididymis and are differentially expressed along the tubule (Viger & Robaire 1996).

Examining the consequences of inhibiting DHT formation is likely to provide important information regarding the regulation of epididymal functions, yet very few inhibitor studies have focused on this tissue (de Larminat & Blaquier 1979, Cohen *et al.* 1981, Cooke & Robaire 1986, Zoppi *et al.* 1992). In order to elucidate specific functions of 5α-reductase in the epididymis using inhibitors, compounds that are specific only to 5α-reductase and that inhibit both isoforms of the enzyme (i.e. dual inhibitors) are required. The latter is necessary as it has not yet been determined if one or both of the isoforms are important for mediating the androgen regulation of epididymal structure and functions. Specificity and toxicity were problems with early compounds such as diethyl-4-methyl-3-oxo-4-aza-5 alpha-androstane-17 beta-carboxamide (4-MA) (Cooke & Robaire 1986, DiSalle *et al.* 1994). Modification of 4-MA yielded finasteride, the first clinically available 5α-reductase inhibitor (Sudduth *et al.* 1993, Li *et al.* 1995, Steers 2001). With the discovery of two isoforms of 5α-reductase, it was shown that finasteride is selective for the type 2 isoform. The lack of specificity of finasteride for the type 1 isoform has been proposed as the reason why this drug is only moderately effective in the treatment of benign prostatic hyperplasia (Steers 2001, Bartsch *et al.* 2002). Drug development efforts to create more

therapeutically effective compounds for the treatment of androgen-dependent disorders such as benign prostatic hyperplasia have provided compounds that fulfill the requirements of specificity and dual action. PNU157706 is a novel dual 5α-reductase inhibitor for the potential treatment of benign prostatic hyperplasia (di Salle *et al.* 1998). It is more potent than finasteride in decreasing DHT levels. This compound is of express interest as it has been reported to have an effect on epididymal weight (di Salle *et al.* 1998).

Hormones often effect changes in their target tissues by altering the expression of specific sets of genes. The expression patterns of several epididymis-specific and/or epididymis-relevant genes have been analyzed along the epididymis (for review see Cornwall *et al.* 2002); however, understanding the structure and function of this complex tissue requires more than analyzing the properties of isolated genes. The biological function of sperm maturation arises from the interactions of many components. cDNA array technology provides a powerful method for large-scale, parallel gene expression analysis that can provide insight into these interactions, and how they are regulated. Our objective in this study is to use cDNA array technology in combination with a novel 5α-reductase inhibitor to help elucidate the DHT-dependent regulatory networks that govern segment-specific gene expression in the epididymis. We demonstrate that the inhibition of DHT formation in the epididymis alters the expression of many genes potentially involved in sperm-related functions. Furthermore, the present studies indicate that DHT-dependent regulation of gene expression is highly segment-specific and involves components of different signaling pathways.

Materials and Methods

Animals

Adult male Sprague-Dawley rats (325-350g) were obtained from Charles River Canada (St. Constant, PQ, Canada), maintained under controlled light (14L:10D) and temperature (22°C), and freely provided with food and water. Caput-corpus epididymides from 90-day old animals that were previously stored at -80°C were used for *in vitro* enzyme assays. For the *in vivo* study, rats were randomly divided into 4 groups of 5 animals each and gavaged with 0.5 ml/kg of 0 (control), 0.1 (low), 1.0 (medium) or 10 mg/kg (high) of PNU157706 (generously provided as a gift by Pharmacia & Upjohn, Italy) suspended in 0.5% methylcellulose solution (BDH, Montreal, QC) containing 0.4% Tween 80 (A&C American Chemicals Ltd, Montreal, QC) for 28 consecutive days. Doses were selected based on reported effects on rat ventral prostate; the dosing regimen with 10 mg/kg/day PNU157706 has been shown to decrease prostatic dihydrotestosterone levels by >90% (di Salle *et al.* 1998). At killing, animals were anesthetized and seminal vesicles, testes, ventral prostates and epididymides from the left side were collected. Epididymides were sectioned into initial segment, caput, corpus and cauda, and immediately frozen in liquid nitrogen. Tissues were stored at -80°C until used for RNA extraction. All animal studies were conducted in accordance with the principles and procedure outlined in the Guide to the Care and Use of Experimental Animals prepared by the Canadian Council on Animal Care.

Statistical Analysis

One-way ANOVA followed by Tukey's test was used to detect significant effects of PNU157706 treatment on tissue weights. The level of significance was taken as $p < 0.05$.

In vitro 5α-reductase assays

Unlabeled steroids were purchased from Steraloids Inc. (Newport, RI). (1,2,6,7-^{3}H) testosterone (74.0 Ci/mmol) was from Dupont/NEN (Boston, MA). Organic solvents were from BDH, (Montreal, QC). Dimethylglutaric acid (DMG), NADPH and dimethyl sulfoxide (DMSO) were from Sigma Chemical Co. (St Louis, MO). Plastic coated Whatman^{TM} PE SIL G silica gel chromatography plates were purchased from Chromatographic Specialties (Montreal, QC). PNU157706 solutions were prepared in DMSO.

Frozen caput-corpus epididymides (only normal, untreated tissues were used) were thawed, homogenized (Polytron) in DMG buffer (50 mM 2,3-dimyristoylglycerol (DMG), 5% glycerol, NaOH, 0.5mM NADPH) pH 6.5, and the nuclear and microsomal fractions were assayed for 5α-reductase activity as previously described (Cooke & Robaire 1987); protein was estimated using the method of Lowry (Lowry *et al.* 1951). Initial studies were done to determine the linearity of nuclear and microsomal activity with time and protein concentration (data not shown). Purification and quantification of steroids was by thin-layer chromatography and scintillation counting (Cooke & Robaire 1987). Reaction

rates were obtained by linear regression analysis ($r > 0.97$). At pH 4.5, no 5α-reductase activity was measurable at any concentration of testosterone.

Kinetic studies of 5α-reductase activity were done at pH 6.5 with testosterone (nine concentrations ranging from 5 to 375 nM). To determine the effects of PNU157706 on 5α-reductase activity, PNU157706 was included at concentrations of 0, 1, 3 and 10 nM. A single incubation time of 1h and an incubation volume of 1 ml were used in kinetic studies. To obtain the apparent Michaelis constant ($K_{m(app)}$) and apparent maximum velocity ($V_{max(app)}$), the Wilkinson method (non-linear least squares analysis of a rectangular hyperbola (Wilkinson 1961)) was used. Slope and intercept replots were obtained from, respectively, $K_{m(app)} / V_{max(app)}$ versus [I] and $1/V_{max(app)}$ versus [I]. Linear regression analysis of the replots was employed to determine the $K_{i(app)}$ values.

RNA extraction

Total RNA was extracted from each sample using guanidine thiocyanate as described previously (Jervis & Robaire 2001). Following isolation, RNA samples were DNase-treated (Atlas Pure Isolation Kit user manual, section IV, Clontech, Palo Alto, CA) to remove contaminating genomic DNA and RNA concentration was assessed by optical density determination at 260 nm (Beckman DU7 spectrophotometer, Montreal, PQ, Canada). In addition to spectrophotometric readings, RNA quality was verified by gel electrophoresis. Each sample consisted of a single epididymal segment obtained from individual rats; no tissues were pooled.

Complementary DNA arrays and hybridization

RNA samples were used to probe cDNA arrays (Atlas Rat 1.2K; Clontech) according to the manufacturer's instructions. Five arrays per epididymal segment per treatment group (control, low, medium and high PNU157706) were probed and referred to as replicates (n=5/treatment/segment), with the following exceptions: 4 replicates per group were used (n=4/group) for the initial segment in the high PNU157706 group, the initial segment and cauda in the medium PNU157706 group and the initial segment and caput in the low PNU157706 group; 3 arrays were used (n=3/group) for the corpus in the medium PNU157706 group. A total of 75 arrays were screened for this experiment. Arrays were exposed to phosphorimager plates (Molecular Dynamics; Sunnyvale, CA) 24 h before scanning with a phosphorimager (Storm, Molecular Dynamics). Analysis of array images with Atlas Image (Version 2.0; Clontech) was performed to quantify the intensity of each cDNA spot, which reflects the relative abundance of RNA in the sample. The raw data for each gene (intensity minus the background) were imported into Genespring 4.0.7 (Silicon Genetics, Redwood, CA) for further analysis. For each replicate array in a given treatment group, a gene was considered detected if its intensity was above threshold, with threshold defined as two times the average background of that individual array. A gene was considered expressed if it was detected in at least three replicates in that group.

To minimize experimental variation and allow for comparison of different treatment groups, data were normalized with the standard experiment-to-experiment normalization (Genespring). Specifically, the median level of

expression on each array was defined as 1 and expression of each gene was normalized relative to 1; this value was averaged for all replicates in a group to generate what is referred to as the relative intensity for a given gene. All individual gene profiles are shown using the standard experiment-to-experiment normalization. Gene expression changes of 2-fold or greater (i.e. 50% decrease or 100% increase) are focused on throughout the text except for genes belonging to specific gene families. The importance of replication for validation of gene expression studies has been documented (Lee *et al.* 2000, Herwig *et al.* 2001). Conventional statistical analysis was not applicable to our large normalized data sets. We include errors of the mean (SEM) of individual genes to show variability among replicates in a given group.

As a means to validate our gene array expression data, we examined the control expression profiles of three genes with well-established epididymal expression profiles (Fig. 1). As expected, the expression of 5α-reductase type 2 was highest in the initial segment and caput epididymidis, angiotensin converting enzyme expression peaked dramatically in the caput epididymidis in comparison to the other segments, and androgen receptor gene expression remained constant throughout the epididymis (Viger & Robaire 1996, Strittmatter & Snyder 1984, Robaire & Viger 1993).

Results

Tissue Weights

There were no significant changes in body weights among any of the groups of animals (data not shown). As expected, the weights of the seminal vesicle and ventral prostate, both DHT-dependent tissues, were significantly decreased with all doses of PNU157706 while there was no effect of treatment on testis weights (Fig. 2A-C). A 10% decrease in epididymal weight was observed with the low and medium PNU157706 treatment groups with a further decrease to 82% of control in the high PNU157706 group (Fig. 2D).

5α-Reductase Activity

The addition of PNU157706 to incubations at concentrations of 0, 1, 3 and 10 nM inhibited epididymal 5α-reductase as shown in Fig. 3A (nuclear) and B (microsomal) fractions. Kinetic analyses demonstrated that PNU157706 caused the $K_{m(app)}$ values to increase (about three to four fold) without affecting the $V_{max(app)}$ values. Confirmation of these findings was achieved with slope ($K_{m(app)}/V_{max(app)}$ vs. [I]) and intercept ($1/V_{max(app)}$ vs.[I]) replots, shown in Fig. 3C (nuclear) and D (microsomal); the slopes of the replots were positive and the slopes of the intercept replots were almost horizontal or exhibited a negative slope. The values for the inhibition constant ($K_{i(app)}$) for PNU157706 from the slope replots were lower than those obtained from the intercept replots (Table 1) indicating that this drug is a competitive inhibitor of 5α-reductase. Furthermore,

since the K_i is lower than the $K_{m(app)}$, the affinity of 5α-reductase for PNU157706 is greater than the affinity for testosterone.

Number of genes expressed

The expression of 1176 genes was examined simultaneously using cDNA arrays. In each epididymal segment, similar numbers of transcripts were detected in the control and high PNU157706 treatment groups. In the initial segment, transcripts for 34% (397) and 31% (368) of genes were detected in tissues from control and treated rats, respectively. Similarly, in the caput epididymidis, transcripts for 35% (411) of genes were detected in tissue from control animals and 31% (360) in those from treated rats. A lower number of transcripts was detected in the corpus epididymidis compared to the other segments: 25% (295) of genes in control, 21% (247) in treated. Slightly more transcripts were detected in the cauda epididymidis; 36% (420) of genes in control, 37% (438) in treated.

Overall changes in gene expression with PNU157706 treatment

The range of gene expression changes in the initial segment following high PNU157706 treatment extended from a greater than 80% decrease (c-met proto-oncogene) to a 6.6-fold increase (set β + set α isoform). In the caput epididymidis, the range was from a 73% decrease in expression (cathepsin B) to a 9.7-fold increase in expression (P2X purinoceptor 1). In addition to having the

lowest number of genes with detected expression, the corpus epididymidis also demonstrated the smallest range of gene expression changes following treatment; expression changes ranged from a 78% decrease (carbonic anhydrase 4 (CA4)) to a 4.8-fold increase (muscarinic acetylcholine receptor M4). The largest range of gene expression changes was observed in the cauda epididymidis which was from a greater than 90% decrease (elastase 2) to a 7.3-fold increase (serine proteinase rPC7).

As expected, using the criterion of 2-fold changes in gene expression, i.e., a 100% increase or 50% decrease, the expression of a large proportion of genes was unaffected by PNU157706 treatment in each epididymal segment. Analysis of the number of genes undergoing a minimum 2-fold change in expression between control and high PNU157706 treatment groups revealed a unique response to treatment in the initial segment (Fig. 4). In this segment, a similar number of genes increased and decreased in expression by 2-fold or greater. In contrast, in more distal epididymal segments, there were more genes decreasing than increasing in expression by 2-fold or more following treatment (Fig. 4).

Segment-specific changes in gene expression

A total of 81 genes changed in expression by 2-fold or greater in the epididymis following treatment with high PNU157706. The expression of the majority of these genes was affected uniquely in one segment. The few transcripts that changed in expression by at least 2-fold in more than one segment are indicated in Table 2. Transcripts of particular interest that changed

in a segment specific manner were examined in the control, low, medium and high PNU157706 treatment groups in order to visualize dose-response profiles.

Initial Segment. A total of 25 transcripts changed by a minimum of 2-fold in the initial segment. Differential expression was observed for 4 genes that are mediators or modulators of signal transduction (Fig. 5A). Two oncogenic proteins (NF-2, retinoblastoma protein pp105) and ras-GTPase activating protein (p120GAP) decreased in expression. The maximum decrease was achieved only with the high dose of PNU157706. The fourth gene, Crk-associated substrate (CAS), a kinase substrate, showed increased expression with medium and high PNU157706 treatment. Another important group of transcripts affected in the initial segment were those involved in fatty acid/lipid metabolism: fatty acid amide hydrolase, corticosteroid 11-β dehydrogenase 1, very long chain acyl-CoA dehydrogenase (VLCAD) and 5-lipoxygenase (5-LO) (Fig. 5B). Transcripts for these 4 genes increased following PNU157706 treatment. For the two dehydrogenases and fatty acid amide hydrolase, the maximum increase was observed with the low dose while for 5-lipoxygenase, an increase in expression was only achieved with the high dose. Interestingly, two genes with roles in regulating ion and water balance in body fluids were also affected by PNU157706 treatment; atrial natriuretic peptide clearance (ANP-C) receptor expression decreased while chloride channel CLC-7 expression increased (Fig. 5C).

Caput. Only 14 genes changed in expression by a minimum of 2-fold in the caput epididymidis. Specifically, the expression of three signaling genes was differentially affected by PNU157706 treatment (Fig. 6A). One transcript, cAMP-dependent protein kinase type II-beta regulatory chain (cAMP dep PK Type 11β), showed a decrease only with the high dose of PNU157706. In contrast, Ehk 3, a tyrosine kinase, and neural thrombospondin 1-like protein (NELL-1), a protein kinase C-binding protein, both increased and the maximum effect was achieved with the low dose of PNU157706. Cathepsins, a family of cysteine proteases that function in protein degradation and processing, have been shown previously to change in expression in the epididymis under certain conditions (McGrath 1999, Jervis & Robaire 2002). In the caput epididymidis, cathepsin B expression decreased following treatment only with the high dose of PNU157706 (Fig. 6B). Also of note in the caput epididymidis was the increase in expression of short chain acyl-CoA dehydrogenase (SCAD), another gene involved in fatty acid/lipid metabolism (Fig. 6C). As seen with other genes, the maximum increase in expression was obtained with the low dose of PNU157706. Other transcripts specifically affected by treatment in the caput epididymidis included two transforming growth factor β family members; TGFβ receptor 1 and TGFβ masking large subunit decreased maximally in expression with the low dose of PNU157706.

Corpus. In the corpus epididymidis, 15 genes showed expression changes of 2-fold or greater. Of particular interest in this segment was the dose-dependent

decrease in expression of carbonic anhydrase 4 (CA-4), a gene implicated in the acidification of the epididymal lumen (Fig. 7A). Three signaling-related genes also decreased in expression in a dose-dependent manner (Fig. 7B). These were ras related protein rab13, a G-protein involved in trafficking and targeting, ephrin B1, a receptor tyrosine kinase ligand, and Jak1 tyrosine-protein kinase.

Cauda. A total of 38 genes changed a minimum of 2-fold in expression in the cauda epididymidis. Once again, transcripts involved in mediating or modulating signal transduction were differentially affected by inhibitor treatment (Fig. 8A). Rab-related GTP-binding protein and muscle/brain cAMP-dependent protein kinase inhibitor (PKI-alpha) showed the same dose-response profile where the maximum decrease in expression was achieved with the low dose of PNU157706. Transcripts for phosphatidylinositol 4-kinase (PI4-K) and the putative protein kinase C regulatory protein, 14-3-3 protein gamma subtype, also similarly decreased in a dose-dependent manner with the maximum decrease achieved with the medium dose. The ras-related GTPase rab4B followed a different profile decreasing in a dose-dependent manner. In addition to these signalling genes, phospholipase expression was also affected. Phospholipases hydrolyze ester bonds in phospholipids to generate second messengers such as diacyl glycerol and inositol triphosphate. Specifically in the cauda epididymidis, three phospholipase C (PLC) family members decreased in expression following PNU157706 treatment (Fig. 8B). PLC gamma-2, PLC delta-1 and PLC gamma-1 expression decreased in a dose-dependent manner, but the expression of the

latter two genes was not affected by the low dose of PNU157706. Three different ion transporters decreased specifically in the cauda epididymidis (Fig. 8C). Hydrogen-potassium transporting ATPase alpha 2a subunit, implicated in the acidification of bodily fluids, decreased in expression maximally with the low dose of PNU157706. In contrast, the sodium/potassium transporting ATPase beta 2 subunit decreased in expression maximally only with the high dose of PNU157706. The anion exchanger, band 3, which contributes to the regulation of cell pH and of cell volume, decreased in expression in a dose-dependent manner. Other genes that changed in expression specifically in the cauda epididymidis after inhibitor treatment included genes with metabolic functions (e.g. aldolase C and ceruloplasmin decreased), proteases (elastase 2 decreased, cathepsin S increased) and growth-factor related genes (e.g. platelet derived growth factor receptor α and vascular endothelial growth factor D decreased).

Expression by gene family

An advantage of cDNA array technology is the capability of examining the effects of 5α-reductase inhibitor treatment on the overall expression of gene families that are known to be of importance in the epididymis. The expression patterns of two functionally relevant gene families, oxidative stress-related genes and proteasomal genes, were examined along the epididymis.

Oxidative stress-related genes. The expression of four glutathione S-transferases (GSTs), one glutathione peroxidase (GPX) and glutathione synthetase (GSH synthetase) was examined along the epididymis following PNU157706 treatment (Fig. 9). GST mu expression decreased by 30% in the initial segment but remained unchanged in the other segments. GST Yrs-Yrs expression also decreased in the initial segment as well as in the cauda epididymidis by approximately 33%, and 48%, respectively. In contrast, expression of this particular GST increased in the corpus by 2.5-fold. Interestingly, GST subunit 5 theta expression was also differentially affected by treatment in a segment-specific manner; expression decreased in the initial segment by 52% and increased in the cauda by 3-fold. GST subunit 13 expression was decreased by 38% in the initial segment and by 67% in the corpus. Also changing in the initial segment was the expression of GPX3 which decreased 57%. Lastly, the expression of GSH synthetase was increased specifically in the caput by approximately 1.6-fold. Clearly, the expression of oxidative-stress related genes was most affected in the proximal epididymis, particularly in the initial segment. Furthermore, with few exceptions, the predominant effect of treatment was decreased expression.

Proteasomal genes. We examined the expression of eight proteasomal proteins along the epididymis following treatment with PNU157706 (Fig. 10). The predominant effect of treatment was a decrease in expression of the proteasomal genes, particularly in the distal epididymis (corpus and cauda epididymidis).

Differential expression of the proteasomal genes was not observed in the initial segment. In this region, the greatest fold-change in expression was a 1.4-fold increase for proteasome component C3. Proteasomal gene transcript levels in the caput were also relatively unaffected by treatment with the exception of proteasome activator rPA28 subunit alpha that decreased 33% in expression and proteasome subunit RC10-II that decreased approximately 52% in expression. In the corpus epididymidis, transcript levels for proteasome subunit R-delta, component C8, subunit R-iota and beta subunit decreased by approximately 33%, 33%, 63% and 30% respectively. The greatest effect of treatment was seen in the cauda epididymidis. In this region, the greatest decrease in expression was observed for proteasome subunit rPA28 (69%). Additionally, proteasome components C2 and C3 both decreased in expression by 33% and proteasome component C8 and beta subunit decreased in expression by nearly 50%.

Discussion

In the present study, we have confirmed that PNU157706 is a competitive inhibitor of microsomal and nuclear epididymal 5α-reductase activity and has effects, in vivo, on the weights of DHT-dependent tissues, including the epididymis. Furthermore, we demonstrate that dual inhibition of 5α-reductase has a highly segment-specific effect on the expression of a wide range of genes in the epididymis, thus indicating that DHT is a global mediator of many epididymal functions.

Perhaps the most important function of the epididymis is the creation of a continuously changing luminal environment that allows for proper sperm concentration, maturation, and storage in different segments of the epididymis (Robaire & Hinton 2002, Turner 1991). A large number of studies have demonstrated that many of the individual processes that contribute to the creation of an optimal luminal microenvironment are regulated by androgens (Ezer & Robaire 2002). These processes include fluid and ion transport, luminal acidification as well as protein and lipid secretion, processing and degradation. Our analysis of 2-fold changes in gene expression following inhibitor treatment revealed the segment-specific increase and decrease in expression of many genes potentially involved in these processes.

The major role of fluid transport across the epididymal epithelium is to concentrate sperm as they travel through the tubule. In addition, this process regulates the concentration of constituents in the luminal fluid to which sperm are exposed. Androgens influence fluid balance in the epididymis by regulating sodium and anion transport across the epithelium (Wong & Yeung 1977, Au *et al.* 1978, Leung *et al.* 2002) and modulate the epididymal expression of specific aquaporins, water channels that mediate water transport in many tissues (Pastor-Soler *et al.* 2002). In this study, we have shown an effect of 5α-reductase inhibition on the expression of several genes involved in fluid and ion transport. In the initial segment, expression of the clearance receptor for atrial natriuretic peptide decreased with treatment. This is of particular interest in light of the fact that atrial natriuretic peptide interacts physiologically with the renin-angiotensin

system to control the fluid balance important for hemodynamic and renal function (Suzuki *et al.* 2001, Melo *et al.* 2000, Oliveira-Souza *et al.* 2002). Furthermore, in the epididymis, androgens regulate the expression of specific components of the renin-angiotensin system, namely angiotensinogen and angiotensin II receptor type 1 (Leung *et al.* 2002, Leung *et al.* 2000). Thus, we propose that DHT-dependent expression of specific components regulates this system mediating fluid transport in the epididymis.

Acidification of the luminal fluid is believed to be necessary for proper sperm maturation as well as storage, particularly in the distal epididymis (Turner & Reich 1985). The anti-androgen flutamide has been shown to elevate epididymal luminal pH in rats, thus indicating that acidification is under the control of androgens (Caflisch 1993). Anion exchangers, ion channels, specific ATPases and carbonic anhydrase are implicated in the acidification process (Turner 2002, Jensen *et al.* 1999, Kaunisto *et al.* 1999). The mRNA for carbonic anhydrase IV, predominantly expressed in the corpus epididymidis, decreases following androgen withdrawal by castration (Kaunisto *et al.* 1999). In the present study, carbonic anhydrase IV is also shown to be expressed predominantly in the corpus epididymidis. Furthermore, carbonic anhydrase IV showed the highest fold decrease in expression with inhibitor treatment, specifically in this segment, suggesting that the androgen regulating the expression of this gene is DHT. In the cauda epididymidis, we describe a further effect of inhibitor treatment on the expression of two other genes involved in pH

regulation; the expression of the alpha 2a subunit of the H^+/K^+ transporting ATPase decreased as did that of band 3 anion exchanger.

Sequential exposure of sperm to epididymal luminal contents, including proteins, is necessary for proper maturation (Turner 1991). While fluid regulation assures the right quantity of proteins present in the epididymal lumen, the qualitative evolution of proteins along the tubule involves protein processing and degradation events. Furthermore, protein processing is required for direct modification of sperm surface proteins, a major feature of epididymal sperm maturation (Phelps *et al.* 1990, Jones 1998). Some of the genes showing the highest fold segment-specific changes in expression following inhibitor treatment are those involved in protein processing/degradation (cathepsin B, elastase 2, serine proteinase rPC7), suggesting that DHT is an important regulator of this function. Of these three genes, only cathepsin B has been characterized previously in the epididymis at the protein and enzyme activity levels (Igdoura *et al.* 1995, Tomomasa *et al.* 1994). Notably, elastase 2 has angiotensin II forming ability (Santos *et al.* 2002) and might therefore represent another DHT-regulated component of the renin-angiotensin system in the epididymis.

A striking effect of inhibitor treatment was the increased expression of five genes involved in fatty acid/lipid metabolism in the proximal epididymis. These results are not altogether surprising since the epididymal lumen is a lipid-rich environment and the modification and exchange of sperm membrane lipids is another major feature of sperm maturation occurring in the proximal epididymis (Jones 1998). Interestingly, modification of the phospholipid environment

regulates 5α-reductase activity (Cooke & Robaire 1985). Lipid metabolizing enzymes have been shown previously to be androgen regulated in the prostate (Swinnen *et al.* 1997) and the epididymis (Beck 1980). Our results suggest that DHT regulates lipid metabolism by endogenously repressing the expression of specific enzymes.

An important advantage of gene expression profiling is the ability to analyze, in an integrated way, the expression of genes that carry out important functions as well as genes that regulate these functions. Inhibitor treatment clearly has a segment-specific effect on the expression of genes involved in signal transduction, indicating possible mechanisms through which DHT signals to promote sperm-related functions. For example, the protein kinase C (PKC) binding protein, NELL2, increased in expression in the caput epididymidis, while expression of 14-3-3 protein gamma subtype, a PKC regulator, decreased in the cauda epididymidis. The PKC family is interesting as various important PKC isoforms have been shown to be androgen-regulated in the rat ventral prostate (Montalvo *et al.* 2002). Inhibitor treatment also altered the expression of several ras/rab related genes in a segment-specific way. These are members or regulators of the small G protein superfamily that mediates cellular processes such as cytoskeletal reorganization, vesicular and nuclear transport, and notably, gene expression (Paduch *et al.* 2001). An example of one such gene is rab13, which decreased in expression in the corpus epididymidis following inhibitor treatment. DHT-dependent regulation of this gene is of note because rab13 has been shown to regulate the assembly of functional tight junctions in epithelial

cells (Marzesco *et al.* 2002) and androgens are known to regulate components of tight junctions in epithelial cells of the epididymis (Cyr *et al.* 1996, Gregory *et al.* 2001). Tight junctions are important components of occluding barriers, including the blood-epididymis barrier, which enables the creation of the optimal luminal microenvironment.

In addition to their functional relevance in the epididymis, lipids also play key roles in signaling (Cantley 2002). Inhibitor treatment decreased the expression of three phospholipase C (PLC) enzymes specifically in the cauda epididymidis. Phosphoinositides are classical signaling lipids and the importance of phospholipases in regulating phosphoinositide metabolism has been well characterized. More recently, a role for lipids in nuclear signal transduction has emerged that requires the activation of phospholipase C (Dygas & Baranska 2001). This nuclear location has implications for direct control of gene expression (Dygas & Baranska 2001, Martelli *et al.* 2002). DHT-dependent regulation of PLC expression might therefore indirectly control the expression of other important genes.

Spermatozoa generate reactive oxygen species required for capacitation and chromatin condensation, however, spermatozoa are also highly susceptible to the damaging effects of reactive oxygen species (Aitken 2002). Antioxidant defense mechanisms are thus of great importance in the epididymis to protect maturing spermatozoa as well as the epididymal epithelium from oxidative damage. Orchidectomy and androgen replacement studies demonstrate that the epididymal glutathione S-transferases are under separate control and are

differentially regulated by androgens (Robaire & Hales 1982). Our results further support the importance of DHT in regulating enzymes of the glutathione cycle, predominantly in the proximal epididymis where the glutathione antioxidant enzymes are concentrated.

The key function of the proteasome, a large, multicatalytic protease, is protein degradation (DeMartino & Slaughter 1999, Coux *et al.* 1996). In the epididymis, controlled protein degradation via the proteasome pathway not only regulates the luminal protein repertoire and contributes to sperm membrane remodeling during maturation, it also provides an important secondary defense against oxidative stress by preventing the accumulation of oxidized and damaged proteins (Mehlhase & Grune 2002, Grune 2000). Furthermore, the regulated degradation of proteins is involved in the control of various signal transduction pathways (Callis & Vierstra 2000). The overwhelming effect of inhibitor treatment was a decrease in proteasomal gene expression predominantly in the corpus and cauda epididymidis, implying that proteasomal gene expression is DHT-dependent in these two segments. Of particular relevance is a recent study in prostate cancer cells indicating an important role of the proteasome system in the regulation of androgen receptor transcriptional activity (Lin *et al.* 2002). Evidence suggests that the proteasome exerts its regulatory role by affecting androgen receptor transactivation, nuclear translocation, degradation and interaction with coregulators. This finding is supported by plant studies where the proteasome has been implicated in hormone signaling (Callis & Vierstra 2000, Gray & Estelle 2000). Thus, it is also plausible that regulation of proteasomal gene expression

by DHT affects the downstream expression of other androgen dependent genes in the epididymis.

In summary, our study of the effects of 5α-reductase inhibition on gene expression in the epididymis has shown DHT to be a major regulator of the segment-specific expression of a wide array of genes and gene families. The nature of the genes affected enhances our understanding of the androgen-regulated processes contributing to optimal sperm maturation in this tissue. Several novel genes were identified and further investigation is required to determine their functional relevance and potential as therapeutic targets in the control of male fertility and contraception.

Acknowledgements

NAH is a recipient of a CIHR Studentship. We should like to thank Dr. T. Zaccheo from Pharmacia-Upjohn (presently Pfizer) for a generous gift of PNU157706.

Funding

NAH is a recipient of a Canadian Institutes of Health Research Studentship. These studies were entirely financed by the CIHR (Canada). No financial contribution was received by the authors from private sources.

References

Aitken RJ 2002 Active oxygen in spermatozoa during epididymal transport. In *The epididymis: From molecules to clinical practice* pp 435-447. Eds Robaire B & Hinton BT. New York: Kluwer Academic/Plenum Publishers.

Andersson S & Russell DW 1990 Structural and biochemical-properties of cloned and expressed human and rat steroid 5-alpha-reductases. *Proceedings of the National Academy of Sciences of the USA* **87** 3640-3644.

Au CL, Ngai HK, Yeung CH & Wong PY 1978 Effect of adrenalectomy and hormone replacement on sodium and water transport in the perfused rat cauda epididymidis. *Journal of Endocrinology* **77** 265-266.

Bartsch G, Rittmaster RS & Klocker H 2002 Dihydrotestosterone and the concept of 5 alpha-reductase inhibition in human benign prostatic hyperplasia. *World Journal of Urology* **19** 413-425.

Beck B 1980 Tissue-specificity of the epididymal androgen dependent phospholipase-A. International Journal of Andrology **3** 349-362.

Blaquier JA, Burgos MH & Cameo MS 1972 Role of androgens in maturation of epididymal spermatozoa in guinea-pig. *Endocrinology* **90** 839-842.

Caflisch CR 1993 Effect of A Nonsteroidal Antiandrogen, Flutamide on Intraluminal Acidification in Rat Testis and Epididymis. *Andrologia* **25** 363-367.

Callis J & Vierstra RD 2000 Protein degradation in signaling. *Current Opinion in Plant Biology* **3** 381-386.

Cantley LC 2002 The phosphoinositide 3-kinase pathway. Science **296** 1655-1657.

Cohen J, Ooms MP & Vreeburg JTM 1981 Reduction of fertilizing-capacity of epididymal spermatozoa by 5-alpha-steroid reductase inhibitors. *Experientia* **37** 1031-1032.

Cooke GM & Robaire B 1985 Modulation of epididymal delta-4-steroid 5-alpha-reductase activity in vitro by the phospholipid environment. *Journal of Biological Chemistry* **260** 7489-7495.

Cooke GM & Robaire B 1986 The effects of diethyl-4-methyl-3-oxo-4-aza-5-alpha-androstane-17-beta-carboxamide (4-Ma) and (4R)-5,10-seco-19-norpregna-4,5-diene-3,10,20-trione (Seco) on androgen biosynthesis in the rat testis and epididymis. *Journal of Steroid Biochemistry* **24** 877-886.

Cooke GM & Robaire B 1987 The mechanism of rat epididymal 4-ene steroid 5-alpha-reductase. *Journal of Steroid Biochemistry and Molecular Biology* **26** 361-368.

Cornwall GA, Lareyre JJ, Matusik RJ, Hinton BT & Orgebin-Crist MC 2002 Gene expression and epididymal function. In *The epididymis: From molecules to clinical practice* pp 169-199. Eds Robaire B & Hinton BT. New York: Kluwer Academic/Plenum Publishers.

Coux O, Tanaka K & Goldberg AL 1996 Structure and functions of the 20S and 26S proteasomes. *Annual Review of Biochemistry* **65** 801-847.

Cyr DG, Hermo L & Laird DW 1996 Immunocytochemical localization and regulation of connexin43 in the adult rat epididymis. *Endocrinology* **137** 1474-1484.

de Larminat MA & Blaquier JA 1979 Effect of in vivo administration of 5 alpha reductase inhibitors on epididymal function. *Acta Physiologica Latin America* **29** 1-6.

DeMartino GN & Slaughter CA 1999 The proteasome, a novel protease regulated by multiple mechanisms. *Journal of Biological Chemistry* **274** 22123-22126.

di Salle E, Giudici D, Radice A, Zaccheo T, Ornati G, Nesi M, Panzeri A, Delos S & Martin PM 1998 PNU 157706, a novel dual type I and II 5 alpha-reductase inhibitor. *Journal of Steroid Biochemistry and Molecular Biology* **64** 179-186.

Disalle E, Briatico G, Giudici D, Ornati G & Panzeri A 1994 Endocrine properties of the testosterone 5-alpha-reductase inhibitor turosteride (Fce-26073). *Journal of Steroid Biochemistry* **48** 241-248.

Dygas A & Baranska J 2001 Lipids and signal transduction in the nucleus. *Acta Biochimica Polonica* **48** 541-549.

Ezer N & Robaire B 2002 Androgen regulation of epididymal structure and function. In *The epididymis: From molecules to clinical practice* pp 297-316. Eds Robaire B & Hinton BT. New York: Kluwer Academic/Plenum Publishers.

Ezer N & Robaire B 2003 Gene expression is differentially regulated in the epididymis after orchidectomy. *Endocrinology* **144** 977-988.

Gloyna RE & Wilson JD 1969 A comparitive study of the conversion of testosterone to 17β-hydroxy-5α-androstan-3-one (dihydrotestosterone) by rat prostate and epididymis. *Journal of Clinical Endocrinology and Metabolism* **29** 970-977.

Gregory M, Dufresne J, Hermo L & Cyr DG 2001 Claudin-1 is not restricted to tight junctions in the rat epididymis. *Endocrinology* **142** 854-863.

Grune T 2000 Oxidative stress, aging and the proteasomal system. Biogerontology **1** 31-40.

Herwig R, Aanstad P, Clark M & Lehrach H 2001 Statistical evaluation of differential expression on cDNA nylon arrays with replicated experiments. *Nucleic Acids Research* **29** e117.

Hess RA, Zhou Q & Nie R 2002 The role of estrogens in the endocrine and paracrine regulation of the efferent ductules, epididymis and vas deferens. In *The epididymis: From molecules to clinical practice* pp 317-337. Eds Robaire B & Hinton BT. New York: Kluwer Academic/Plenum Publishers.

Hinton BT, Lan ZJ, Rudolph DB, Labus JC & Lye RJ 1998 Testicular regulation of epididymal gene expression. *Journal of Reproduction and Fertility* **53 (suppl)** 47-57.

Igdoura SA, Morales CR & Hermo L 1995 Differential expression of cathepsin-B and cathepsin-D in testis and epididymis of adult-rats. *Journal of Histochemistry and Cytochemistry* **43** 545-557.

Jensen LJ, Stuart-Tilley AK, Peters LL, Lux SE, Alper SL & Breton S 1999 Immunolocalization of AE2 anion exchanger in rat and mouse epididymis. *Biology of Reproduction* **61** 973-980.

Jervis KM & Robaire B 2001 Dynamic changes in gene expression along the rat epididymis. *Biology of Reproduction* **65** 696-703.

Jervis KM & Robaire B 2002 Changes in gene expression during aging in the Brown Norway rat epididymis. *Experimental Gerontology* **37** 897-906.

Jervis KM & Robaire B 2003 Effects of caloric restriction on gene expression along the epididymis of the Brown Norway rat during aging. *Experimental Gerontology* **38** 549-560.

Jones R 1998 Plasma membrane structure and remodelling during sperm maturation in the epididymis. *Journal of Reproduction and Fertility* **53 (Suppl)** 73-84.

Kaunisto K, Fleming RE, Kneer J, Sly WS & Rajaniemi H 1999 Regional expression and androgen regulation of carbonic anhydrase IV and II in the adult rat epididymis. *Biology of Reproduction* **61** 1521-1526.

Lan ZJ, Labus JC & Hinton BT 1998 Regulation of gamma-glutamyl transpeptidase catalytic activity and protein level in the initial segment of the rat epididymis by testicular factors: Role of basic fibroblast growth factor. *Biology of Reproduction* **58** 197-206.

Lee MLT, Kuo FC, Whitmore GA & Sklar J 2000 Importance of replication in microarray gene expression studies: Statistical methods and evidence from

repetitive cDNA hybridizations. *Proceedings of the National Academy of Sciences of the USA* **97** 9834-9839.

Leung PS, Wong TP, Chung YW & Chan HC 2002 Androgen dependent expression of AT1 receptor and its regulation of anion secretion in rat epididymis. *Cell Biology International* **26** 117-122.

Leung PS, Wong TP, Lam SY, Chan HC & Wong PYD 2000 Testicular hormonal regulation of the renin-angiotensin system in the rat epididymis. *Life Sciences* **66** 1317-1324.

Li X, Chen CL, Singh SM & Labrie F 1995 The Enzyme and Inhibitors of 4-Ene-3-Oxosteroid 5-Alpha-Oxidoreductase (Vol 60, Pg 430, 1995). *Steroids* **60** 540.

Lin HK, Altuwaijri S, Lin WJ, Kan PY, Collins LL & Chang C 2002 Proteasome activity is required for androgen receptor transcriptional activity via regulation of androgen receptor nuclear translocation and interaction with coregulators in prostate cancer cells. *Journal of Biological Chemistry* **277** 36570-36576.

Lowry OF, Rosebrough NJ, Farr AL & Randall RJ 1951 Protein estimation with the Folin phenol reagent. *Journal of Biological Chemistry* **193** 265-271.

Martelli AM, Bortul R, Tabellini G, Bareggi R, Manzoli L, Narducci P & Cocco L 2002 Diacylglycerol kinases in nuclear lipid-dependent signal transduction pathways. *Cellular and Molecular Life Sciences* **59** 1129-1137.

Marzesco AM, Dunia I, Pandjaitan R, Recouvreur M, Dauzonne D, Benedetti EL, Louvard D & Zahraoui A 2002 The small GTPase Rab13 regulates assembly of functional tight junctions in epithelial cells. *Molecular Biology of the Cell* **13** 1819-1831.

McGrath ME 1999 The lysosomal cysteine proteases. *Annual Review of Biophysics and Biomolecular Structure* **28** 181-204.

Mehlhase J & Grune T 2002 Proteolytic response to oxidative stress in mammalian cells. *Biological Chemistry* **383** 559-567.

Melo LG, Pang SC & Ackermann U 2000 Atrial natriuretic peptide: Regulator of chronic arterial blood pressure. *News in Physiological Sciences* **15** 143-149.

Montalvo L, Sanchez-Chapado M, Prieto JC & Carmena MJ 2002 Regulation of the expression of protein kinase C isoenzymes in rat ventral prostate: effects of age, castration and flutamide treatment. *Life Sciences* **71** 2257-2266.

Normington K & Russell DW 1992 Tissue distribution and kinetic characteristics of rat steroid 5-alpha-reductase isozymes - evidence for distinct physiological functions. *Journal of Biological Chemistry* **267** 19548-19554.

Oliveira-Souza M, Malnic G & Mello-Aires M 2002 Atrial natriuretic peptide impairs the stimulatory effect of angiotensin II on H+-ATPase. *Kidney International* **62** 1693-1699.

Orgebin-Crist MC & Tichenor PL 1973 Effect of testosterone on sperm maturation in-vitro. *Nature* **245** 328-329.

Orgebin-Crist MC 1967 Sperm Maturation in Rabbit Epididymis. *Nature* **216** 816-818.

Orgebin-Crist MC, Jahad N & Hoffman LH 1976 Effects of testosterone, 5-alpha-dihydrotestosterone, 3-alpha-androstanediol, and 3-beta-androstanediol on maturation of rabbit epididymal spermatozoa in organ-culture. *Cell and Tissue Research* **167** 515-525.

Orgebin-Crist MC, Lareyre JJ, Suzuki K, Araki Y, Fouchecourt S, Matusik RJ & Ong DE 2002 Retinoids and epididymal function. In *The epididymis: From molecules to clinical practice* pp 339-352. Eds Robaire B & Hinton BT. New York: Kluwer Academic/Plenum Publishers.

Paduch M, Jelen F & Otlewski J 2001 Structure of small G proteins and their regulators. *Acta Biochimica Polonica* **48** 829-50.

Pastor-Soler L, Isnard-Bagnis C, Herak-Kramberger C, Sabolic I, Van Hoek A, Brown D & Breton S 2002 Expression of aquaporin 9 in the adult rat epididymal epithelium is modulated by androgens. *Biology of Reproduction* **66** 1716-1722.

Phelps BM, Koppel DE, Primakoff P & Myles DG 1990 Evidence That Proteolysis of the Surface Is An Initial Step in the Mechanism of Formation of Sperm Cell-Surface Domains. *Journal of Cell Biology* **111** 1839-1847.

Robaire B & Hales BF 1982 Regulation of Epididymal Glutathione S-Transferases - Effects of Orchidectomy and Androgen Replacement. *Biology of Reproduction* **26** 559-565.

Robaire B & Hinton BT 2002 *The Epididymis From Molecules to Clinical Practice*. New York; Kluwer Academic/Plenum Publishers.

Robaire B & Viger RS 1993 Regulation of epididymal epithelial cell functions. In *Understanding Male Infertility: Basic and Clinical Approaches*, pp 183-210. Eds RW Whitcomb and B Zirkin.

Santos CF, Oliveira EB, Salgado MCO & Greene AS 2002 Molecular cloning and sequencing of the cDNA for rat mesenteric arterial bed elastase-2, an

angiotensin II-forming enzyme. *Journal of Cardiovascular Pharmacology* **39** 628-635.

Steers WD 2001 5Aplha-reductase activity in the prostate. *Urology* **58 suppl 1** 17-24.

Strittmatter SM & Snyder SH 1984 Angiotensin-converting enzyme in the male reproductive system: autoradiographic visualization with [^{3}H]captopril. *Endocrinology* **115** 2332-2341.

Sudduth SL, Koronkowski MJ, Sharifi R, Lee M, Cali TJ & Chamberlain TM 1993 Finasteride - the 1St 5-alpha-reductase inhibitor. *Pharmacotherapy* **13** 309-329.

Suzuki T, Yamazaki T & Yazaki Y 2001 The role of the natriuretic peptides in the cardiovascular system. *Cardiovascular Research* **51** 489-494.

Swinnen JV, Ulrix W, Heyns W & Verhoeven G 1997 Coordinate regulation of lipogenic gene expression by androgens: Evidence for a cascade mechanism involving sterol regulatory element binding proteins. *Proceedings of the National Academy of Sciences of the USA* **94** 12975-12980.

Tindall DJ, Nayfeh SN & French FS 1972 Androgen uptake and binding in rat epididymal nuclei, in-vivo. *Biochemical and biophysical research communications* **49** 1391-1397.

Tomomasa H, Waguri S, Umeda T, Koiso K, Kominami E & Uchiyama Y 1994 Lysosomal Cysteine Proteinases in Rat Epididymis. *Journal of Histochemistry and Cytochemistry* **42** 417-425.

Turner TT & Reich GW 1985 Cauda epididymal sperm motility: a comparison among five species. *Biology of Reproduction* **32** 120-128.

Turner TT 1991 Spermatozoa are Exposed to a Complex Microenvironment as They Traverse the Epididymis. *Annals of the New York Academy of Sciences* **637** 364-383.

Turner TT 2002 Necessity's potion: Inorganic ions and small organic molecules in the epididymal lumen. In *The epididymis: From molecules to clinical practice* pp 131-150. Eds Robaire B & Hinton BT. New York: Kluwer Academic/Plenum Publishers.

Turner TT, Jones CE, Howards SS, Ewing LL, Zegeye B & Gunsalus GL 1984 On the androgen microenvironment of maturing spermatozoa. *Endocrinology* **115** 1925-1932.

Viger RS & Robaire B 1996 The mRNAs for the steroid 5 alpha-reductase isozymes, types 1 and 2, are differentially regulated in the rat epididymis. *Journal of Andrology* **17** 27-34.

Wilkinson GN 1961 Statistical estimations in enzyme kinetics. *Biochemical Journal* **80** 324-332.

Wong PYD & Yeung CH 1977 Hormonal-regulation of fluid reabsorption in isolated rat cauda epididymidis. *Endocrinology* **101** 1391-1397.

Zoppi S, Lechuga M & Motta M 1992 Selective-Inhibition of the 5-Alpha-Reductase of the Rat Epididymis. *Journal of Steroid Biochemistry and Molecular Biology* **42** 509-514.

Figures and Legends

Figure 1. Expression of 5α-reductase type 2 (dotted line), angiotensin-converting enzyme (solid line) and androgen receptor (dashed line) in the initial segment (IS), caput (CA), corpus (CO) and cauda (CD) epididymides of vehicle treated (control) rats. Gene expression is presented as relative intensity (mean ± SEM, 3-5 replicates/group).

Figure 1

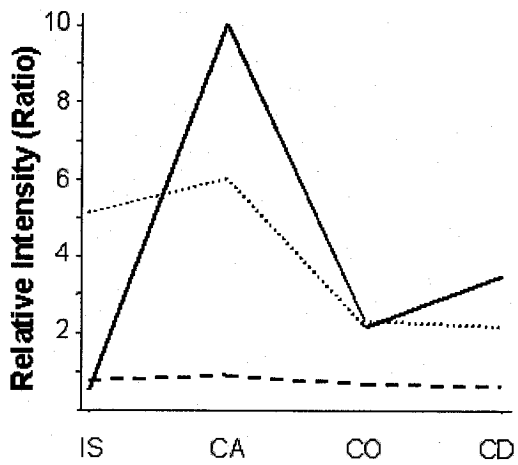


Figure 2. Effect of PNU157706 treatment on weights of seminal vesicles (A), left ventral prostates (B), testes (C) and epididymides (D). Data are represented as the mean ± SEM for each treatment group on the x-axis: Control/0 mg/kg (0), Low dose/0.1mg/kg (0.1), Medium dose/1.0 mg/kg (1), High dose/10 mg/kg (10). * indicates $p < 0.05$.

Figure 2

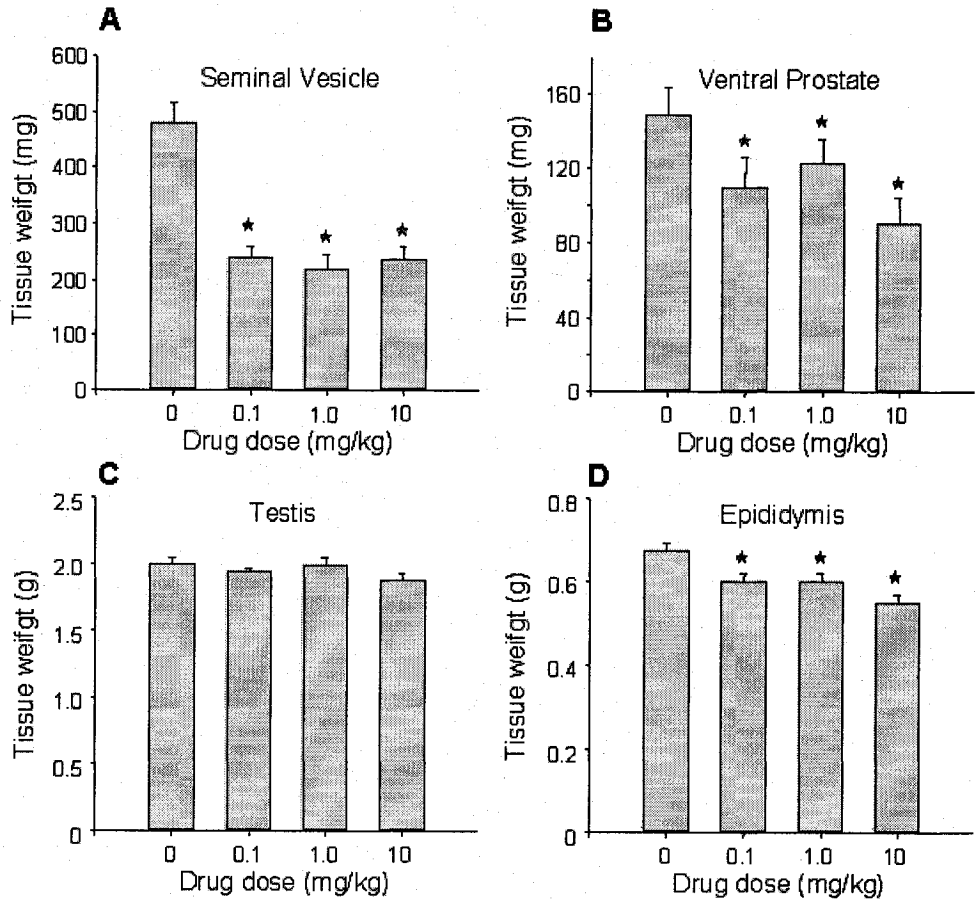

A
Seminal Vesicle
Tissue weifgt (mg)
600
500
400
300
200
100
0
0
0.1
1.0
10
Drug dose (mg/kg)
B
Ventral Prostate
Tissue weifgt (mg)
160
120
80
40
0
0
0.1
1.0
10
Drug dose (mg/kg)
C
Testis
Tissue weifgt (g)
2.5
2.0
1.5
1.0
0.5
0
0
0.1
1.0
10
Drug dose (mg/kg)
D
Epididymis
Tissue weifgt (g)
0.8
0.6
0.4
0.2
0
0
0.1
1.0
10
Drug dose (mg/kg)

Figure 3. Inhibition of rat nuclear (A) and microsomal (B) 5α-reductase activity *in vitro* by different concentrations of PNU157706 (PNU). For nuclear (C) and microsomal (D) 5α-reductase, slope replots (squares) were obtained from $K_{m(app)}/V_{max(app)}$ vs. (PNU) and intercept replots (triangles) were obtained from $1/V_{max(app)}$ vs. (PNU).

Figure 3

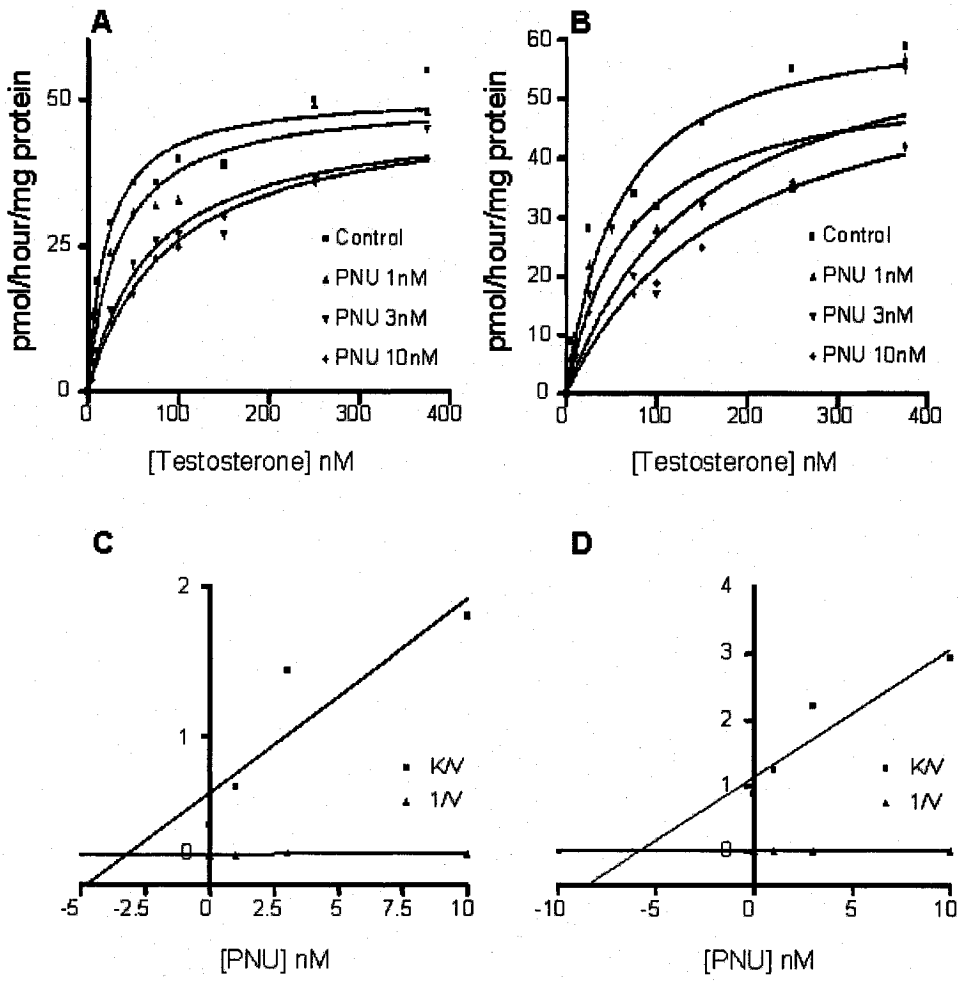

A
B
pmol/hour/mg protein
[Testosterone] nM
Control
PNU 1nM
PNU 3nM
PNU 10nM
C
D
K/V
1/V
[PNU] nM

Figure 4. Number of genes in each epididymal segment showing changes in gene expression following 10 mg/kg/day PNU157706 treatment. The vertical scale represents the total number of genes that showed at least a 2-fold change in expression in either direction (100% increase or 50% decrease). The dark grey bars indicate the number of genes increasing in expression (above x-axis); the light grey bars indicate the number of genes decreasing in expression (below x-axis). Initial segment (IS), Caput (CA), Corpus (CO), Cauda (CD).

Figure 4

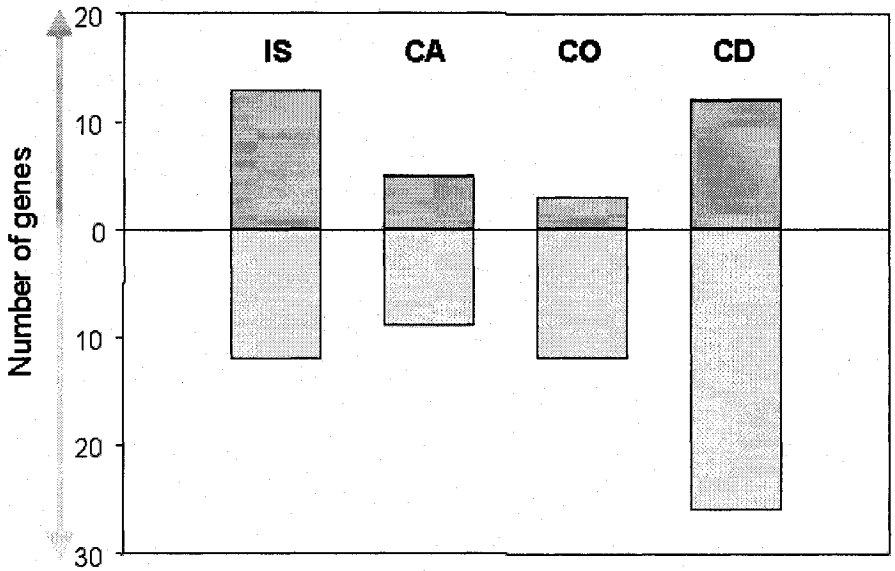


Figure 5. Genes with altered expression in the initial segment following PNU157706 treatment for 28 days. Each line represents one gene. (A) Signalling-related genes: NF-2, retinoblastoma protein pp105 (RBpp105), ras-GTPase activating protein (p120GAP), Crk-associated substrate (CAS). (B) Fatty acid/lipid metabolism genes: Fatty acid amide hydrolase (f.a. amide hydrolase), corticosteroid 11β-hydroxysteroid dehydrogenase (corticosteroid 11β-HSD), very long chain acyl-CoA dehydrogenase (VLCAD), 5-lipoxygenase (5-LO). (C) Fluid/ion balance related genes: atrial natriuretic peptide clearance receptor (ANP-C), chloride channel CLC-7 (CLC-7). Gene expression is presented as relative intensity (mean ± SEM, 3-5 replicates/group) on the y-axis for each treatment group on the x-axis: Control/0 mg/kg (0), Low dose/0.1mg/kg (0.1), Medium dose/1.0 mg/kg (1), High dose/10 mg/kg (10).

Figure 5

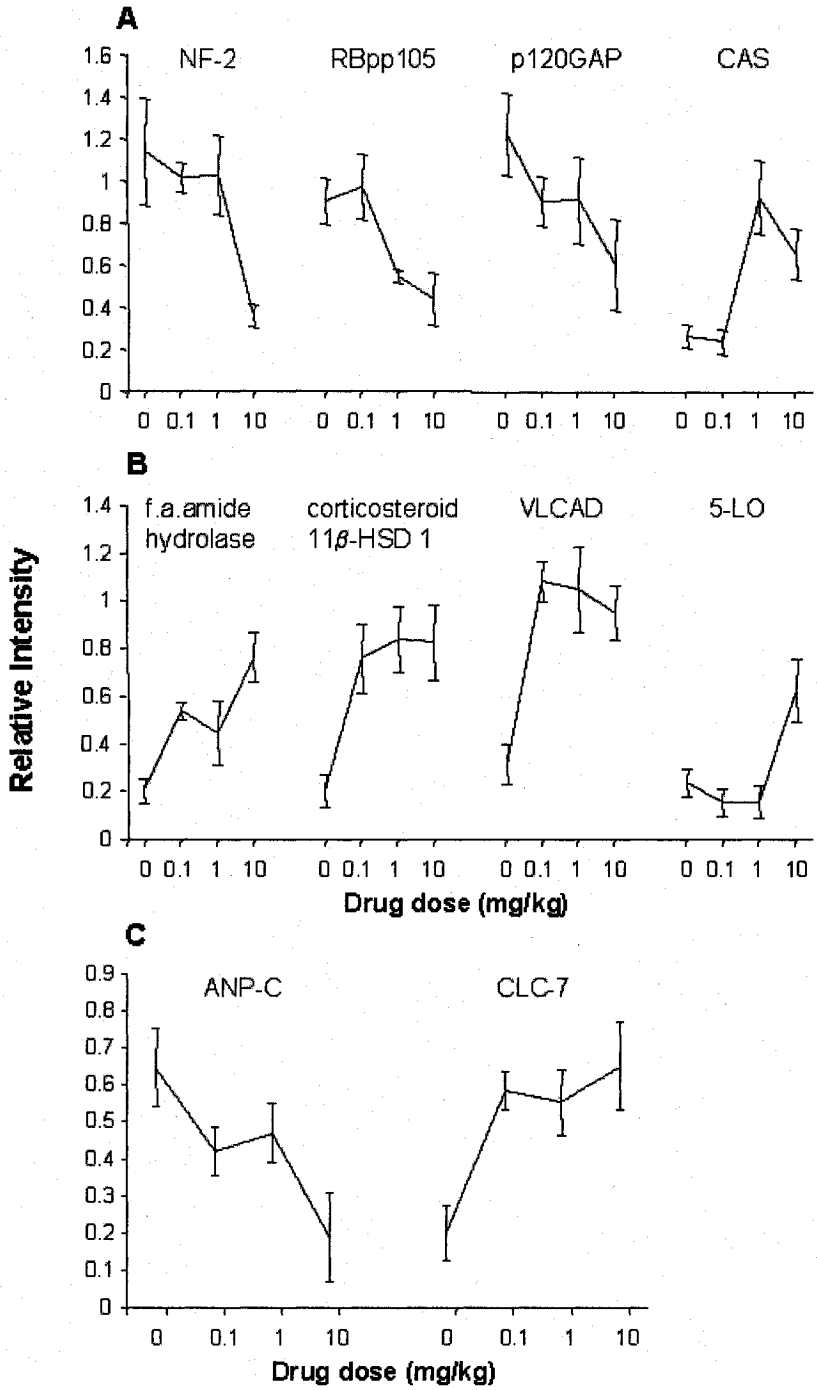

A
NF-2
RBpp105
p120GAP
CAS
B
f.a.amide hydrolase
corticosteroid 11β-HSD 1
VLCAD
5-LO
Relative Intensity
Drug dose (mg/kg)
C
ANP-C
CLC-7
Drug dose (mg/kg)

Figure 6. Genes with altered expression in the caput epididymidis following PNU157706 treatment for 28 days. Each line represents one gene. (A) Signalling-related genes: cAMP-dependent protein kinase type II-beta regulatory chain (camp dep. PK type IIβ), Ehk3, neural thrombospondin1-like protein (NELL-1). (B) Cathepsin B. (C) Short chain acyl-CoA dehydrogenase (SCAD). Gene expression is presented as relative intensity (mean ± SEM, 3-5 replicates/group) on the y-axis for each treatment group on the x-axis: Control/0 mg/kg (0), Low dose/0.1mg/kg (0.1), Medium dose/1.0 mg/kg (1), High dose/10 mg/kg (10).

Figure 6

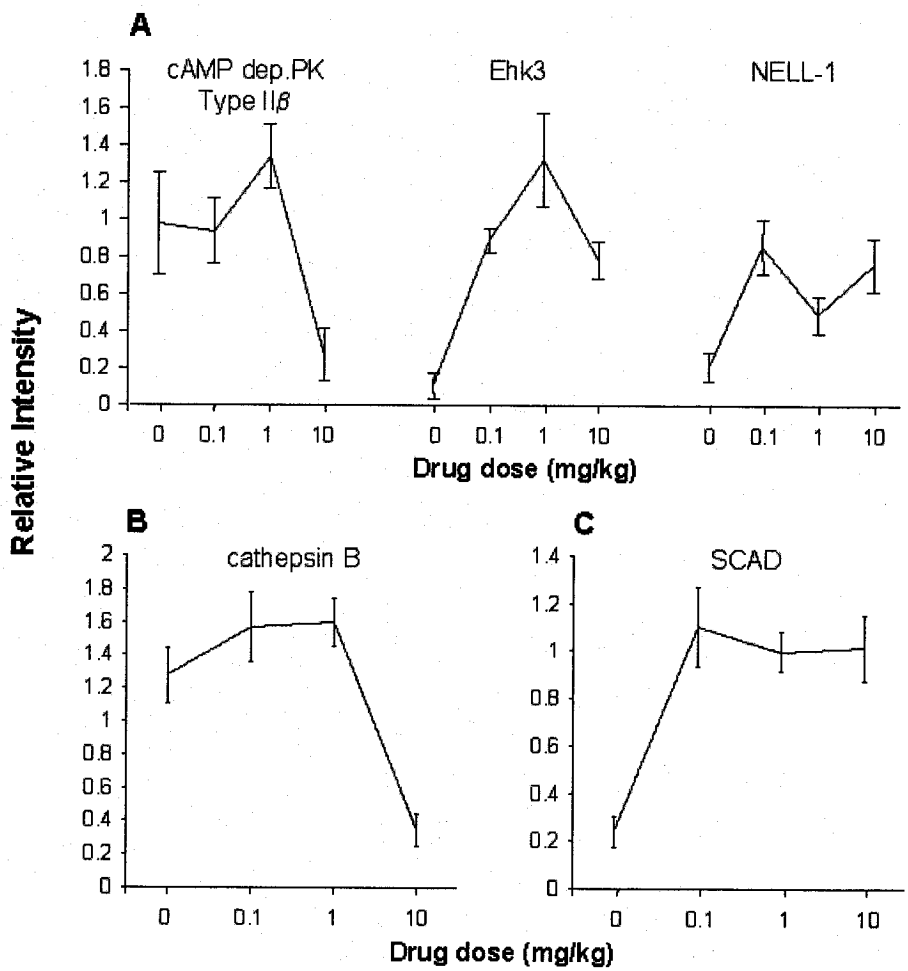

A
cAMP dep.PK Type IIβ
Ehk3
NELL-1
Relative Intensity
Drug dose (mg/kg)
B
cathepsin B
C
SCAD
Drug dose (mg/kg)

Figure 7. Genes with altered expression in the corpus epididymidis following PNU157706 treatment for 28 days. Each line represents one gene. (A) Carbonic anhydrase 4 (CA4). (B) Signalling-related genes: ras related protein RAB13 (rab13), ephrinB1, Jak1 tyrosine-protein kinase (Jak1). Gene expression is presented as relative intensity (mean ± SEM, 3-5 replicates/group) on the y-axis for each treatment group on the x-axis: Control/0 mg/kg (0), Low dose/0.1mg/kg (0.1), Medium dose/1.0 mg/kg (1), High dose/10 mg/kg (10).

Figure 7

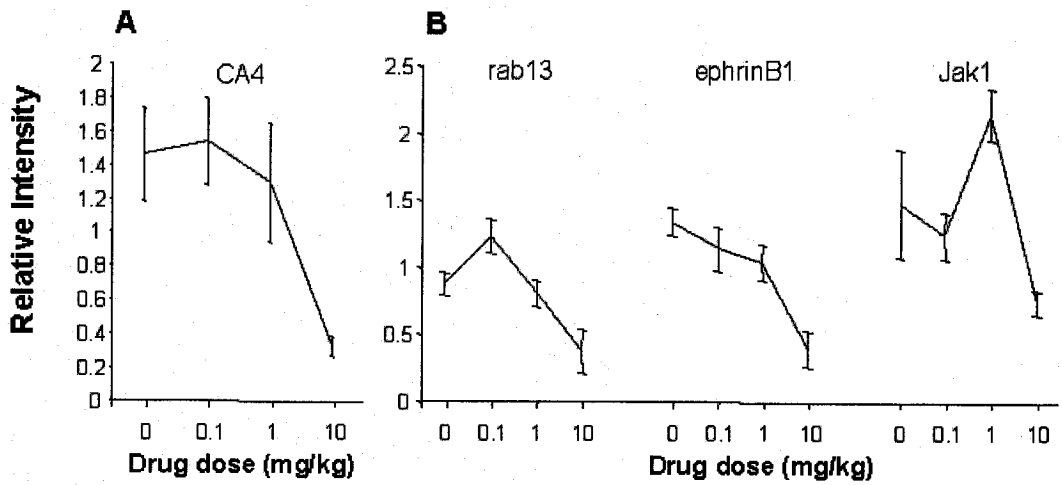

A
B
CA4
rab13
ephrinB1
Jak1
Relative Intensity
Drug dose (mg/kg)
Drug dose (mg/kg)
0
0.1
1
10

Figure 8. Genes with altered expression in the cauda epididymis following PNU157706 treatment (10mg/kg) for 28 days. Each line represents one gene. (A) Signalling-related genes: Rab-related GTP-binding protein (Rab-related GBP), muscle/brain cAMP-dependent protein kinase inhibitor (PKI-alpha), phosphatidylinositol 4-kinase (PI4-K), 14-3-3 protein gamma subtype, ras-related GTPase rab4B (rab4B). (B) Phospholipase genes: Phospholipase C gamma 2 (PLC gamma 2), phospholipase C delta 1 (PLC delta 1), phospholipase C gamma 1 (PLC gamma 1). (C) Ion transporter genes: Hydrogen-potassium transporting ATPase alpha 2a subunit (H^+/K^+ ATPase alpha 2a sub.), sodium/potassium transporting ATPase beta 2 subunit (Na^+/K^+ ATPase beta 2 sub.), band 3 anion exchanger (Band 3). Gene expression is presented as relative intensity (mean ± SEM, 3-5 replicates/group) on the y-axis for each treatment group on the x-axis: Control/0 mg/kg (0), Low dose/0.1mg/kg (0.1), Medium dose/1.0 mg/kg (1), High dose/10 mg/kg (10).

Figure 8

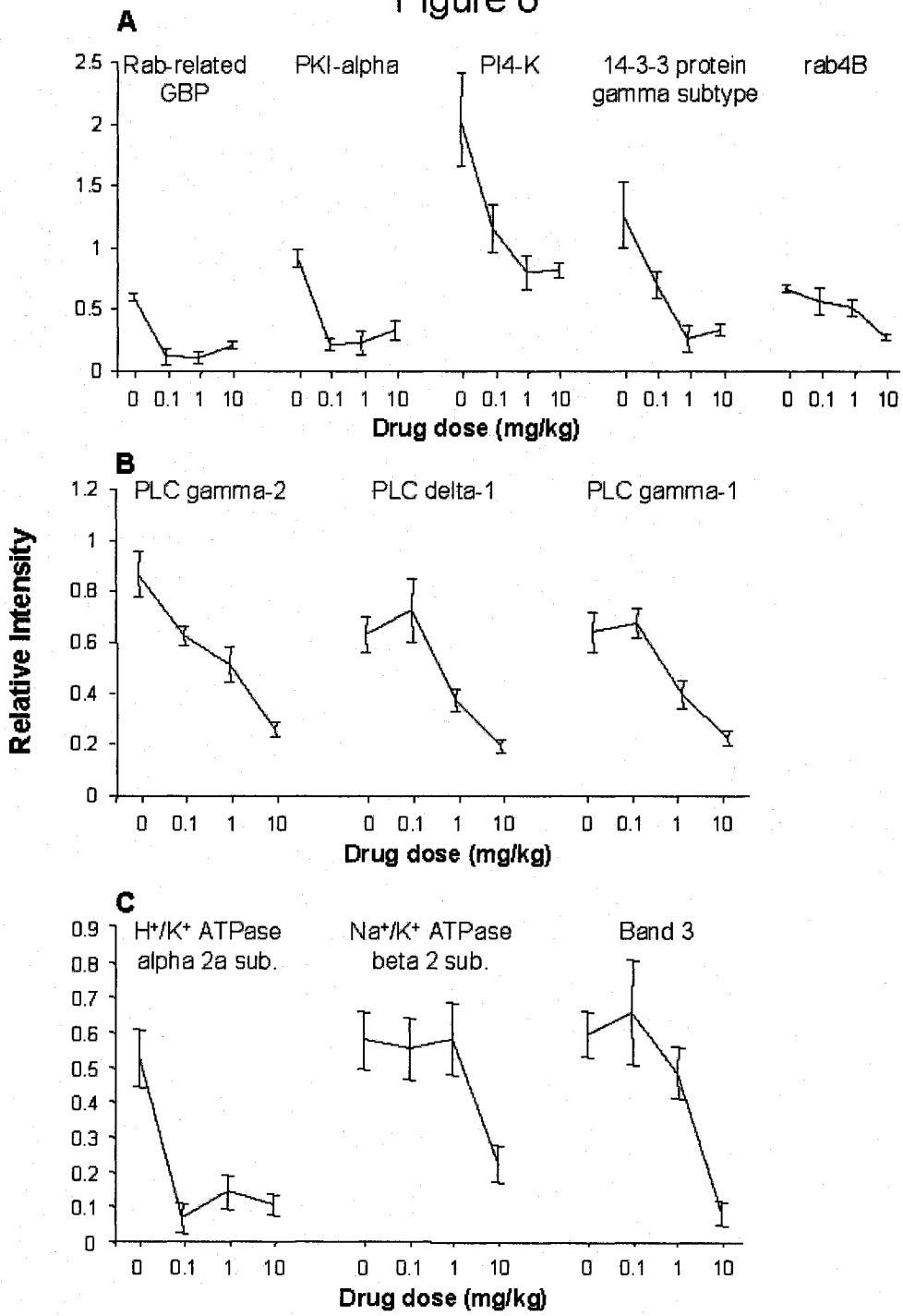

A
Rab-related GBP
PKI-alpha
PI4-K
14-3-3 protein gamma subtype
rab4B
2.5
2
1.5
1
0.5
0
0 0.1 1 10
Drug dose (mg/kg)
B
PLC gamma-2
PLC delta-1
PLC gamma-1
Relative Intensity
1.2
1
0.8
0.6
0.4
0.2
0
0 0.1 1 10
Drug dose (mg/kg)
C
H+/K+ ATPase alpha 2a sub.
Na+/K+ ATPase beta 2 sub.
Band 3
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0 0.1 1 10
Drug dose (mg/kg)

Figure 9. Expression of oxidative stress related genes in the control (light grey bars) and high PNU157706 treatment (dark grey bars, 10mg/kg/day) groups in the initial segment (IS), caput (CA), corpus (CO) and cauda (CD) epididymidis. Gene expression is presented as relative intensity (mean ± SEM, 3-5 replicates/group) on the y-axis for each specific gene on the x-axis: GST mu (mu), GST Yrs-Yrs (Yrs-Yrs), GST subunit 5-theta (5-theta), GST subunit 13 (13), GPX3, and glutathione synthetase (GSH synth).

Figure 9

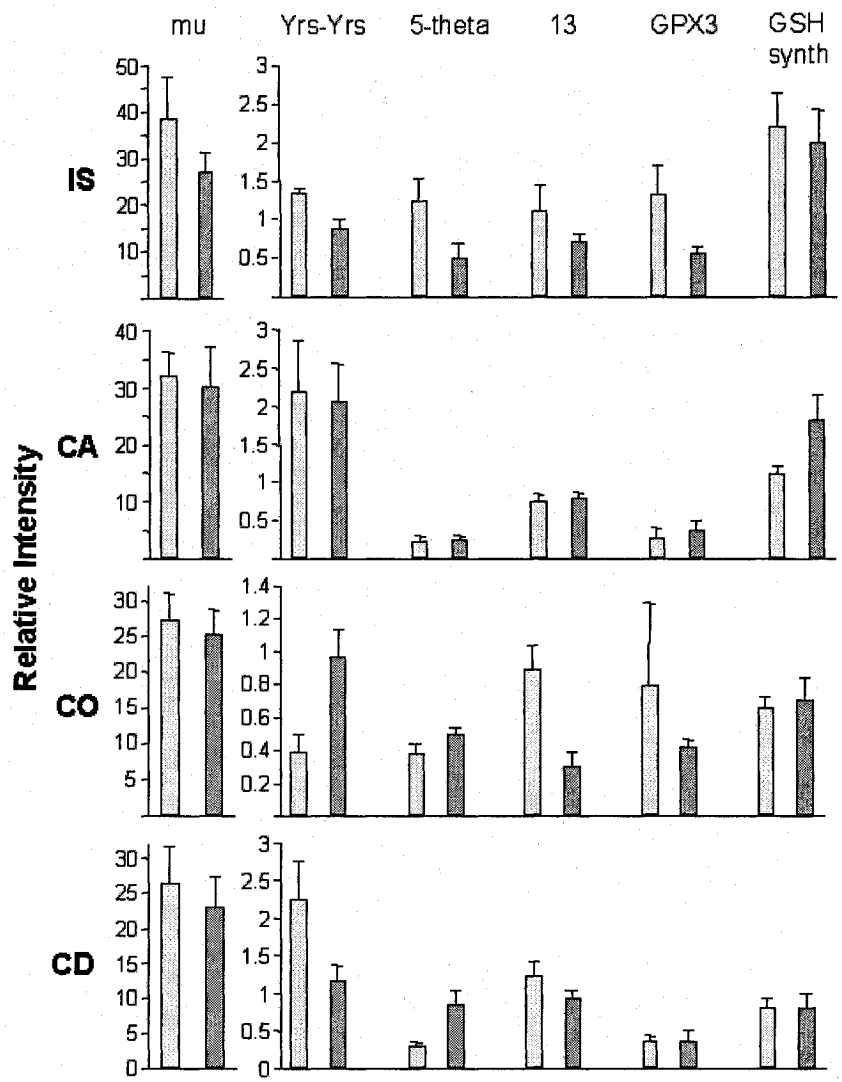
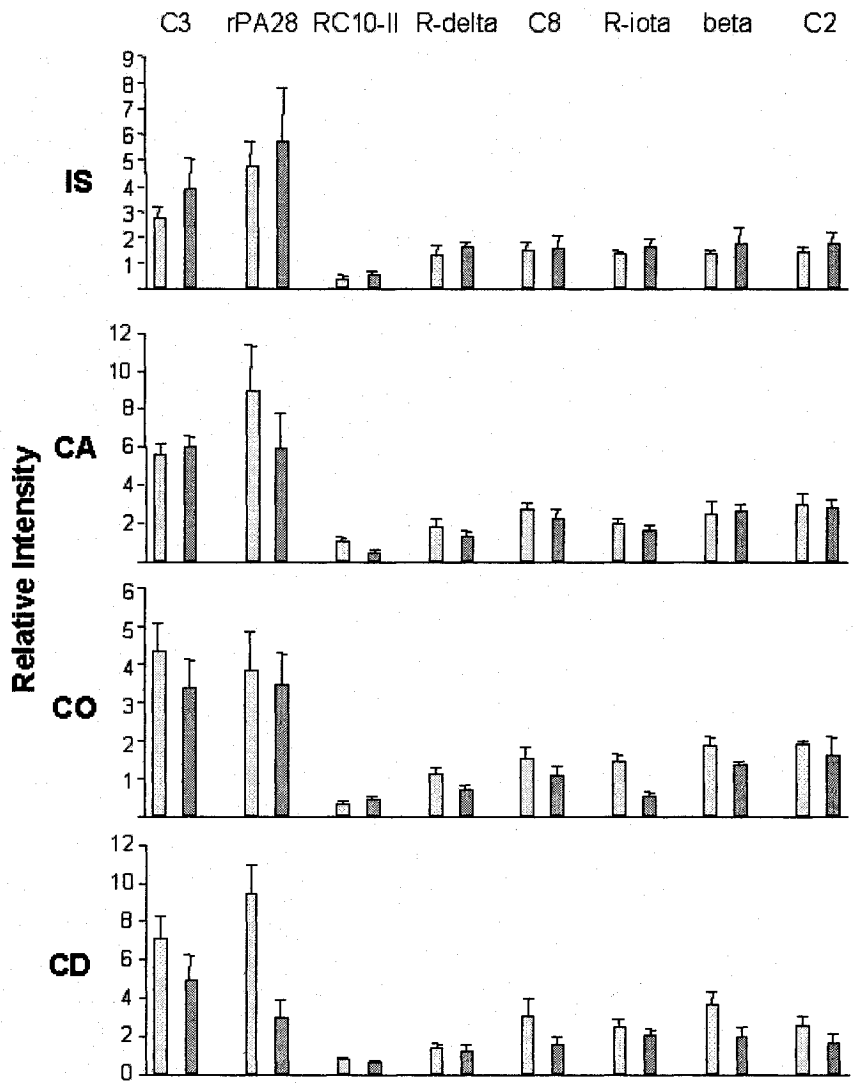

mu
Yrs-Yrs
5-theta
13
GPX3
GSH synth
IS
CA
CO
CD
Relative Intensity

Figure 10. Expression of proteasomal genes in the control (light gray bars) and high PNU157706 treatment (dark grey bars, 10mg/kg/day) groups in the initial segment (IS), caput (CA), corpus (CO) and cauda (CD) epididymidis. Gene expression is presented as relative intensity (mean ± SEM, 3-5 replicates/group) on the y-axis for each specific gene on the x-axis: proteasome component C3 (C3), proteasome activator rPA28 subunit alpha (rpA28), proteasome subunit RC10-II (RC10-II), proteasome subunit R-delta (R-delta), proteasome component C8 (C8), proteasome subunit R-iota (R-iota), proteasome subunit beta (beta), and proteasome component C2 (C2).

Figure 10


C3
rPA28
RC10-II
R-delta
C8
R-iota
beta
C2
IS
CA
CO
CD
Relative Intensity

Tables and Legends

Table 1. Summary of the in vitro effect of PNU157706 on epididymal nuclear and microsomal 5α-reductase enzyme kinetics. Non-linear least squares analysis was used to obtain the $K_{m(app)}$ and $V_{max(app)}$ values. Linear regression analysis of the slope and intercept replots was used to obtain the $K_{i(app)}$ values.

	Experiment 1					Experiment 2				
	$K_{m(app)}$	$V_{max(app)}$	r	$K_{i(app)}$	r	$K_{m(app)}$	$V_{max(app)}$	r	$K_{i(app)}$	r
Nuclear 5α-reductase										
0 nM PNU	20.5	51.1	0.95	-	-	20.7	45.7	0.86	-	-
1 nM PNU	33.1	50.4	0.97	-	-	40.9	48.5	0.93	-	-
3 nM PNU	68.7	47.8	0.96	-	-	64.4	53.5	0.90	-	-
10 nM PNU	88.2	49.1	0.99	-	-	96.7	48.6	0.96	-	-
Slope replot ($K_{m(app)}/V_{max(app)}$ vs [PNU])	-	-	-	3.13	0.90	-	-	-	4.02	0.98
Intercept replot ($1/V_{max(app)}$ vs [PNU])	-	-	-	74.5	0.51	-	-	-	-ve	0.23
Microsomal 5α-reductase										
0 nM PNU	57.1	64.4	0.94	-	-	32.4	63.9	0.95	-	-
1 nM PNU	67.7	54.4	0.88	-	-	62.2	64.5	0.83	-	-
3 nM PNU	145.3	65.7	0.91	-	-	60.8	49.7	0.91	-	-
10 nM PNU	174.6	59.7	0.95	-	-	172.7	66.8	0.94	-	-
Slope replot ($K_{m(app)}/V_{max(app)}$ vs [PNU])	-	-	-	4.76	0.94	-	-	-	3.1	0.99
Intercept replot ($1/V_{max(app)}$ vs [PNU])	-	-	-	-ve	0.01	-	-	-	-ve	0.13

Table 2. Genes with minimum 2-fold changed expression in more than 1 epididymal segment following 10 mg/kg PNU157706 treatment. The arrows represent the direction of change in expression. The dash (-) indicates no change in expression by 2-fold or greater. Accession numbers are for the GenBank database. Functional classifications are according to Clontech™.

Accession Number	Name	Change in gene expression				Functional classification
		Initial Segment	Caput	Corpus	Cauda	
U65007	c-met proto-oncogene	↓81%	↑564%	-	-	Growth factor/chemokine/protein kinase receptor
M95735	Syntaxin B	↓73%	-	-	↓77%	Trafficking & targeting protein
L11651	Eukaryotic translation initiation factor 5	↓72%	-	↓71%	↑458%	Translation factor
M76767	Fatty acid synthase	↓68%	-	-	↑469%	Fatty acid metabolism
M31602	Carboxypeptidase E	↓64%	↓66%	-	-	Metalloproteinase
X67654	Glutathione-S-transerase sub. 5 theta	↓58%	-	-	↑196%	Oxidative metabolism
X14977	Aldehyde dehydrogenase 2	↑140%	-	-	↑100%	Other metabolism
X80477	P2X purinoceptor 1	-	↑873%	↑156%	↑220%	ATP-gated ion channel

Connecting Text

In the previous chapter, cDNA array studies revealed changes in the expression of numerous genes following 5α-reductase inhibitor treatment, indicating that DHT is an important regulator of epididymal gene expression. The nature of the affected genes suggested their involvement in processes that are essential for the formation of the dynamic luminal microenvironment that is required for proper sperm maturation in the epididymis. Therefore, the next objective was to determine if the observed changes in epididymal gene expression following 5α-reductase inhibitor treatment actually translated to effects on epididymal sperm functions.

CHAPTER 3

Effects of PNU157706, a Dual 5α-Reductase Inhibitor, on Rat Epididymal Sperm Maturation and Fertility

Natali Anne Henderson and Bernard Robaire

Published in Biology of Reproduction

Biol Reprod. 2005 Feb;72(2):436-443.

Abstract

Sperm entering the epididymis gain progressive motility and fertilizing ability in a process termed maturation. The functional dependence of the epididymis on dihydrotestosterone (DHT) is well established, yet few studies have examined the consequences on the epididymis of inhibiting DHT formation. We have shown that inhibition of both isoforms of 5α-reductase (types 1 and 2), the enzyme that converts testosterone to DHT, has pronounced effects on epididymal gene expression. In the present study, we investigate whether inhibiting 5α-reductase has consequences on epididymal sperm maturation. Rats were treated with vehicle or 10 mg/kg/day PNU157706, a dual-type inhibitor, for 28 days. Fertility and several key facets of sperm maturation were analyzed. Changes in sperm motility were assessed by computer-assisted sperm analysis (CASA). Changes in sperm morphology were assessed by CASA and electron microscopy. The motility of spermatozoa from the cauda epididymidis of treated animals showed a significant decrease in both the percentage of motile and progressively motile sperm as well as altered motion parameters. The morphology of cauda epididymal spermatozoa was also adversely affected by the treatment; the most prominent effect was a markedly elevated proportion of sperm that retained their cytoplasmic droplet. Matings with treated males resulted in fewer successful pregnancies and a higher rate of pre-implantation loss. Progeny outcome was unaffected. The compromised sperm motility and morphology likely contribute to the subfertility of inhibitor-treated rats. Our results indicate a role for dual 5α-reductase inhibitors in further

studies of epididymal physiology and as a potential component of a male contraceptive.

Introduction

Within the testis, several complex sequential stages of germ cell differentiation give rise to spermatozoa [1, 2]. Sperm are then introduced to the epididymis, a single highly convoluted tubule structurally divided into four main segments (initial segment, caput, corpus and cauda). This tissue is the site of sperm maturation and additionally functions in sperm transport, protection and storage [3, 4]. Sperm maturation in the epididymis involves various morphological and biochemical changes, the initiation of progressive motility and the acquisition of fertilizing ability [3, 5-7]. In comparison with spermatogenesis in the testis, an elaborate process requiring approximately 2 mo in the adult male primate [8], sperm maturation in the epididymis does not involve cell division and is accomplished in a significantly shorter period of time [9]. These features of sperm maturation have generated significant interest in the epididymis as an advantageous posttesticular target for the development of safe, rapid and reversible male contraceptives. Achieving this therapeutic goal as well as elucidating epididymal causes of male infertility require an increased understanding of epididymal physiology.

The maintenance of epididymal structure and functions is known to be highly dependent on the presence of androgens [10, 11]. Furthermore, several in vivo and in vitro studies have confirmed that it is not testosterone, but rather

dihydrotestosterone (DHT), that is the main androgen acting in this tissue [12-15]. In contrast, testicular spermatogenesis is a testosterone-dependent process [16, 17, reviewed in 18]. Therefore, the inhibition of DHT production is an interesting experimental approach for studying the regulation of sperm maturation in the epididymis while presumably having little or no effect on sperm production in the testis.

5α-Reductase (EC 1.3.1.22) is the enzyme that catalyzes the conversion of testosterone into DHT. Two isoforms of 5α-reductase have been identified and are termed type 1 and type 2 [19, 20]; the contributions of each isozyme to male reproductive biology have not been fully elucidated. It is known that both 5α-reductase isozymes are present in the epididymis, where they are differentially distributed and regulated [21]. Though the importance of each isozyme in the epididymis remains unclear, a synergistic or additive contribution of both isozymes to epididymal functions is a plausible likelihood. Supporting this concept is the observation of a more pronounced virilization defect in dual 5α-reductase knockout mice when compared to the single 5α-reductase type 2 knockout animals [22] as well as greater decreases in serum and prostate DHT levels when both isozymes are inhibited simultaneously [23-25].

The utility of 5α-reductase type 2 inhibitors (e.g. finasteride) for treatment of androgen-dependent disorders such as benign prostatic hyperplasia and alopecia has been demonstrated [reviewed in 24-26]. Recently, drug development efforts to create even more therapeutically effective compounds have led to the production and testing of a novel class of 5α-reductase inhibitors,

termed dual inhibitors, that concurrently inhibit both 5αreductase type 1 and type 2. PNU157706 is one such inhibitor developed for the potential treatment of benign prostatic hyperplasia. PNU157706, *in vitro*, has been shown to be a specific competitive inhibitor of epididymal 5α-reductase activity and it decreases DHT-dependent tissue weights (i.e., seminal vesicle and ventral prostate) in vivo [23, 27]. Furthermore, PNU157706 was shown to be more potent than finasteride in decreasing DHT levels [23]. In a previous study, we have shown that treatment of adult male rats with PNU157706 has a highly segment-specific effect on epididymal gene expression [27]. Importantly, some of the more dramatically affected genes are potentially involved in essential processes contributing to the formation of an optimal luminal microenvironment required for proper sperm maturation. For example, genes involved in fatty acid and lipid metabolism, regulation of ion and fluid transport, luminal acidification, oxidative defense, and protein processing and degradation were affected [27].

Studying the consequences of inhibiting DHT formation is likely to provide important information regarding the androgen-regulation of epididymal functions; however, very few recent studies have examined the effects of 5α-reductase inhibition, specifically in the epididymis. The present study was designed to evaluate the effects of an altered androgen environment on epididymal sperm maturation by taking advantage of a novel drug and sperm analysis technology to determine the effects of 5α-reductase inhibition on sperm morphology, motility and fertilizing ability.

Materials and Methods

Animals and Treatment Protocol

Adult Sprague-Dawley rats were obtained from Charles River Canada (St. Constant, PQ, Canada), maintained under controlled light (14L:10D) and temperature (22˚C), and provided with food and water ad libitum. Male rats (325-350g) were randomly divided into 2 groups and gavaged with 0.5 ml/kg of 0 (control) or 10 mg/kg (treated) of PNU157706 (Pharmacia & Upjohn, Italy) suspended in 0.5% methylcellulose solution (BDH, Montreal, QC) containing 0.4% Tween 80 (A&C American Chemicals Ltd, Montreal, Quebec) for 28 consecutive days. The dose was selected based on reported effects on rat ventral prostate; the dosing regimen with 10 mg/kg/day PNU157706 has been shown to decrease prostatic dihydrotestosterone levels by >90% [23]. Virgin female rats (200-250g) were used for the mating study. All animal studies were conducted in accordance with the principles and procedure outlined in the Guide to the Care and Use of Experimental Animals prepared by the Canadian Council on Animal Care.

Sperm Motility

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. At the time of killing, animals were anesthetized and seminal vesicles, testes, ventral prostates and one epididymis, sectioned into initial segment, caput, corpus and cauda segments; were collected and

immediately frozen in liquid nitrogen. Tissues were stored at -80°C for future use. Sperm samples were collected from the distal caput and distal cauda of the remaining epididymis and used for computer-assisted sperm analysis (CASA) as previously described [28, 29], with the exception that the medium used for motility analysis was as follows: Hanks balanced salts solution (Gibco Invitrogen Co., Grand Island, NY), buffered with 4.2 g/L Hepes and 0.35 g/L $NaHCO_3$ and containing 2.0 g/L BSA, 0.9 g/L D-glucose, 0.1 g/L sodium pyruvate and 0.025 g/L soybean trypsin inhibitor, pH 7.4, at 37°C [30]. Briefly, the epididymis was trimmed free of fat and clamped at the caput-corpus and corpus-cauda junctions, then severed on the corpus side of the clamp and blotted. While still clamped, the caput and cauda epididymides were rinsed in separate 35-mm Petri dishes containing 2 ml of motility media and then transferred to separate Petri dishes containing 3 ml of fresh media. A #11 scalpel blade was used to pierce several tubules of the distal caput and distal cauda regions allowing sperm to diffuse into the medium. The tissue was removed and sperm were allowed to disperse for approximately 5 min. Sperm concentrations were optimized prior to aliquoting into an 80-μm-deep glass cannula for CASA on the HTM-IVOS, (Hamilton-Thorne Research, Beverly, MA) using version 12 of the Toxicology software. Approximately 3500 caput epididymal sperm and 6000 cauda epididymal sperm were analyzed for each treatment group (n=5-7). The percentages of motile and progressively motile sperm and the following kinematic parameters were determined by CASA: average-path velocity (VAP), curvilinear velocity (VCL), straight-line velocity (VSL), amplitude of lateral head displacement (ALH), beat

cross frequency (BCF), linearity (LIN=VSL/VCL x 100) and straightness (STR=VSL/VAP x 100).

Sperm Morphology

The morphology of spermatozoa from the cauda epididymidis was evaluated by phase-contrast microscopy using the HTM-IVOS. Sperm from the original dilution for motility analysis were fixed with 10% neutral buffered formalin (1:3, Sigma-Aldrich, Oakville, Ontario). Samples were aliquoted onto regular slides and sperm were analyzed using the 10x phase contrast objective. This system allowed clear visualization of cytoplasmic droplets [28]. Approximately 300 to 500 sperm per sample were analyzed and the percentage of sperm retaining their cytoplasmic droplet was calculated. Similarly, broken sperm (head only, tail only, other breakages) and angulated sperm (bent at midpiece) were also clearly detectable using this system and the frequency of these abnormalities was calculated.

In addition to the HTM-IVOS analysis of sperm morphology, spermatozoa from the cauda epididymidis of four control and four treated rats were also processed for electron microscopic analysis. A #11 scalpel blade was used to pierce the cauda several times and sperm were collected by shaking in Hanks minimum essential medium (Invitrogen Canada Inc., Burlington, Ontario). Spermatozoa were washed in Hanks media, fixed in the same media containing 1% glutaraldehyde (Mecalab Ltd., Montréal, Québec), and embedded for electron microscope analysis. Briefly the samples were washed three times in sodium

cacodylate buffer (0.1M) containing 3% sucrose, pH 7.4, postfixed in 1% osmium tetroxide and 1.5% potassium ferrocyanide and embedded in epoxy resin. Sperm ultrastructure was then analyzed on the electron microscope (Philips 410 electron microscope, Eindhoven, The Netherlands). For analysis of cytoplasmic droplet retention, a minimum of 100 midpiece sperm tail (flagellum) cross sections per animal were photographed, and the number of sperm tails with cytoplasmic droplets recorded.

Mating Study

A mating study was designed to determine the effects of PNU157706 treatment on male fertility. Vaginal smears from female rats were analyzed daily for a minimum of 2 weeks to confirm normal estrous cycles. On the last day of treatment, each male rat was paired overnight with 2 female rats in proestrus. Female rats were examined the next morning for the presence of sperm in vaginal smears; this was defined as day 0 of gestation for sperm-positive animals. The male reproductive indices that were calculated are: copulation index (number of sperm positive females / number of pairings), pregnancy index (number of pregnancies / number of sperm positive females), and fertility index (number of pregnancies / number of pairings).

Pregnancy Outcome

To examine the effect of inhibitor treatment on pregnancy outcome, female rats underwent a cesarean section on Day 20 of gestation: The ovaries

were removed and the number of corpora lutea was counted; uteri were opened, and the numbers of implantation sites were counted. The status of each implantation site was classified as resorption, dead or live fetus. For each treatment group, the overall implantation rate (number of implantations / number of corpora lutea), preimplantation loss per female [(number of corpora lutea - number of implantation sites) / number of corpora lutea], overall resorption rate (number of resorptions / number of total implantations) and postimplantation loss per female [(number of implantations – number of live fetuses)/ number of implantations] were calculated. Fetuses were sexed, weighed and examined for external malformations.

Statistical analysis

Statistical analysis of frequency data (reproductive indices, fetal sex ratio) was done by the Fisher Exact test. The implantation and resorption rates were analyzed by the test of proportions (Z-test). The remaining data assessing male fertility and pregnancy outcome, sperm motility and sperm morphology were analyzed for differences between the control and treated groups by Student's *t* test or the Mann-Whitney U test (in cases of failed normality or equal variance tests). Data are presented as mean ± SEM. For all analyses, the level of significance was set at $p < 0.05$.

Results

Effects of PNU157706 Treatment on Sperm Motility

In the control group, a pattern of spermatozoal motility from circular, in the caput epididymidis, to progressively forward, in the cauda epididymidis, was observed. This progression was reflected in the sperm motion parameters measured by CASA where sperm from the cauda epididymides had higher straight line velocity (VSL) and straightness (STR) (Table 1), as well as an increase in the percent that was progressively motile (Fig. 1). For sperm taken from the caput epididymidis, there was no effect of PNU157706 treatment on any of the motion parameters (Table 1), or on the percentage of motile and progressively motile sperm (Fig. 1). In contrast, treatment with the 5α-reductase inhibitor caused a significant decrease in the VSL, STR and linearity (LIN) of sperm from the cauda epididymidis (Table 1). There was also a significant decrease in the percentage of motile and progressively motile sperm from the cauda epididymides of PNU157706-treated rats when compared to control rats (Fig. 1).

Effects of PNU157706 Treatment on Sperm Morphology

A major morphological feature of epididymal sperm maturation is the displacement of the cytoplasmic droplet along the midpiece of sperm travelling from the caput to the cauda epididymal regions [7, 31]. Analysis of sperm from the cauda epididymidis under phase-contrast microscopy using the CASA

system allowed clear visualization of the cytoplasmic droplet (Fig. 2A). As expected, a low percentage (approximately 12%) of sperm from this segment of the tissue of control males retained their cytoplasmic droplet. However, sperm samples from the cauda epididymides of males treated with PNU157706 had a significantly higher percentage of retained cytoplasmic droplets compared to controls, doubling to nearly 25% (Fig. 2B).

Some sperm abnormalities such as bent (angulated) sperm and sperm breakages (i.e., headless, tailless) were also easily visualized using the CASA system; examples of these defects are shown in Figure 3. Sperm samples from the cauda epididymides of control and treated males had similar percentages of bent sperm (Table 2). In contrast, there was a higher incidence of sperm breakage in sperm from the cauda epididymides of males treated with the inhibitor; the percentages of headless, tailless and other breaks all increased significantly with treatment, as did the percentage of overall abnormalities observed using CASA (Table 2).

Analysis of the ultrastructure of sperm from the cauda epididymidis revealed an apparent increase in the proportion of mid-piece spermatozoa having cytoplasmic droplets; examples of sperm cross-sections with and without the droplet are shown in Figure 4. The droplet appears as a membrane-bound structure surrounding the sperm tail. There was a significant effect of treatment on the percentage of mid-piece sperm cross-sections surrounded by cytoplasmic droplets (Fig. 4C). This is consistent with our CASA analysis of cytoplasmic droplet retention in sperm from the cauda epididymidis. Very few ultrastructural

abnormalities were observed in sperm samples from either control or treated rats.

Effects of PNU157706 Treatment on Male Fertility

In this study, each male (n=8 / treatment group) was paired with 2 virgin females in proestrus for a total of 16 pairings for each treatment group. The effects of 5α-reductase inhibitor treatment on indices of male reproduction are shown in Table 3. All of the control males successfully mated with at least one female, with the majority mating with both females. All of these successful matings resulted in pregnancies. In contrast, there was a decrease in the number of sperm-positive (successfully mated) females exposed to PNU157706-treated males. In some cases, approximately 50 or more sperm were present and easily detected in the vaginal smears of females exposed to treated males (comparable to controls), while in other cases as few as 2 sperm were detected. Furthermore, not all of the sperm-positive females exposed to treated males became pregnant. Overall, the number of females with successful pregnancies that resulted from pairings with treated males was significantly reduced.

Effects of PNU157706 Treatment on Pregnancy Outcome

As expected, females exposed to either control or PNU157706-treated males had similar numbers of corpora lutea (17.1 ± 0.7 and 17.2 ± 1.8, respectively). In contrast, the implantation rate, reflecting the number of released

ova that were successfully fertilized and implanted in the uterus, was significantly decreased in females exposed to treated males (80.6%) compared to those exposed to control males (95%). Approximately half of the pregnant females that were mated with control males had some degree of preimplantation loss (7/13) compared to 83% of the pregnant females exposed to treated males (5/6). The mean preimplantation loss per litter increased from 5.3% in females exposed to control males to nearly 19% in females exposed to treated males (Fig. 5). Upon examination of the data for individual pregnant females, it is apparent that there is a heterogeneous effect of treatment on pre-implantation loss. Of the females mated with PNU157706-treated males, three had low rates of preimplantation loss (<15%) that were comparable to females mated with control males. Of the three remaining females, one had a higher rate of preimplantation loss (>18%) while two females had exceedingly high preimplantation loss at approximately 37 and 41%. In addition, three females exposed to treated males showed evidence of successful copulation (sperm-positive vaginal smears) that failed to result in pregnancy. This occurrence can be considered as 100% preimplantation loss due to failure of sperm to fertilize the released ova. Analysis of preimplantation loss based on the number of sperm positive females showed a significant increase in females exposed to males treated with PNU157706 (nearly 46%) when compared to control-mated females.

There was no significant effect of inhibitor treatment on postimplantation loss (Fig. 5). There were no late fetal deaths (non-live fetuses) observed in this study, therefore the postimplantation loss observed was due solely to

resorptions. Approximately half of the pregnant females exposed to either control or treated males had resorptions (control 6/13; treated 3/6) and the resorption rate was not significantly higher for those exposed to treated males.

The fetuses sired by control and treated males showed no gross external malformations. There was no difference in ano-genital distance between fetuses sired by PNU157706 treated males (male 3.69 ± 0.12 mm; female 1.97 ± 0.02 mm) and control males (male 3.92 ± 0.04 mm; female 1.95 ± 0.02 mm). The sex ratio (male/female) was also unaffected by treatment (control 96/107; treated 39/40). Fetuses sired by males treated with PNU157706 did not differ in weight (males 3.95 ± 0.36 g; females 3.81 ± 0.41 g) when compared to those sired by control males (males 4.23 ± 0.23 g; females 3.98 ± 0.23 g).

Discussion

Finasteride, a type 2 selective 5α-reductase inhibitor, was first marketed for the treatment of benign prostatic hyperplasia in 1992 [32]. Consequently, the long-term efficacy and safety of finasteride have been extensively studied for this indication [reviewed in 24, 25]. Finasteride treatment decreases serum and prostate DHT levels by approximately 65-70% and 85-90% respectively [25]. Efforts to discover more therapeutically effective compounds, i.e., that decrease DHT levels to a greater extent, have led to the development of a novel class of dual 5α-reductase inhibitors. In early 2003, dutasteride (GI198745) became the first dual 5α-reductase inhibitor available for the treatment of BPH [33]. In

comparison to finasteride, dutasteride and other dual compounds achieve almost total suppression of DHT levels [23, 33, 34].

There are a limited number of studies that examine the effects of finasteride on other reproductive tissues and/or fertility [35-38] and fewer still that examine the extraprostatic effects of the novel class of dual inhibitors [33, 34]. In 1991, Cukierski *et al.* [35] reported a partial decrease in fertility following chronic finasteride treatment (80 mg/kg/day for 24 weeks) of adult male rats. Wise *et al.* [36] reported a similar effect in a related study. In these earlier studies, testis weight and histology were unaffected by treatment and the subfertility was attributed solely to a deficit in the formation of copulatory plugs due to the effects of treatment on seminal vesicle and prostate weights. However, any data pertaining to the epididymis were lacking; therefore it is possible that the compromised fertility could have been due to an effect on this tissue as well.

In recent years, Mahendroo et al. [22] have developed and studied type-specific and dual 5α-reductase null mutation mice. The dual knockout mice were fertile (pregnancy rates of 60% compared to 96% for wild-type animals). Unexpectedly, type 2 or dual knockout male mice showed only a mild virilization defect such that their internal and external genitalia were fully formed, but their prostates and seminal vesicles were smaller. These findings were markedly different from the phenotype of type 2 5α-reductase deficiency in humans that results in a form of male pseudohermaphroditism [for review, see 39]. The authors concluded that, in mice, testosterone alone is required for differentiation of the male urogenital tract and that the synthesis of DHT serves largely as a

signal amplification mechanism. In contrast, both testosterone and DHT are required for proper development of the reproductive system in men, and studies in male rats with pharmacological inhibitors also support a two-androgen model of sexual differentiation [41-42]. Thus, the rat appears to be a more suitable animal model for understanding the roles of DHT within the human male reproductive system and comparisons with the mouse should be made with caution.

With the recent novel use of finasteride for the treatment of androgenic alopecia in young men of reproductive age [26] and the emergence of dual inhibitors that decrease DHT levels to a greater extent [33, 34], a renewed focus on the effects of 5α-reductase inhibition on the testis and fertility has emerged. To date, no adverse effects of 5α-reductase inhibitor treatment on testis weight, histology and/or sperm counts, and hence spermatogenesis, have been reported [24, 38, 43, 44], nor do 5α-reductase knockout mice show any testicular phenotype [22]; however, epididymal studies still remain scarce. Given the important role of the epididymis in sperm maturation, and the dependence of this tissue on DHT, a closer examination of the effects of 5α-reductase inhibition on epididymal sperm was considered necessary.

In the current study, we show that PNU157706 treatment of adult male rats for 28 days resulted in decreased fertility. It is unlikely that the effects of PNU157706 treatment on male fertility were due to an effect on sexual behaviour since 100% of the treated males showed evidence of successful copulation and 75% sired at least one litter. Current evidence does not suggest a role for DHT

in regulating sexual behavior. While the physiological functions of brain 5α-reductase activity have not been fully elucidated, evidence points to roles predominantly during brain ontogenesis and the sexual differentiation of specific brain regions, as well as possible anesthetic/anxiolytic actions evoked when progesterone and/or corticoid levels are high, i.e. during stress, and neuroprotective roles mediated via the catabolism of potentially neurotoxic levels of steroids [reviewed in 45]. Additionally, the very low incidence of sexual side effects in men treated with finasteride or the dual inhibitor dutasteride provides support for a minimal or nonexistent role for DHT in regulating sexual behavior [32-34].

Examination of pregnancy outcome demonstrated an effect of treatment on implantation rate and preimplantation loss per pregnant female. Preimplantation loss represents either failure of sperm to fertilize the released ova or the death of the fertilized embryo before implantation. The latter suggests involvement of genetic damage (chromosomal aberrations) leading to early death and is proposed as the reason for increased preimplantation loss and decreased fetal weight seen with increased paternal age and long-term paternal exposure to the alkylating agent cyclophosphamide [46, 47]. It is unlikely that the genetic integrity of spermatozoa in this study is compromised since the testis, the site of continuous sperm cell division, has not been shown to be affected by 5α-reductase inhibitor treatment [24, 38, 43, 44]. Furthermore, treatment had no effect on any of the fetal parameters measured, including fetal weight, which might be indicative of genetic impairment, nor were there any abnormal or dead

fetuses sired by treated males. It is more plausible that the sperm were unable to reach and fertilize the released ova due to compromised sperm motility and/or morphology. Supporting this is the observation of fewer sperm in the vaginal smears of some females mated with treated males and a lower pregnancy index.

We showed a decrease in both overall motility and progressive motility of sperm from the cauda epididymidis, accompanied by decreases in some motion parameters. The sperm tail encompasses the structural components directly involved in sperm motility [for review, see 48]; consequently, compromised tail ultrastructure could lead to impaired motility. In the present study, however, electron microscopic analysis of mid-piece flagellar ultrastructure revealed no effects of treatment that could account for the reduced sperm motility. Again, these findings are consistent with a lack of effect of 5α-reductase inhibitor treatment on the testis, where sperm ultrastructure is established. The acquisition of sperm motility is a key element of epididymal sperm maturation [3]. By the time sperm reach the distal regions of the epididymis, maximum progressive motility is achieved that enables sperm to reach and penetrate the egg [3, 49]. This transition to progressive motility requires interactions between sperm and the surrounding epididymal luminal environment [3, 4, 50]. In fact, in reproductive toxicology studies, altered sperm motility is a valuable indicator of toxicity arising from an exclusive effect on the epididymis or sperm within the epididymis [51]. In the current study, it is likely that the reduced sperm motility observed is due to exposure of sperm to a compromised epididymal luminal environment. Notably, in a previous study we have shown that PNU157706

treatment affects the segment-specific expression of many genes in the epididymis potentially involved in the creation of the luminal environment that is critical for proper sperm maturation [27].

While the characteristic head, midpiece and tail structures of spermatozoa are already present before sperm leave the testis, other morphological changes occur during sperm transit through the epididymis. One prominent change is the migration of the cytoplasmic droplet along the midpiece of the sperm and its eventual shedding in the distal regions of the epididymis [7, 31]. The percentage of sperm retaining their cytoplasmic droplet was increased in rats treated with PNU157706. Interestingly, while the reason for shedding of the droplet remains unclear, some evidence suggests that cytoplasmic droplet retention can be correlated with altered epididymal function and decreased fertility [52-55]. Shedding of the droplet is thought to be related to the well-characterized changes in sperm plasma membrane lipid composition and fluidity that occur during epididymal transit [5, 56]. It is tempting to speculate that the increased cytoplasmic droplet retention, and possibly the increased sperm breakages observed in the current study are due to altered sperm membrane composition and dynamics arising from exposure to a suboptimal epididymal milieu. In fact, we have shown that PNU157706 treatment alters the epididymal expression of genes involved in oxidative defense that protect the highly susceptible sperm membrane lipids from oxidative damage [27]. Indeed, proper redox chemistry is essential within the epididymis as it regulates pathways involved in important sperm functions such as motility and sperm-egg interaction [52]; thus any

perturbation of the redox system likely has manifold consequences on epididymal sperm. Additionally, PNU157706 treatment also alters the expression of genes involved in fatty acid and lipid metabolism within the epididymis [27]. Of relevance to the current study is the reported reproductive impairment observed in mouse models of sphingolipid storage disorders (Niemann-Pick, Tay-Sachs, and Sandhoff diseases) that exhibit gross lipid accumulation within cells and tissues, including those of the male reproductive tract [58-60]. Interestingly, there do not appear to be any changes in testis weight, morphology or sperm counts in these animals; hence, the reproductive pathologies are believed to result from lipid accumulation in lysosomes of the epididymis that give rise to a dysfunctional sperm maturation environment. These models not only highlight the importance of lipid regulation within the epididymis, but also clearly support an important role for the epididymis in sperm-related functions that are unique and separate from the testis.

In conclusion, while an effect on sperm fertilizing ability was reported in earlier 5α-reductase inhibitor studies, to our knowledge, this is the first study that clearly demonstrates an effect of inhibitor treatment on sperm maturation in the epididymis. It is highly likely that the compromised sperm motility and morphology observed in the current study contribute to the subfertility observed in 5α-reductase inhibitor-treated rats. Furthermore, our results support a role for both 5α-reductase isozymes within the epididymis and indicate the potential of dual 5α-reductase inhibition as an experimental model for more in-depth study of

epididymal sperm-related functions separate from testicular sperm-related functions.

Acknowledgements

We thank Dr. T. Zaccheo from Pharmacia & Upjohn (presently Pfizer) for a generous gift of PNU157706. Johanne Ouellette is gratefully acknowledged for her assistance with the electron microscope analysis. These studies were supported by a grant from the Canadian Institutes of Health Research. NAH is a recipient of a Canadian Institutes of Health Research Studentship.

References

1. Leblond CP, Clermont Y. Definition of the stages of the cycle of the seminiferous epithelium in the rat. Ann N Y Acad Sci 1952; 55:548-573.
2. Clermont Y. Kinetics of spermatogenesis in mammals: seminiferous epithelium cycle and spermatogonial renewal. Physiol Rev 1972; 52:198-236.
3. Orgebin-Crist MC. Sperm Maturation in Rabbit Epididymis. Nature 1967; 216:816-818.
4. Hermo L, Robaire B. Epididymal cell types and their functions. In: Robaire B, Hinton BT (eds.), The Epididymis: From Molecules to Clinical Practice. New York: Kluwer Academic/Plenum Publishers; 2002: 81-102.
5. Jones R. Plasma membrane structure and remodelling during sperm maturation in the epididymis. J Reprod Fertil Suppl 1998; 53:73-84.
6. Blandau RJ, Rumery RE. The relationship of swimming movements of epididymal spermatozoa to their fertilizing capacity. Fertil Steril 1964; 15:571-579.
7. Fawcett DW, Phillips DM. Observations on the release of spermatozoa and on changes in the head during the passage through the epididymis. J Reprod Fertil 1969; 6:405-418.
8. Heller CG, Clermont Y. Spermatogenesis in man: an estimation of its duration. Science 1963; 140:184 –186.
9. Amann RP, Johnson L, Thompson DL Jr, Pickett BW. Daily spermatozoal production, epididymal spermatozoal reserves and transit time of

spermatozoa through the epididymis of the rhesus monkey. Biol Reprod 1976; 5:586-592.

10. Orgebin-Crist MC, Tichenor PL. Effect of testosterone on sperm maturation in-vitro. Nature 1973; 245:328-329.
11. Blaquier JA, Burgos MH, Cameo MS. Role of androgens in maturation of epididymal spermatozoa in guinea-pig. Endocrinology 1972; 90:839-842.
12. Orgebin-Crist MC, Jahad N, Hoffman LH. Effects of testosterone, 5-alpha-dihydrotestosterone, 3-alpha-androstanediol, and 3-beta-androstanediol on maturation of rabbit epididymal spermatozoa in organ-culture. Cell Tissue Res 1976; 167:515-525.
13. Tindall DJ, Nayfeh SN, French FS. Androgen uptake and binding in rat epididymal nuclei, in-vivo. Biochem Biophys Res Commun 1972; 49:1391-1397.
14. Turner TT, Jones CE, Howards SS, Ewing LL, Zegeye B, Gunsalus GL. On the androgen microenvironment of maturing spermatozoa. Endocrinology 1984; 115:1925-1932.
15. Gloyna RE, Wilson JD. A comparative study of the conversion of testosterone to 17β-hydroxy-5α-androstan-3-one (dihydrotestosterone) by rat prostate and epididymis. J Clin Endocrinol Metab 1969; 29:970-977.
16. Walsh PC, Swerdloff RS. Biphasic effect of testosterone on spermatogenesis in the rat. Invest Urol 1973; 11:190-193.
17. Zirkin BR, Santulli R, Awoniyi CA, Ewing LL. Maintenance of advanced spermatogenic cells in the adult rat testis: quantitative relationship to

testosterone concentration within the testis. Endocrinology 1989; 124:3043-3049.

18. McLachlan RI, O'Donnell L, Meachem SJ, Stanton PG, de Kretser DM, Pratis K, Robertson DM. Identification of specific sites of hormonal regulation in spermatogenesis in rats, monkeys, and man. Recent Prog Horm Res 2002; 57:149-179.
19. Andersson S, Russell DW. Structural and biochemical properties of cloned and expressed human and rat steroid 5 alpha-reductases. Proc Natl Acad Sci U S A 1990; 87:3640-3644.
20. Normington K, Russell DW. Tissue distribution and kinetic characteristics of rat steroid 5 alpha-reductase isozymes. Evidence for distinct physiological functions. J Biol Chem 1992; 267:19548-19554.
21. Viger RS, Robaire B. The mRNAs for the steroid 5 alpha-reductase isozymes, types 1 and 2, are differentially regulated in the rat epididymis. J Androl 1996; 17:27-34.
22. Mahendroo MS, Cala KM, Hess DL, Russell DW. Unexpected virilization in male mice lacking steroid 5 alpha-reductase enzymes. Endocrinology 2001; 142:4652-4662.
23. di Salle E, Giudici D, Radice A, Zaccheo T, Ornati G, Nesi M, Panzeri A, Delos S, Martin PM. PNU 157706, a novel dual type I and II 5alpha-reductase inhibitor. J Steroid Biochem Mol Biol 1998; 64:179-186.
24. Steers WD. 5alpha-reductase activity in the prostate. Urology 2001; 58:17-24.

25. Bartsch G, Rittmaster RS, Klocker H. Dihydrotestosterone and the concept of 5alpha-reductase inhibition in human benign prostatic hyperplasia. World J Urol 2002; 19:413-425.
26. Libecco JF, Bergfeld WF. Finasteride in the treatment of alopecia. Expert Opin Pharmacother 2004; 5:933-940.
27. Henderson NA, Cooke GM, Robaire B. Effects of PNU157706, a dual 5alpha-reductase inhibitor, on gene expression in the rat epididymis. J Endocrinol 2004; 181:245-261.
28. Syntin P, Robaire B. Sperm structural and motility changes during aging in the Brown Norway rat. J Androl 2001; 22:235-244.
29. Slott VL, Suarez JD, Poss PM, Linder RE, Strader LF, Perreault SD. Optimization of the Hamilton-Thorn computerized sperm motility analysis system for use with rat spermatozoa in toxicological studies. Fundam Appl Toxicol 1993; 21:298-307.
30. Klinefelter GR, Gray LE Jr, Suarez JD. The method of sperm collection significantly influences sperm motion parameters following ethane dimethanesulphonate administration in the rat. Reprod Toxicol 1991; 5:39-44.
31. Hermo L, Dworkin J, Oko R. Role of epithelial clear cells of the rat epididymis in the disposal of the contents of cytoplasmic droplets detached from spermatozoa. Am J Anat 1988; 183:107-124.
32. Gormley GJ, Stoner E, Bruskewitz RC, Imperato-McGinley J, Walsh PC, McConnell JD, Andriole GL, Geller J, Bracken BR, Tenover JS, et al. The

effect of finasteride in men with benign prostatic hyperplasia. The Finasteride Study Group. N Engl J Med 1992; 327:1185-1191.

33. Roehrborn CG, Boyle P, Nickel JC, Hoefner K, Andriole G; ARIA3001 ARIA3002 and ARIA3003 Study Investigators. Efficacy and safety of a dual inhibitor of 5-alpha-reductase types 1 and 2 (dutasteride) in men with benign prostatic hyperplasia. Urology 2002; 60:434-441.
34. Foley CL, Kirby RS. 5 alpha-reductase inhibitors: what's new? Curr Opin Urol 2003; 13:31-37.
35. Cukierski MA, Sina JL, Prahalada S, Wise LD, Antonello JM, MacDonald JS, Robertson RT. Decreased fertility in male rats administered the 5 alpha-reductase inhibitor, finasteride, is due to deficits in copulatory plug formation. Reprod Toxicol 1991; 5:353-362.
36. Wise LD, Minsker DH, Cukierski MA, Clark RL, Prahalada S, Antonello JM, MacDonald JS, Robertson RT. Reversible decreases of fertility in male Sprague-Dawley rats treated orally with finasteride, a 5 alpha-reductase inhibitor. Reprod Toxicol 1991; 5:337-346.
37. Iguer-Ouada M, Verstegen JP. Effect of finasteride (Proscar MSD) on seminal composition, prostate function and fertility in male dogs. J Reprod Fertil Suppl 1997; 51:139-149.
38. George FW, Johnson L, Wilson JD. The effect of a 5 alpha-reductase inhibitor on androgen physiology in the immature male rat. Endocrinology 1989; 125:2434-2438.

39. Imperato-McGinley J, Zhu YS. Androgens and male physiology the syndrome of 5alpha-reductase-2 deficiency. Mol Cell Endocrinol 2002; 198:51-59.

40. Imperato-McGinley J, Binienda Z, Arthur A, Mininberg DT, Vaughan ED Jr, Quimby FW. The development of a male pseudohermaphroditic rat using an inhibitor of the enzyme 511α-reductase. Endocrinology 1985; 16:807-812.

41. George FW, Johnson L, Wilson JD. The effect of a 5 alpha-reductase inhibitor on androgen physiology in the immature male rat. Endocrinology 1989; 125:2434-2438.

42. Anderson CA, Clark RL. External genitalia of the rat: normal development and the histogenesis of 5α-reductase inhibitor-induced abnormalities. Teratology 1990; 42:483-496.

43. Overstreet JW, Fuh VL, Gould J, Howards SS, Lieber MM, Hellstrom W, Shapiro S, Carroll P, Corfman RS, Petrou S, Lewis R, Toth P, Shown T, Roy J, Jarow JP, Bonilla J, Jacobsen CA, Wang DZ, Kaufman KD. Chronic treatment with finasteride daily does not affect spermatogenesis or semen production in young men. J Urol 1999; 162:1295-1300.

44. Rhoden EL, Gobbi D, Menti E, Rhoden C, Teloken C. Effects of the chronic use of finasteride on testicular weight and spermatogenesis in Wistar rats. BJU Int 2002; 89:961-963.

45. Poletti A, Coscarella A, Negri-Cesi P, Colciago A, Celotti F, Martini L. 5 Alpha-reductase isozymes in the central nervous system. Steroids 1998; 63:246-251.

46. Serre V, Robaire B. Paternal age affects fertility and progeny outcome in the Brown Norway rat. Fertil Steril 1998; 70:625-631.

47. Trasler JM, Hales BF, Robaire B. Chronic low dose cyclophosphamide treatment of adult male rats: effect on fertility, pregnancy outcome and progeny. Biol Reprod 1986; 34:275-283.

48. Turner RM. Tales from the tail: what do we really know about sperm motility? J Androl 2003; 24:790-803.

49. Yeung CH, Oberlander G, Cooper TG. Characterization of the motility of maturing rat spermatozoa by computer-aided objective measurement. J Reprod Fertil 1992; 96:427-441.

50. Turner TT. Spermatozoa are exposed to a complex microenvironment as they traverse the epididymis. Ann N Y Acad Sci 1991; 637:364-383.

51. Perreault SD, Cancel AM. Significance of incorporating measures of sperm production and function into rat toxicology studies. Reproduction 2001; 121:207-216.

52. Dadoune JP. Ultrastructural abnormalities of human spermatozoa. Hum Reprod 1988; 3:311-318.

53. Keating J, Grundy CE, Fivey PS, Elliott M, Robinson J. Investigation of the association between the presence of cytoplasmic residues on the human

sperm midpiece and defective sperm function. J Reprod Fertil 1997; 110:71-77.

54. Gomez E, Buckingham DW, Brindle J, Lanzafame F, Irvine DS, Aitken RJ. Development of an image analysis system to monitor the retention of residual cytoplasm by human spermatozoa: correlation with biochemical markers of the cytoplasmic space, oxidative stress, and sperm function. J Androl 1996; 17:276-287.

55. Akbarsha MA, Latha PN, Murugaian P. Retention of cytoplasmic droplet by rat cauda epididymal spermatozoa after treatment with cytotoxic and xenobiotic agents. J Reprod Fertil 2000; 120:385-390.

56. Jones R. Plasma membrane composition and organization during maturation of spermatozoa in the epididymis. In: Robaire B, Hinton BT (eds.), The Epididymis: From Molecules to Clinical Practice. New York: Kluwer Academic/Plenum Publishers; 2002: 405-416.

57. Baker MA, Aitken RJ. The importance of redox regulated pathways in sperm cell biology. Mol Cell Endocrinol 2004; 216:47-54.

58. Butler A, He X, Gordon RE, Wu HS, Gatt S, Schuchman EH. Reproductive pathology and sperm physiology in acid sphingomyelinase-deficient mice. Am J Pathol 2002; 161:1061-1075.

59. Adamali HI, Somani IH, Huang JQ, Gravel RA, Trasler JM, Hermo L. II. Characterization and development of the regional- and cellular-specific abnormalities in the epididymis of mice with beta-hexosaminidase A deficiency. J Androl 1999; 20:803-824.

60. Trasler J, Saberi F, Somani IH, Adamali HI, Huang JQ, Fortunato SR, Ritter G, Gu M, Aebersold R, Gravel RA, Hermo L. Characterization of the testis and epididymis in mouse models of human Tay Sachs and Sandhoff diseases and partial determination of accumulated gangliosides. Endocrinology 1998; 139:3280-3288.

Figures and Legends

Figure 1. Effect of PNU157706 treatment on sperm motility. The percentages of motile sperm (top) and progressively motile sperm (bottom) from the caput (left) and cauda (right) are indicated for the control (0 mg/kg/day, light grey bars) and treated (10 mg/kg/day, dark grey bars) groups. * $P < 0.05$ versus the control group, n = 5-7.

Figure 1

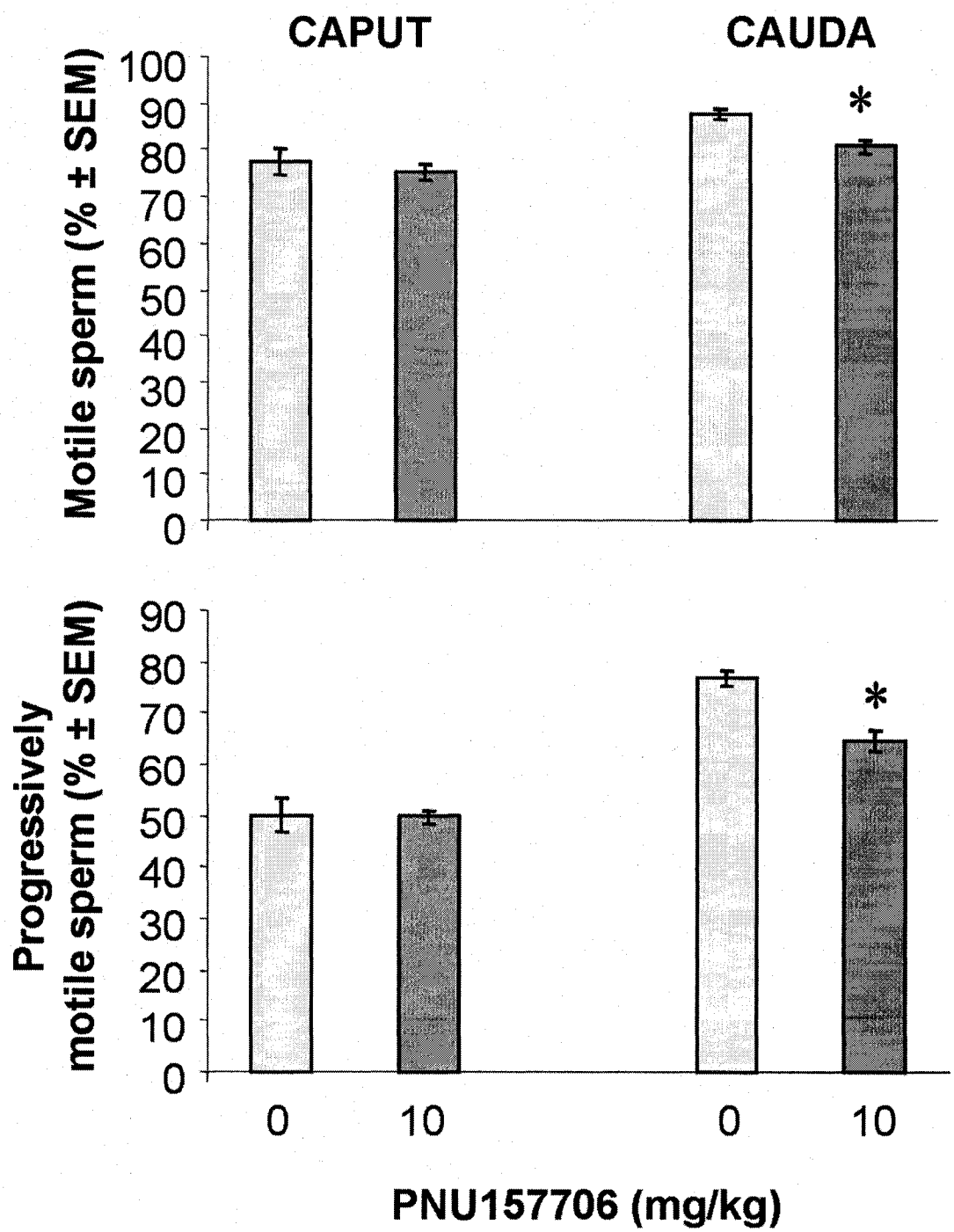
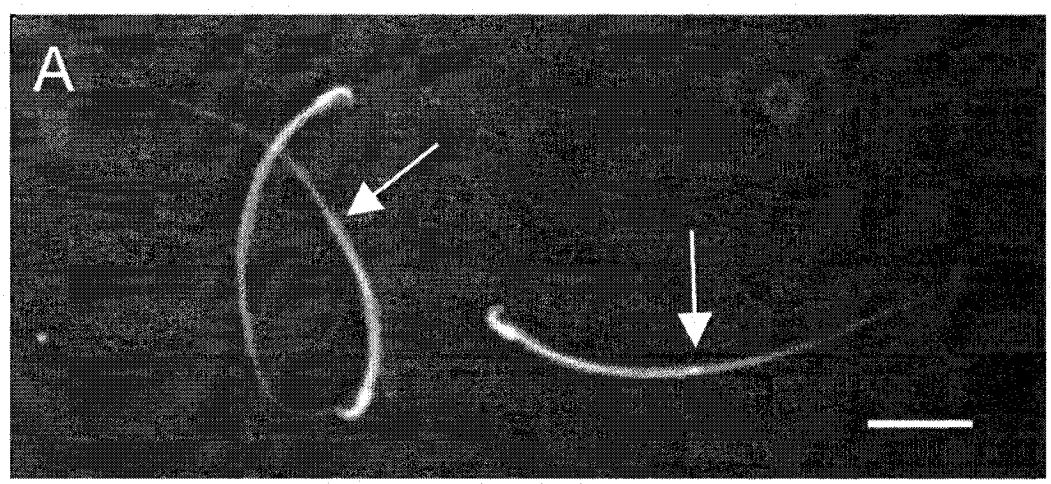

CAPUT
CAUDA
Motile sperm (% ± SEM)
100
90
80
70
60
50
40
30
20
10
0
*
Progressively motile sperm (% ± SEM)
90
80
70
60
50
40
30
20
10
0
*
0
10
0
10
PNU157706 (mg/kg)

Figure 2. HTM-IVOS (phase-contrast microscopy) analysis of the effect of PNU157706 treatment on cytoplasmic droplet retention in cauda sperm. **A**) Example of a phase-contrast image from the HTM-IVOS system showing the absence or presence (white arrows) of the cytoplasmic droplet on cauda sperm. Bar = 16 µm. **B**) Calculation from phase-contrast images of the percentage of cauda sperm retaining the cytoplasmic droplet in the control (0 mg/kg/day, light grey bars) and treated (10 mg/kg/day, dark grey bars) groups. 300-500 sperm per sample (n=5) were analyzed. * $P < 0.05$ versus the control group.

Figure 2


A

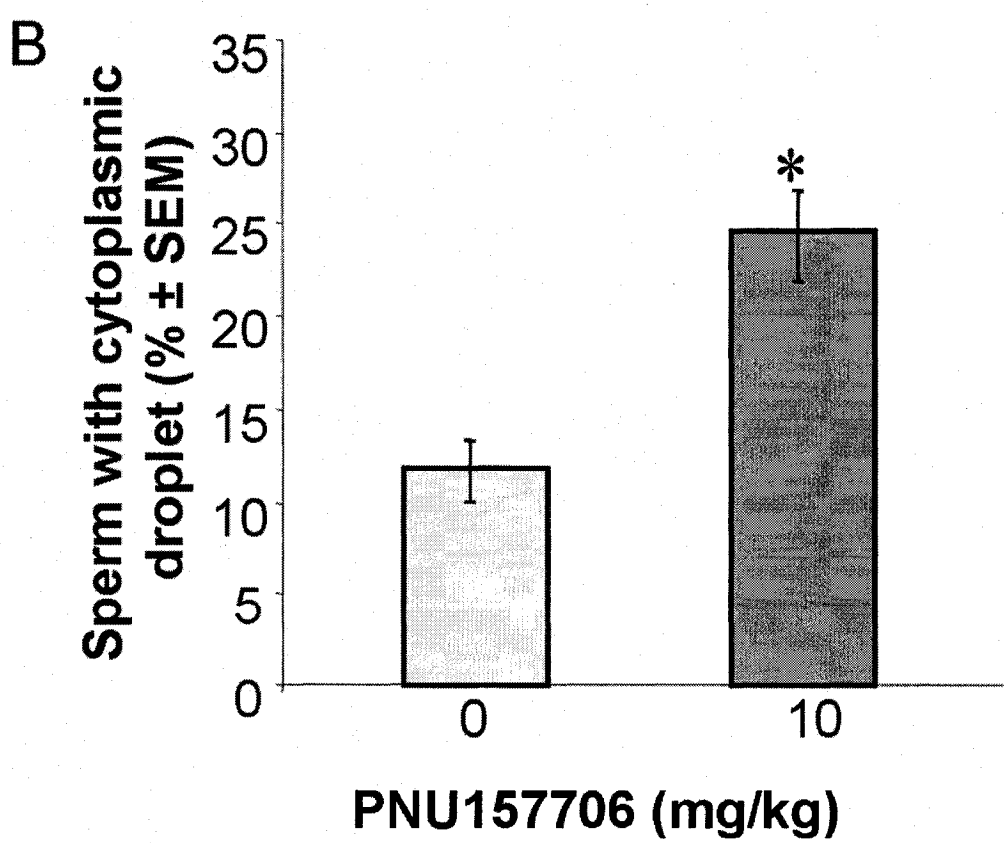

B
Sperm with cytoplasmic droplet (% ± SEM)
35
30
25
20
15
10
5
0
*
0
10
PNU157706 (mg/kg)

Figure 3. Examples of phase-contrast images of abnormal sperm morphologies detected using the HTM-IVOS system. **A**) Tail only. **B**) Bent. **C**) Broken. **D**) Head only. Bar = 16 µm.

Figure 3

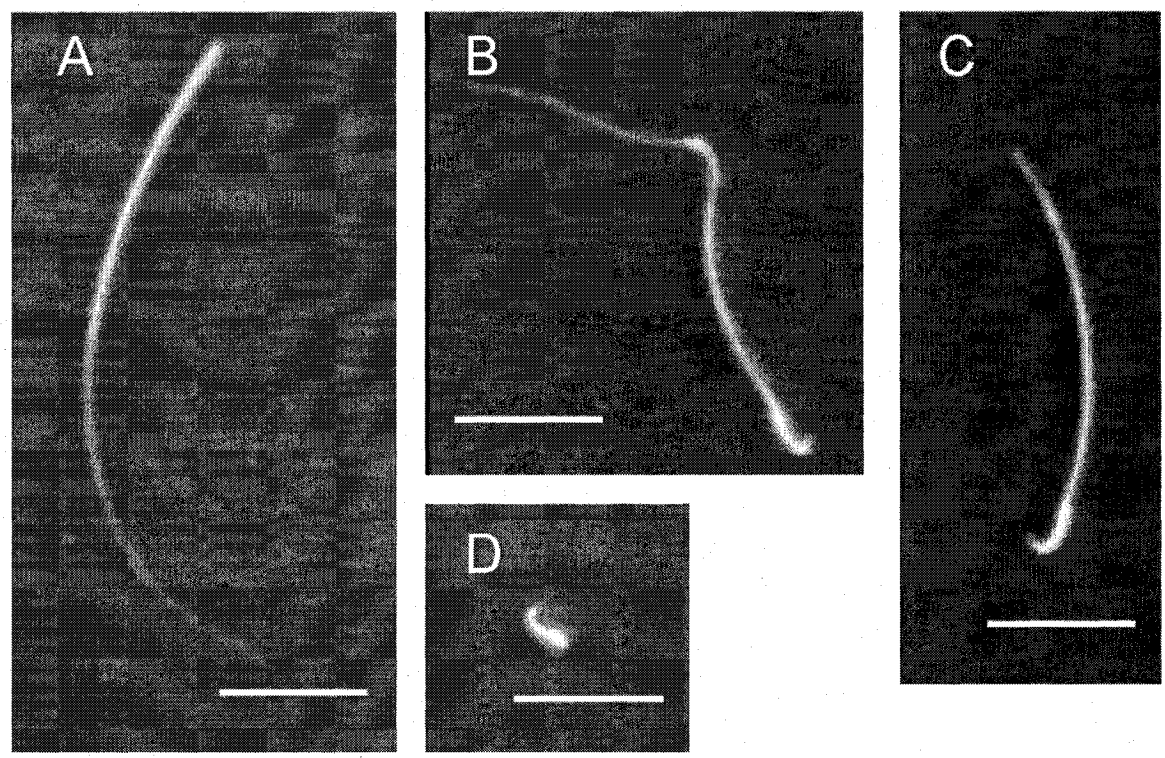
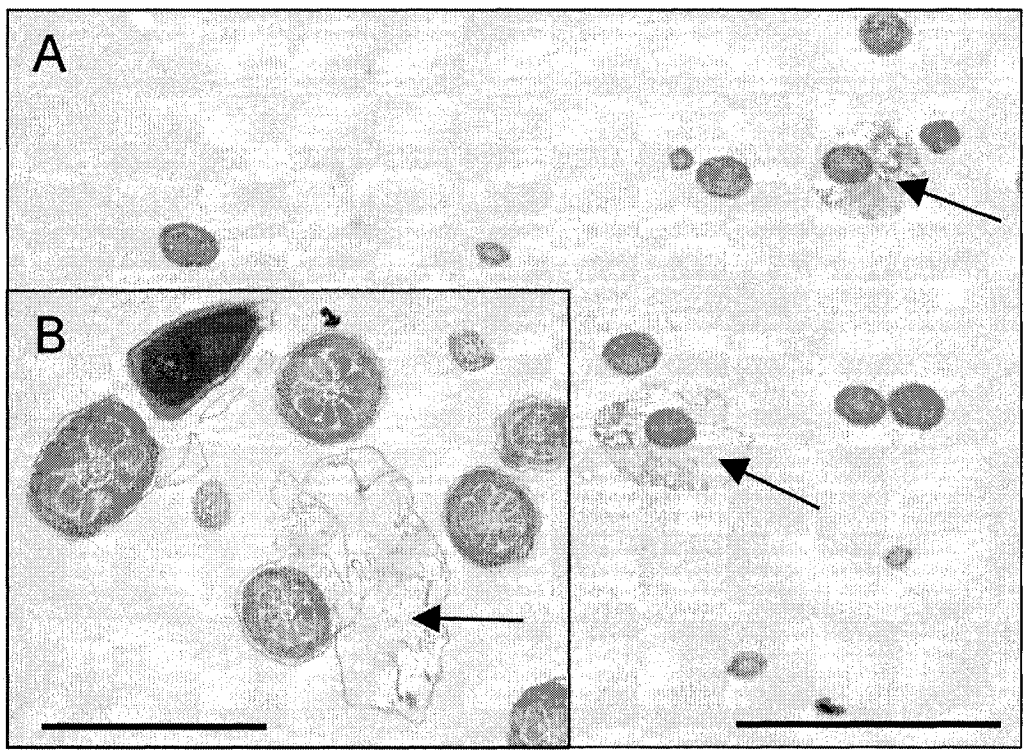


Figure 4. Electron microscopic analysis of the effect of PNU157706 treatment on cytoplasmic droplet retention in cauda sperm. **A**) Example of an electron micrograph showing several tail mid-piece cross-sections of cauda sperm. Cytoplasmic droplets surrounding the sperm midpiece are indicated by black arrows. Bar = 0.5 µm. **B**) Higher magnification of the cytoplasmic droplet. Bar = 2 µm. **C**) Calculation from electron micrographs of the percentage of cauda sperm retaining the cytoplasmic droplet in the control (0 mg/kg/day, light grey bars) and treated (10 mg/kg/day, dark grey bars) groups. A minimum of 100 midpiece sperm tail (flagellum) cross-sections per animal (n=4) were analyzed. * $P < 0.05$ versus the control group.

Figure 4


A
B

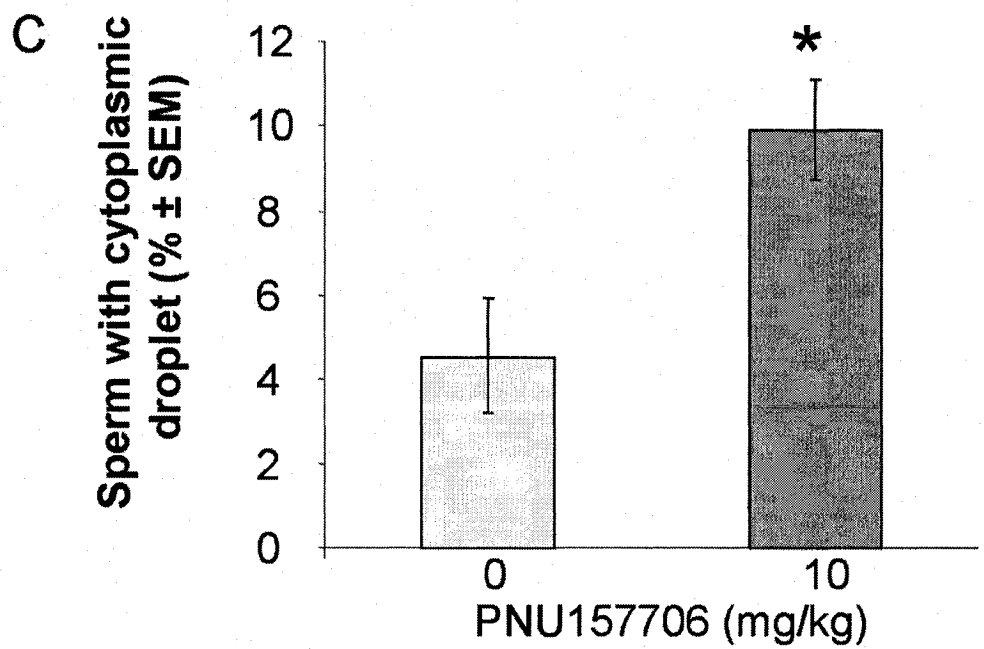
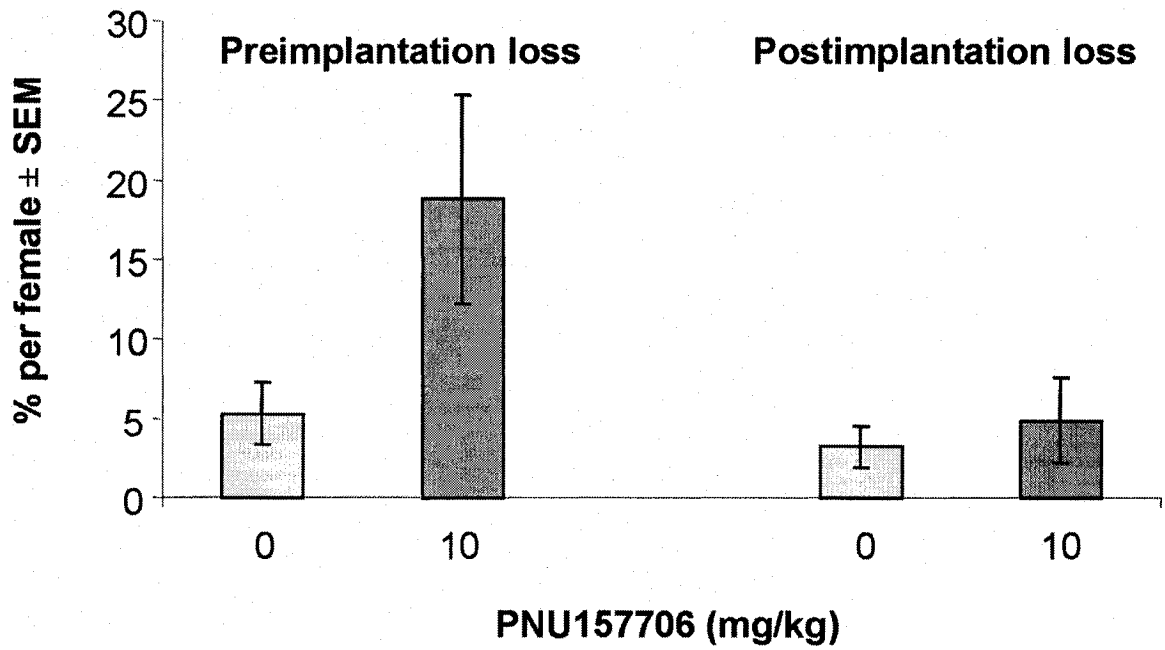

C
Sperm with cytoplasmic droplet (% ± SEM)
12
10
8
6
4
2
0
*
0
10
PNU157706 (mg/kg)

Figure 5. Effect of PNU157706 treatment on pregnancy outcome on Day 20 of gestation. The rates of preimplantation and postimplantation loss are shown as percentage per female on the y-axis for the control (0 mg/kg/day, light grey bars) and treated (10 mg/kg/day, dark grey bars) groups on the x-axis.

Figure 5


Preimplantation loss
Postimplantation loss
% per female ± SEM
30
25
20
15
10
5
0
0
10
0
10
PNU157706 (mg/kg)

Tables and Legends

Table 1. Effect of PNU157706 treatment on motion parameters of caput and cauda epididymal sperm.[a]

[a] Values represent mean ± SEM (n = 5-7). Average-path velocity (VAP), straight-line velocity (VSL), curvilinear velocity (VCL), amplitude of lateral head displacement (ALH), beat cross frequency (BCF), straightness (STR), linearity (LIN).

[b] $P < 0.05$ versus control group.

PNU157706 (mg/kg)	Caput		Cauda	
	0	10	0	10
Velocity parameters				
VAP (μm/sec)	143.8 ± 5.3	143.7 ± 7.3	157.7 ± 3.1	155.5 ± 4.9
VSL (μm/sec)	81.5 ± 3.2	82.1 ± 3.5	103.3 ± 2.6	92.4 ± 3.7[b]
VCL (μm/sec)	309.6 ± 9.2	307.8 ± 12.4	348.1 ± 4.8	335.6 ± 8.1
Head motion parameters				
ALH (μm)	19.9 ± 0.6	20.1 ± 0.7	22.1 ± 1.0	23.4 ± 0.7
BCF (Hz)	27 ± 0.3	25.8 ± 0.7	22.7 ± 1.1	21.5 ± 0.6
Derived parameters				
STR (%)	59.2 ± 0.8	59.5 ± 1.3	66.8 ± 2.2	60.7 ± 1.5[b]
LIN (%)	28.5 ± 0.2	28.4 ± 0.4	30.4 ± 0.7	28.0 ± 0.6[b]

Table 2. Effect of PNU157706 treatment on cauda epididymal sperm morphology.[a]

[a] Values represent mean ± SEM (n = 5-7).

[b] $P < 0.05$ versus control group.

PNU157706 (mg/kg)	Abnormalities observed with HTM-IVOS				
	Head only (%)	Tail only (%)	Bent (%)	Other (%)	Total (%)
0	0.43 ± 0.3	0.38 ± 0.1	2.4 ± 0.4	0.18 ± 0.1	3.4 ± 0.7
10	2.03 ± 0.5[b]	1.17 ± 0.3[b]	4.21 ± 0.8	0.74 ± 0.2[b]	8.2 ± 0.8[b]

Table 3. Effect of PNU157706 treatment on male reproductive indices.

[a] Copulation index = number of sperm positive females / number of pairings (% value in parentheses)

[b] Pregnancy index = number of pregnancies / number of sperm positive females (% value in parentheses)

[c] Fertility index = number of pregnancies / number of pairings (% value in parentheses)

[d] $p<0.05$ versus control group.

	Treatment group	
	Control	PNU157706
Copulation index[a]	13/16 (81.3%)	9/16 (56.2%)
Pregnancy index[b]	13/13 (100 %)	6/9 (66.7%)
Fertility index[c]	13/16 (81.3%)	6/16 (37.5%)[d]

Connecting Text

Thus far, the studies presented clearly demonstrated that DHT has an important role in regulating gene expression and sperm maturation in the epididymis and it does so in a highly segment-specific manner. What remained unclear was the identity of segment-specific signaling/regulatory molecules and pathways acting upstream and/or downstream of DHT to mediate its actions in the epididymis. Therefore, the elucidation of potential DHT signaling mechanisms in the epididymis formed the basis of the final objective. Two main groups of genes were chosen for gene expression analysis: 1) The isozymes of 5α-reductase and the androgen receptor, which are directly involved in mediating androgen action in the epididymis and 2) Different growth factor families, which were chosen based on increasing evidence for the involvement of growth factor signaling in other reproductive tissues such as the prostate.

CHAPTER 4

Segment-Specific Expression of Signaling Pathway Genes in the Rat Epididymis and the Effects of Dual 5α-Reductase Inhibition

Natali Anne Henderson, Gerard M. Cooke[1] and Bernard Robaire

Submitted to Endocrinology

[1] G.M. Cooke did the in vitro 5α-reductase enzyme assays described in this chapter.

Abstract

Dihydrotestosterone (DHT) is the primary androgen acting in the epididymis, the site of sperm maturation. Previously, we showed that treatment of male rats with PNU157706, an inhibitor that acts on both isoforms of 5α-reductase to prevent DHT formation, has effects on the expression of genes implicated in processes that create the optimal luminal microenvironment required for sperm maturation, and on sperm maturation itself. However, signaling pathways involved in regulating or mediating DHT actions in the epididymis remain largely unknown. The goals of this study were to determine the expression profiles of different signaling genes in the epididymis and assess their DHT-dependence by using two different dual 5α-reductase inhibitors. Rats were untreated or gavaged with vehicle, 10 mg/kg/day of PNU157706 or 32 mg/kg/day of FK143 for 28 days and epididymal gene expression was analyzed. Gene array analysis revealed analogous effects of FK143 on overall epididymal gene expression when compared to previous PNU157706 studies. Quantitative RT-PCR analysis of the expression of the 5α-reductase isozymes, androgen receptor and members of the IGF, FGF, TGF and VEGF families revealed novel segment-specific expression profiles in the epididymis that were differentially affected by 5α-reductase inhibition; the two inhibitors had parallel effects. Specifically, in proximal regions, 5α-reductase 1, androgen receptor and TGF-β1 expression increased after treatment while in distal segments expression of IGF-1, IGFBP-5, IGFBP-6, and FGF-10 decreased. These results provide novel insight into epididymal signaling mechanisms and indicate potential candidates

acting either upstream or downstream of DHT to regulate and/or mediate its actions in the epididymis.

Introduction

The epididymis is a highly specialized tissue within the male excurrent duct system that functions in the transport, maturation, protection and storage of spermatozoa (1, 2). There are four distinct regions of the epididymis that carry out the sperm-related functions: the initial segment, caput, corpus and cauda epididymidis. Importantly, the acquisition of progressive motility and fertilizing ability (i.e. sperm maturation) requires the successive exposure of spermatozoa to the particular luminal environment created by the different epididymal segments (1, 2). The functional segmentation of the epididymis is reflected at the molecular level by complex segment-specific gene expression profiles (3, 4). The unique patterns of gene expression along the duct contribute to the evolving repertoire of components that make up the highly specialized luminal fluid.

The structure and functions of the epididymis are highly dependent on androgens (5, 6). More specifically, several studies have clearly shown that the primary androgen acting in this tissue is dihydrotestosterone (DHT) (7-10). 5α-Reductase (EC 1·3·1·22) is the enzyme that catalyzes the conversion of testosterone to DHT. Both isoforms of 5α-reductase (type 1 and type 2) are present in the epididymis and are differentially expressed along the tubule (11).

Previously, using gene expression profiling, we have shown that treatment of adult male rats with the dual 5α-reductase inhibitor, PNU157706, has a highly

segment-specific effect on epididymal gene expression (12). The affected genes are involved in fatty acid and lipid metabolism, regulation of ion and fluid transport, luminal acidification, oxidative defense, and protein processing and degradation; these are essential processes that contribute to the formation of the optimal luminal microenvironment (12).

Importantly, distinctive segment-specific epididymal gene expression changes also occur during aging and in response to other experimental manipulations such as orchidectomy, caloric restriction and vitamin E treatments (13-16). These unique responses clearly demonstrate the differential regulation of this tissue, however, surprisingly little is known about how this regulation is achieved. While DHT clearly has a prominent role in the epididymis, the involvement of unique signaling molecules and pathways acting upstream and/or downstream of DHT remains to be deciphered. Therefore, the main objective of the present study was to examine the effects of 5α-reductase inhibition on the expression of specific signaling genes in the epididymis in order to elucidate possible mechanisms of DHT action in this tissue.

In the present study, gene arrays were used to analyze the effects of a second 5α-reductase inhibitor, FK143, on gene expression; FK143 is a non-steroidal, non-competitive inhibitor of both isozymes of 5α-reductase (17, 18). The cumulative array data from this study and our previous PNU157706 study (12), as well as the current literature, were used as a guide in the selection of genes potentially involved in epididymal regulatory mechanisms for further characterization in the epididymis. The genes chosen include both isozymes of

5α-reductase and the androgen receptor, which are directly involved in mediating androgen action in the epididymis, as well as those involved in different growth factor signaling systems. Interestingly, there is recent evidence supporting a role for various growth factors in epididymal function; for example, the overexpression of VEGF in the testis and epididymis of transgenic mice results in infertility (19). In a different animal model, the GH-deficient dwarf (dw/dw) rat, the administration of insulin-like growth factor 1 increases the motility and improves the morphology of immature spermatozoa (20). Whether an epididymal mechanism contributes fully or in part to the effects of these growth factors in such animal models has not yet been determined, but it is certainly possible that these and other growth factor systems are involved in epididymal regulation.

The steady-state epididymal expression profiles of the selected genes were analyzed using the sensitive technique of quantitative real-time RT-PCR. We then assessed the effects of both 5α-reductase inhibitors (PNU157706 and FK143) on the epididymal expression of these signaling genes to determine whether they are dependent on DHT, and thus potentially acting as upstream (i.e. the 5α-reductase isozymes) or downstream regulators of DHT-dependent gene expression and function.

Materials and Methods

Animals

Adult male Sprague-Dawley rats (325-350g) were obtained from Charles River Canada (St. Constant, PQ, Canada), maintained under controlled light

(14L:10D) and temperature (22°C), and provided with food and water ad libitum. Caput-corpus epididymides from 90-day old animals that were previously stored at -80°C were used for *in vitro* enzyme assays. For *in vivo* studies, adult male rats were left untreated (for determination of steady-state mRNA profiles, n=5) or they were randomly divided into 3 treatment groups (n=5) and gavaged with 0.5 ml/kg of either the vehicle alone (control group) or 10 mg/kg PNU157706 or 32 mg/kg FK143 (inhibitor treatment groups) suspended in vehicle (0.5% methylcellulose solution (BDH, Montréal, QC) containing 0.4% Tween 80 (A&C American Chemicals Ltd, Montreal, QC)) for 28 consecutive days. Doses were selected based on reported effects on rat ventral prostate; the dosing regimen with 10 mg/kg/day PNU157706 or 32 mg/kg/day of FK143 has been shown to decrease prostatic dihydrotestosterone levels by >90% (18, 21). Epididymides from untreated and treated rats were sectioned into initial segment, caput, corpus and cauda epididymides and immediately frozen in liquid nitrogen. Tissues were stored at -80°C until used for RNA extraction. The changes in reproductive tissue weights with treatment were consistent what has previously been reported for both compounds (12, 18, 21). All animal studies were conducted in accordance with the principles and procedures outlined in the Guide to the Care and Use of Experimental Animals prepared by the Canadian Council on Animal Care (McGill Animal care Committee protocol #206).

In vitro 5α-Reductase Assays

Unlabelled steroids were purchased from Steraloids Inc. (Newport, RI). (1,2,6,7-^{3}H) testosterone (74.0 Ci/mmol) was from Dupont/NEN (Boston, MA). Organic solvents were from BDH (Montréal, QC). Dimethylglutaric acid (DMG), NADPH and dimethyl sulfoxide (DMSO) were from Sigma Chemical Co. (St Louis, MO). Plastic coated Whatman^{TM} PE SIL G silica gel chromatography plates were purchased from Chromatographic Specialties (Montréal, QC). FK143 solutions were prepared in DMSO.

Frozen caput-corpus epididymides (only control, untreated tissues were used) were thawed, homogenized (Polytron) in DMG buffer (50 mM DMG, 5% glycerol, NaOH, 0.5mM NADPH) pH 6.5, and the nuclear and microsomal fractions were assayed for 5α-reductase activity as previously described for PNU157706 (12). Briefly, kinetic studies of 5α-reductase activity were done at pH 6.5 with testosterone (9 concentrations ranging from 5-375 nM). To determine the effects of FK143 on 5α-reductase activity, FK143 was included at concentrations of 0, 3, 15 and 45 nM. A single incubation time of 1h and an incubation volume of 1 ml were used in kinetic studies. To obtain the apparent Michaelis constant ($K_{m(app)}$) and apparent maximum velocity ($V_{max(app)}$), the Wilkinson method (non-linear least squares analysis of a rectangular hyperbola) was used (22). Slope and intercept replots were obtained from, respectively, $K_{m(app)}$ / $V_{max(app)}$ versus [I] and $1/V_{max(app)}$ versus [I]. Linear regression analysis of the replots was employed to determine the $K_{i(app)}$ values.

RNA Extraction

From each sample, total RNA was extracted using the RNeasy extraction kit with DNase1 treatment (Qiagen Inc., Mississauga, ON, Canada) according to the manufacturer's protocol with the exception that the on-column DNase digestion was extended to 1 hour. RNA concentration was assessed by optical density determination at 260nm (Beckman DU7 spectrophotometer, Montréal, PQ, Canada). In addition to spectrophotometric reading, RNA quality was verified by conventional gel electrophoresis. Each sample consisted of a single epididymal segment obtained from individual rats, i.e., no tissues were pooled.

Complementary DNA Arrays and Hybridization

The effects of FK143 treatment on epididymal gene expression were analyzed with cDNA arrays as described previously (12). Briefly, RNA samples were used to probe cDNA arrays (BD Biosciences Atlas Rat 1.2K array) according to the manufacturer's instructions. Four arrays per epididymal segment per treatment group (control, FK143) were probed and referred to as replicates (n=4/treatment/segment). Arrays were exposed to phosphorimager plates (Molecular Dynamics, Sunnyvale, CA) 24 hours before scanning with a phosphorimager (Storm, Molecular Dynamics). Analysis of array images with Atlas Image (Version 2.0, BD Biosciences) was done to quantify the intensity of each cDNA spot, which reflects the relative abundance of RNA in the sample. The raw data for each gene (intensity minus the background) were imported into GeneSpring 4.0.7 (Silicon Genetics, Redwood, CA) for further analysis. For each

replicate array in a given treatment group, a gene was considered detected if its intensity was above threshold, with threshold defined as two times the average background of that individual array. A gene was considered expressed if it was detected in at least three replicates in that group.

To minimize experimental variation and allow for comparison of different treatment groups, data were normalized with the standard experiment-to-experiment normalization (GeneSpring^{TM}). Specifically, the median level of expression on each array was defined as 1 and expression of each gene was normalized relative to 1; this value was averaged for all replicates in a group to generate what is referred to as the relative intensity for a given gene. A transcript was considered differentially expressed if the change in relative intensity between the control and treatment group was 2-fold or greater (i.e. 50% decrease or 100% increase). The importance of replication for validation of gene expression studies has been documented (23, 24).

Quantitative Real-Time RT-PCR

The expression of selected genes (Table 1) was analyzed by quantitative real-time RT-PCR. Analysis of the steady-state expression profiles for the selected genes along the epididymis was done using RNA samples extracted from the epididymides of untreated adult rats (n=4-5). Analysis of the effects of 5α-reductase inhibition on the epididymal expression profiles of selected genes was done using RNA samples extracted from the epididymides of vehicle, PNU157706 and FK143 treated rats (n=4-5). As expected, the steady-state

(untreated) and vehicle-treated expression profiles for all the genes examined were indistinguishable. For simplicity, only the vehicle-treated profiles are shown which are representative of the steady-state expression profiles discussed in the results section. The steady-state real-time RT-PCR expression profiles generated from untreated epididymides are available as a supplemental figure (suppl Fig. 1).

First strand complementary DNA (cDNA) was synthesized from 1 µg of total RNA using SuperScript-II reverse transcriptase (Invitrogen-Life Technologies, Carlsbad, CA) and oligo $dT_{(12\text{-}18)}$ primers (Invitrogen-Life Technologies) according to manufacturer's instructions, with the exception that only half the concentration of DTT was used (25). The reverse transcription (RT) reactions were then diluted 1:3 and 1 µl aliquots of the diluted RT samples were used for subsequent quantitative real-time PCR analysis.

Quantitative real-time PCR was carried out on the LightCycler system (Roche, Indianapolis, IN) using the Quantitect SYBR green PCR kit (Qiagen) according to the manufacturer's protocol. The gene-specific primer sequences (Table 1) were obtained from the literature or designed using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi/). Briefly, each PCR reaction contained 1 µl of cDNA (RT sample), 0.5 µM of gene-specific primers and 10 µl of the SYBR green master mix. The PCR cycling conditions were as follows: initial denaturation/enzyme activation for 15 minutes at 95°C followed by 40-60 cycles of denaturation at 95°C for 10 sec, annealing at 57–60°C for 5 sec and elongation

at 72°C for 10 sec. The production of a single PCR product was confirmed by melting curve analysis and conventional gel electrophoresis.

The relative quantification of mRNA was done according to the standard curve method (refer to Applied Biosystems User Bulletin # 2, reviewed in 26). The same RT sample was used to assay each target gene and the reference gene cyclophilin in separate (LightCycler) PCR runs. Cyclophilin was chosen as the endogenous control because of its invariant expression throughout the epididymis and its lack of response to androgen status (27). A stock of standard curve cDNA was made as follows: total RNA was extracted from the 4 epididymal segments (initial segment, caput, corpus and cauda epididymides) of an untreated adult rat and then equal amounts of RNA from each segment were mixed together to create a standard 1µg/µl RNA solution. Subsequently, several 1 µl aliquots of RNA standard were reverse-transcribed into cDNA as described previously and then pooled together to create one standard cDNA stock that was used in all PCR experiments. For each analysis, 6 serial dilutions of the standard cDNA were run as a calibration curve in parallel to the samples. The levels of mRNA for each gene (target and reference genes) were determined by comparing each sample to the appropriate standard curve. All standards and samples were assayed in duplicate. Average values for the target gene RNA concentrations determined from each RT sample preparation were normalized to the average value for cyclophilin RNA concentration determined from the same RT sample preparation.

Statistical Analysis

For the steady-state real-time PCR gene expression profiles, differences between epididymal segments were analyzed by ANOVA followed by Tukey's test. To analyze the differences in gene expression between the vehicle (control) and inhibitor treatment groups, ANOVA followed by Dunnett's test was done. The level of significance was set at $p \leq 0.05$ for all analyses.

Results

Effect of FK143 on Epididymal 5α-Reductase Activity

The inhibitory effect of FK143 on 5α-reductase was assessed *in vitro* by examining the effects of FK143 on the 5α-reductase activity of nuclear and microsomal epididymal fractions. The addition of FK143 to incubations at concentrations of 0, 3, 15 and 45 nM inhibited epididymal 5α-reductase as shown in Fig. 1A (nuclear fraction) and B (microsomal fraction). Kinetic analyses demonstrated that FK143 caused the $V_{max(app)}$ values to decrease (by about 75%) with no consistent effect on the $K_{m(app)}$ values. Confirmation of these findings was achieved with slope ($K_{m(app)}/V_{max(app)}$ vs. [I]) and intercept ($1/V_{max(app)}$ vs.[I]) replots, shown in Fig. 1C (nuclear) and D (microsomal); the slopes of the slope replots were negative or almost horizontal whereas the slopes of the intercept replots were positive. The values for the inhibition constant ($K_{i(app)}$) for FK143 determined from the slope and intercept replots respectively were 56.6 and 26.5 for the nuclear 5α-reductase fraction and 44.5 and 53.4 for the microsomal 5α-

reductase fraction. Since the $K_{i(app)}$ values from both slope and intercept replots were almost identical, it was clear that the $K_{m(app)}$ did not influence the $K_{i(app)}$ values determined; these findings were consistent with FK143 being a non-competitive inhibitor of 5α-reductase, as has been reported for other tissues (17).

Effects of FK143 on Epididymal Gene Expression

Comparison of the gene array data from the present FK143 study to our previous study using PNU157706 (12) revealed analogous effects of the different inhibitors on epididymal gene expression; both the nature of the affected genes and the direction of expression changes (i.e. increased or decreased expression) were very similar (refer to Suppl Table 1). In the current study, the expression of a greater number of genes met our detection analysis criteria (i.e. were detectable above the set background expression threshold) therefore, a greater number of genes were analyzed. Novel changes in gene expression (2-fold minimum) that were revealed in the current gene array study are reported in a supplemental table (Suppl Table 2).

The comparison of the effects of two different inhibitors added greater power to our analyses such that we could identify genes with expression changes that were less than 2-fold but were consistently affected by treatment with both inhibitors. Among these genes was 5α-reductase type 2, which is of particular relevance to the present study as it is one of the isozymes that catalyzes the formation of DHT; the averaged response of this gene to 5α-reductase inhibitor treatment (determined from the array studies) was 1.4-, 1.6- and 1.8-fold

decreased expression in the initial segment, caput and corpus epididymides, respectively. Also among these genes were several insulin-like growth factor (IGF) family members. For example, following inhibitor treatment, the expression of IGF binding protein-5 (IGFBP-5) decreased by an average of 1.1-, 1.4- and 1.5-fold in the caput, corpus and cauda epididymides, respectively, and IGFBP-6 expression increased by an average of 1.1-fold in the initial segment and decreased by an average of 1.4-fold in the corpus epididymidis. Lastly, the expression of IGF-1 and IGF-1 receptor (IGF-1R) decreased by an average of 1.4- and 1.6-fold, respectively, in the cauda epididymidis following 5α-reductase inhibitor treatment.

In accordance with the main objective of this study to identify potential signaling systems involved in epididymal regulation, we undertook to characterize the expression of the above-mentioned genes as well as other mediators of androgen action (5α-reductase type 1, androgen receptor) and other growth factor family members (chosen based on the current literature) using the more sensitive technique of quantitative real-time RT-PCR.

Expression of 5α-Reductases and the Androgen Receptor in the Epididymis

We examined the steady-state expression levels of 5α-reductase types 1 and 2 and of the androgen receptor in the epididymis using real-time RT-PCR (Fig. 2, black bars). These three genes have been previously characterized in the epididymidis by Northern blot analysis (11, 28). The gene expression results obtained using these two techniques were analogous for all three genes. 5α-

Reductase type 1 was expressed in the initial segment at levels approximately 4-fold higher than the other regions of the epididymis. 5α-Reductase type 2 was also highly expressed in the initial segment, peaked slightly in the caput epididymidis and then decreased in the corpus and cauda epididymides. Androgen receptor gene expression was relatively constant throughout the epididymis.

Dual 5α-reductase inhibitor treatment had differential effects on the expression of 5α-reductase type 1 and 2 and the androgen receptor in the epididymis (Fig. 2). Both compounds had similar effects on the different gene expression profiles. 5α-Reductase type 1 expression was uniquely affected in the initial segment where it increased maximally with FK143 treatment by approximately 2.5-fold. Androgen receptor gene expression also increased following treatment uniquely in the caput epididymidis. In contrast, the expression of 5α-reductase type 2 remained essentially unaffected by treatment except for an approximate 66% decrease in the corpus epididymidis. The corpus epididymidis was also the segment where the greatest decrease in 5α-reductase type 2 expression was detected using gene array technology.

Expression of IGF Family Members in the Epididymis

We examined the steady-state expression of four IGF family members along the epididymis (Fig. 3, black bars). The expression of IGF-1 was significantly lower in the caput epididymidis compared to the other segments where it was expressed at similar levels. The expression of IGF-1R escalated

longitudinally, increasing nearly 3-fold between the initial segment and the cauda epididymidis. The expression of IGFBP-5 was also highest in the cauda epididymidis, being on average 3-fold higher than in the other segments. The high relative expression values for IGFBP-5 suggest that it is more highly expressed in the epididymis compared to the other IGF family members. However, direct comparisons between the different genes cannot be made conclusively since different target gene primer pairs may not exhibit the same amplification efficiency in PCR experiments. Lastly, the expression profile of IGFBP-6 in the epididymis was very similar to that of IGF-1 such that it was significantly lower in the caput epididymidis compared to the remaining segments.

The effects of dual 5α-reductase inhibition on the expression profiles of the four IGF family members are shown in Figure 3. Again, treatment with the two different dual inhibitors had similar effects on the expression profiles of all the IGF family members examined, with FK143 having a more pronounced effect. Interestingly, the effects of 5α-reductase inhibitor treatment were gene- and segment-specific. The expression of all four genes remained unaffected by treatment in the initial segment. In the more distal segments of the epididymis, treatment with either inhibitor caused a general decrease in the expression of most IGF family members examined. More specifically, the expression of IGF-1 and IGFBP-5 was the most affected by treatment, maximally decreasing by 60% and 84% in the cauda epididymidis, respectively. Decreased expression was also observed for IGFBP-6, reaching significance in the corpus epididymidis.

These changes in gene expression reflect what has been found using gene array technology.

Expression of FGF Family Members in the Epididymis

The steady-state expression profiles for the ligand, fibroblast growth factor-10 (FGF-10), and the receptor, fibroblast growth factor receptor-2 (FGFR-2), were examined in the epididymis (Fig. 4, black bars). The steady-state expression of FGF-10 was significantly lower in the caput epididymidis compared to all other segment. The steady-state expression of FGFR-2 was significantly higher in the cauda epididymidis compared to the more proximal regions. The high relative expression values for FGFR-2 suggest that it is quite highly expressed in the epididymis compared to the other genes analyzed.

The effects of PNU157706 and FK143 treatment on the epididymal expression of FGF-10 and FGFR-2 expression were analyzed (Fig. 4). FGF-10 gene expression was significantly affected by both inhibitor treatments; it decreased maximally with FK143 treatment in the corpus and cauda epididymides by approximately 45% and 31%, respectively. Inhibitor treatments did not result in a significant decrease in FGFR-2 expression.

Expression of TGF Family Members in the Epididymis

The steady-state expression profiles for the ligand, transforming growth factor-β1 (TGF-β1), and the receptor, transforming growth factor-β1 receptor

(TGF-β1R), were examined in the epididymis (Fig. 5, black bars). The expression of TGF-β1 was relatively constant in the initial segment, caput and corpus epididymides and peaked in the cauda epididymidis to nearly double the levels in the proximal regions. In contrast, TGF-β1R was expressed in the in the initial segment and corpus epididymidis at approximately twice the levels in the caput and cauda epididymides. The relative expression values for TGF-β1R were the highest of all the genes examined, suggesting that it is very highly expressed in the epididymis.

The effects of dual 5α-reductase inhibitor treatment on epididymal TGF-β1 and TGF-β1R expression were analyzed (Fig. 5). The only significant effects of 5α-reductase inhibitor treatment were on the expression of TGF-β1 in the caput epididymidis, which nearly doubled following treatment, and TGF-β1R in the cauda epididymidis, which decreased following treatment.

Expression of VEGF Family Members in the Epididymis

The steady-state expression profiles for the ligand, vascular endothelial growth factor (VEGF), and the receptor, vascular endothelial growth factor receptor-2 (VEGFR-2), were examined in the epididymis (Fig. 6, black bars). Both genes were most highly expressed in the initial segment. VEGF expression was lowest in the caput epididymidis and increased slightly towards the cauda epididymidis. VEGFR-2 was expressed in the caput, corpus and cauda epididymides at approximately 63-74% of the initial segment expression level.

The effects of dual 5α-reductase inhibitor treatment on epididymal VEGF and VEGFR-2 expression were analyzed (Fig. 6). In contrast to all the other growth factors analyzed, there were no significant effects of treatment with either inhibitor in any segment of the epididymis.

Discussion

Previously we demonstrated that the dual 5α-reductase inhibitor PNU157706 inhibited epididymal 5α-reductase activity *in vitro* and had extensive effects on epididymal gene expression *in vivo* (12). In the present study, we confirmed that a second compound, FK143, is a noncompetitive inhibitor of nuclear and microsomal epididymal 5α-reductase activity *in vitro*. Furthermore, it was found that treatment with FK143 elicited changes in epididymal gene expression that were analogous to those observed with PNU157706, as evidenced by gene array studies. These corroborating findings clearly indicate that the observed changes in gene expression are due to the common effect of both compounds on the inhibition of DHT formation, as opposed to a mechanism different than 5α-reductase inhibition.

Recently, we showed that treatment of male rats with PNU157706 affects epididymal sperm maturation as evidenced by changes in epididymal sperm morphology, motility and fertilizing ability (29). It is highly likely that the effects of dual 5α-reductase inhibition on epididymal sperm maturation are linked to the effects of inhibitor treatment on the expression of genes that can be functionally categorized according to important epididymal epithelial processes (i.e.,

processes that contribute to the formation of the epididymal luminal environment). What remains unclear is the identity of segment-specific signaling/regulatory molecules and pathways involved in mediating the upstream and/or downstream effects of DHT on epididymal gene expression and function.

In the present study we focused specifically on signaling mechanisms in the epididymis and therefore chose to analyze the expression of both 5α-reductase isozymes and the androgen receptor, as well as several components of different growth factor systems. Moreover, we determined whether the expression of these genes was dependent on DHT by assessing the effects of dual 5α-reductase inhibition. For the most part, these genes have not been extensively characterized in the epididymis, with the exception of the 5α-reductase isozymes and the androgen receptor. Thus, this is the first study demonstrating the differential distribution and regulation of many of these genes.

Expression of 5α-Reductases and the Androgen Receptor in the Epididymis

The expression of both 5α-reductase isozymes has been previously characterized in the epididymis (11, 30, 31). Moreover, the segment-specific epididymal expression profiles of these genes show different patterns during development and aging and respond differently to efferent duct ligation and bilateral orchidectomy (11, 28, 30-32). However, this is the first study to employ 5α-reductase inhibitors to specifically examine the DHT regulation of the expression of these genes in the epididymis. Our results are consistent with the differential regulation of the 5α-reductase isozymes in this tissue. Type 1 5α-

reductase expression was specifically increased in the initial segment following inhibitor treatment, suggesting a negative feedback mechanism of regulation by DHT in this segment. In contrast, the expression of 5α-reductase type 2 was affected only in a limited manner by treatment in the corpus epididymidis. Interestingly, androgen status has been shown to regulate the expression of both 5α-reductase isozymes differently in different tissues. For example, in the rat brain, type 1 5α-reductase expression increases following castration, and androgen replacement with DHT reverses this increase to a larger extent than testosterone replacement (33). In contrast, 5α-reductase type 2 expression falls in the rat prefrontal cortex after castration and increases dramatically with testosterone replacement compared to DHT replacement (33). In fact, the exogenous administration of testosterone, and to a lesser extent DHT, increased type 2 5α-reductase expression even in intact animals (33). Testosterone has also been shown to control the expression of 5α-reductase type 2 in the rat prostate while both testosterone and DHT positively regulate 5α-reductase type 1 expression in the rat liver (34, 35). The differential regulation of both 5α-reductase isozymes suggests that they play physiologically distinct roles in different tissues. Further studies are needed to fully characterize these distinct roles.

As expected, the present real-time PCR expression results were analogous to what has been previously described for the androgen receptor in the epididymis; androgen receptor gene expression is relatively constant throughout the tissue with levels slightly higher in the caput epididymidis

compared to those in the cauda epididymidis (28, 36, 37). Studies have also shown that circulating androgens regulate the expression of the androgen receptor in the epididymis (36, 37); however, as with most of the genes previously examined in the epididymis, no differentiation was made between total androgens (i.e., testosterone plus DHT) and DHT alone. Our results suggest that androgen receptor expression is regulated by DHT specifically in the caput epididymidis.

Growth Factor Signaling Pathways in the Epididymis

Studies using prostate cell lines have provided evidence to support androgen receptor cross talk with growth factor signaling pathways, resulting in both enhanced androgen responses and receptor ligand-independent activation and modulation of androgen receptor transactivation (38-41). Thus, it seems plausible that similar interactions occur in other reproductive tissues, including the epididymis; however, this remains to be examined. The characterization of recently established epididymal cell-lines should facilitate further investigation into the interactions between androgen and growth factor signaling pathways in the epididymis (42, 43).

A physiological role for IGF-1 in the regulation of epididymal functions was hypothesized over ten years ago due to the varied immunohistochemical localization of IGF-1 protein in the rat epididymis (44). IGF-1R protein and mRNA have also been localized to the epididymis (45, 46). Adult mice with a homozygous null mutation of the IGF-1 gene are infertile dwarfs with reduced

testosterone production, and consequently reduced spermatogenesis and reproductive organ size (45). Interestingly, the epididymal phenotype of IGF-1 null mice is more severe in the distal regions of the epididymis with greater reductions in weight and decreased coiling. In the present study, the expression of IGF-1 and IGF-1R was found to be highest in the distal epididymal segments. These results are consistent with a role for IGF signaling in the distal regions of the epididymis (corpus and cauda epididymides). Further corroborating this conclusion are results from the current study demonstrating the unique effects of 5α-reductase inhibitor treatment on the expression of IGF-1 and possibly IGF-1R, predominantly in the corpus and cauda epididymides.

The IGF network also includes numerous IGF binding proteins that modulate the bioavailability and cellular functions of IGFs (47). We analyzed the epididymal expression of two high-affinity binding proteins, IGFBP-5 and IGFBP-6. Again, these genes were more highly expressed in the distal regions of the epididymis and were mainly affected by 5α-reductase inhibitor treatment in these regions. IGFBP-5 expression was particularly affected by treatment, decreasing dramatically in the cauda epididymidis.

Various FGF receptors have been characterized exclusively in principal cells of the initial segment of the rat epididymis (48). Our results also indicate a role for FGF signaling in the distal part of the epididymis since the highest expression levels for FGF-10 and its receptor FGFR-2 were in the corpus and cauda epididymides. Furthermore, the effects of 5α-reductase inhibitor treatment were most pronounced in these regions.

Differential expression profiles for TGF-β isoforms (TGF-β1, TGF-β2, TGF-β3) have previously been characterized in the rat epididymis by Northern blot (49, 50). In these studies, substantial TGF-β1 expression was observed compared to the other isoforms; TGF-β1 expression was upregulated in the caput, corpus and cauda epididymides following total androgen ablation by orchidectomy. In the present study, we also observed the upregulation of TGF-β1 expression most predominantly in the caput epididymidis with no changes in the distal regions. Taken together, these data suggest that TGF-β1 is uniquely regulated by different androgens in the epididymis; our results indicate that there is a DHT-dependent repression of TGF-β1 in the proximal epididymis while castration induced increases in TGF-β1 expression in the corpus and cauda epididymides, indicating a specific role for total androgens or testosterone in these regions. Roles for the TGF-β family have also been implicated in other DHT-dependent reproductive tissues such as the seminal vesicle and ventral prostate (51, 52). TGFβ-1 in particular was also shown to be repressed by androgens in the regressing rat ventral prostate (53), similar to what was observed in the epididymis, further supporting a role for androgens in regulating the expression of this gene.

TGF-β is postulated to be involved in the regulation of programmed cell death in tissues that regress following androgen ablation. Interestingly, in the present study TGF-β1 is induced despite the absence of tissue regression, thus indicating a novel role for TGF-β1 in the epididymis that is separate from its role in apoptosis. To the best of our knowledge, this is the first study demonstrating the differential distribution of any TGF-β receptor mRNA in the epididymis. The

presence of both ligand and receptor strongly supports a role for the TGF-β system in the epididymis; however, DHT only appears to regulate this system at the level of the ligand.

Vascular endothelial growth factor (VEGF) protein was shown to be localized in the rat epididymal epithelium in a region- and cell-specific pattern (54); for example clear cells in the caput, corpus, and cauda epididymides were immunoreactive for VEGF. In humans, VEGF protein has been localized to peritubular and basal cells of the epididymal duct and VEGFR-2 protein has been localized to vascular endothelial cells of the epididymis (55). Interestingly, the overexpression of VEGF in the testis and epididymis of transgenic mice causes infertility (19). These findings suggest a role for VEGF in the epididymis, likely as a paracrine regulator of the epididymal vasculature as opposed to the epididymal epithelium. In the present study, we confirmed the differential distribution of both the VEGF and VEGFR-2 mRNAs in the rat epididymis. Interestingly, unlike the other growth factor families examined, 5α-reductase inhibitor treatment had no significant effect on the expression of VEGF or VEGFR-2, reflecting a lack of DHT regulation of this growth factor system in the epididymis. This may be due to the predominantly vascular as opposed to epithelial localization of this system in the epididymis, which is unique from other growth factor signaling systems.

It is of interest to note that the testis has been shown to produce and secrete many different growth factors, including components of the IGF, TGF, EGF, FGF, PDGF, and NGF systems (reviewed in 56). It has been well established that the epididymis, in particular the initial segment, is dependent on

the testicular input of factors, other than androgens, directly to the epididymal lumen (57, 58); depriving the epididymis of these testicular factors (i.e. via efferent duct ligation) has well-documented effects on gene expression in the initial segment (reviewed in 59). This type of paracrine regulatory mechanism has been coined "lumicrine" regulation since it occurs via a ductal system (59). Thus, it is plausible that growth factors originating in the testis can act as "lumicrine" regulators of the epididymis by acting on their corresponding receptors present in the epididymal epithelium. Similarly, it is also likely that growth factors secreted in one segment of the epididymis can act on the receptors located in the same or more distal regions of the epididymis representing autocrine or paracrine regulatory mechanisms.

In summary, the collective findings of the present study suggest that sex steroid and growth factor signaling become dysregulated following 5α-reductase inhibition, leading to altered epididymal gene expression and function. Furthermore, the results of this study support the involvement of differential signaling mechanisms in regulating and/or mediating the actions of androgens (predominantly DHT) in the different segments of the epididymis. This regulatory diversity is likely to be important in controlling segment-specific sperm-related functions.

Acknowledgements

We thank Dr. T. Zaccheo from Pharmacia and Upjohn (presently Pfizer) and Dr. Yasutaka Shigi from Fujisawa Pharmaceutical Co. for the generous gifts

of PNU157706 and FK143 respectively. Melanie Deslauriers is gratefully acknowledged for her assistance in developing the real-time PCR assays for the 5α-reductase isozymes. These studies were supported by a grant from the Canadian Institutes of Health Research. NAH is a recipient of a Canadian Institutes of Health Research Studentship.

References

1. **Robaire B, Hinton BT** 2002 The Epididymis From Molecules to Clinical Practice. New York; Kluwer Academic/Plenum Publishers.
2. **Orgebin-Crist MC** 1967 Sperm Maturation in Rabbit Epididymis. Nature 216 :816-818
3. **Cornwall GA, Lareyre JJ, Matusik RJ, Hinton BT, Orgebin-Crist MC** 2002 Gene expression and epididymal function. In: Robaire B, Hinton BT, eds. The epididymis: From molecules to clinical practice. New York: Kluwer Academic/Plenum Publishers; 169-199
4. **Jervis KM, Robaire B** 2001 Dynamic changes in gene expression along the rat epididymis. Biol Reprod 65:696-703
5. **Blaquier JA, Burgos MH, Cameo MS** 1972 Role of androgens in maturation of epididymal spermatozoa in guinea-pig. Endocrinology 90:839-842
6. **Orgebin-Crist MC, Tichenor PL** 1973 Effect of testosterone on sperm maturation in-vitro. Nature 245:328-329
7. **Gloyna RE, Wilson JD** 1969 A comparitive study of the conversion of testosterone to 17β-hydroxy-5α-androstan-3-one (dihydrotestosterone) by rat prostate and epididymis. J Clin Endocrinol Metab 29:970-977
8. **Tindall DJ, Nayfeh SN, French FS** 1972 Androgen uptake and binding in rat epididymal nuclei, in-vivo. Biochem Biophys Res Commun 49:1391-1397
9. **Orgebin-Crist MC, Jahad N, Hoffman LH** 1976 Effects of testosterone, 5-alpha-dihydrotestosterone, 3-alpha-androstanediol, and 3-beta-androstanediol

on maturation of rabbit epididymal spermatozoa in organ-culture. Cell Tissue Res 167:515-525

10. **Turner TT, Jones CE, Howards SS, Ewing LL, Zegeye B, Gunsalus GL** 1984 On the androgen microenvironment of maturing spermatozoa. Endocrinology 115:1925-1932
11. **Viger RS, Robaire B** 1996 The mRNAs for the steroid 5 alpha-reductase isozymes, types 1 and 2, are differentially regulated in the rat epididymis. J Androl 17:27-34
12. **Henderson NA, Cooke GM, Robaire B** 2004 Effects of PNU157706, a dual 5alpha-reductase inhibitor, on gene expression in the rat epididymis. J Endocrinol 181:245-261
13. **Jervis KM, Robaire B** 2002 Changes in gene expression during aging in the Brown Norway rat epididymis. Exp Gerontol 37:897-906
14. **Jervis KM, Robaire B** 2003 Effects of caloric restriction on gene expression along the epididymis of the Brown Norway rat during aging. Exp Gerontol 38:549-60
15. **Ezer N, Robaire B** 2003 Gene expression is differentially regulated in the epididymis after orchidectomy. Endocrinology 144:977-988
16. **Jervis KM, Robaire B** 2004 The effects of long-term vitamin E treatment on gene expression and oxidative stress damage in the aging Brown Norway rat epididymis. Biol Reprod 71:1088-1095
17. **Hirosumi J, Nakayama O, Fagan T, Sawada K, Chida N, Inami M, Takahashi S, Kojo H, Notsu Y, Okuhara M** 1995 FK143, a novel nonsteroidal

inhibitor of steroid 5 alpha-reductase: (1) In vitro effects on human and animal prostatic enzymes. J Steroid Biochem Mol Biol 52:357-363

18. **Hirosumi J, Nakayama O, Chida N, Inami M, Fagan T, Sawada K, Shigematsu S, Kojo H, Notsu Y, Okuhara M** 1995 FK143, a novel nonsteroidal inhibitor of steroid 5 alpha-reductase: (2) In vivo effects on rat and dog prostates. J Steroid Biochem Mol Biol 52:365-373
19. **Korpelainen EI, Karkkainen MJ, Tenhunen A, Lakso M, Rauvala H, Vierula M, Parvinen M, Alitalo K** 1998 Overexpression of VEGF in testis and epididymis causes infertility in transgenic mice: evidence for nonendothelial targets for VEGF. J Cell Biol 143:1705-1712
20. **Vickers MH, Casey PJ, Champion ZJ, Gravance CG, Breier BH** 1999 IGF-I treatment increases motility and improves morphology of immature spermatozoa in the GH-deficient dwarf (dw/dw) rat. Growth Horm IGF Res 9:236-240
21. **di Salle E, Giudici D, Radice A, Zaccheo T, Ornati G, Nesi M, Panzeri A, Delos S, Martin PM** 1998 PNU 157706, a novel dual type I and II 5 alpha-reductase inhibitor. J Steroid Biochem Mol Biol 64:179-186
22. **Wilkinson GN** 1961 Statistical estimations in enzyme kinetics. Biochem. J. 80: 324-332
23. **Lee MLT, Kuo FC, Whitmore GA, Sklar J** 2000 Importance of replication in microarray gene expression studies: Statistical methods and evidence from repetitive cDNA hybridizations. Proc Natl Acad Sci U S A 97:9834-9839

24. **Herwig R, Aanstad P, Clark M, Lehrach H** 2001 Statistical evaluation of differential expression on cDNA nylon arrays with replicated experiments. Nucleic Acids Res 29:e117

25. **Lekanne Deprez RH, Fijnvandraat AC, Ruijter JM, Moorman AF** 2002 Sensitivity and accuracy of quantitative real-time polymerase chain reaction using SYBR green I depends on cDNA synthesis conditions. Anal Biochem 307:63-69

26. **Bustin SA** 2002 Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. J Mol Endocrinol 29:23-39

27. **Palladino MA, Hinton BT** 1994 Expression of multiple gamma-glutamyl transpeptidase messenger ribonucleic acid transcripts in the adult rat epididymis is differentially regulated by androgens and testicular factors in a region-specific manner. Endocrinology 135:1146-1156

28. **Viger RS, Robaire B** 1995 Gene expression in the aging brown Norway rat epididymis. J Androl 16:108-117

29. **Henderson NA, Robaire B** 2005 Effects of PNU157706, a dual 5alpha-reductase inhibitor, on rat epididymal sperm maturation and fertility. Biol Reprod 72:436-443

30. **Viger RS, Robaire B** 1991 Differential regulation of steady-state 4-ene steroid 5 alpha-reductase messenger ribonucleic acid levels along the rat epididymis. Endocrinology 128:2407-2414

31. **Viger RS, 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

32. **Viger RS, Robaire B** 1992 Expression of 4-ene steroid 5 alpha-reductase messenger ribonucleic acid in the rat epididymis during postnatal development.
Endocrinology 131:1534-1540

33. **Torres JM, Ortega E** 2003 Differential regulation of steroid 5alpha-reductase isozymes expression by androgens in the adult rat brain. FASEB J 17:1428-1433

34. **Torres JM, Gomez-Capilla JA, Ruiz E, Ortega E** 2003 Semiquantitative RT-PCR method coupled to capillary electrophoresis to study 5alpha-reductase mRNA isozymes in rat ventral prostate in different androgen status. Mol Cell Biochem 250:125-130

35. **Torres JM, Ortega E** 2003 Precise quantitation of 5alpha-reductase type 1 mRNA by RT-PCR in rat liver and its positive regulation by testosterone and dihydrotestosterone. Biochem Biophys Res Commun 308:469-473

36. **Pujol A, Bayard F** 1979 Androgen receptors in the rat epididymis and their hormonal control. J Reprod Fertil 56:217-222

37. **Tezon JG, Blaquier JA** 1983 Androgens control androgen-binding sites in rat epididymis. Endocrinology 113:1025-1030

38. **Culig Z, Hobisch A, Cronauer MV, Radmayr C, Trapman J, Hittmair A, Bartsch G, Klocker H** 1994 Androgen receptor activation in prostatic tumor

cell lines by insulin-like growth factor-I, keratinocyte growth factor, and epidermal growth factor.

Cancer Res 54:5474-5478

39. **Culig Z, Hobisch A, Cronauer MV, Hittmair A, Radmayr C, Bartsch G, Klocker H** 1995 Activation of the androgen receptor by polypeptide growth factors and cellular regulators. World J Urol 13:285-289
40. **Orio F Jr, Terouanne B, Georget V, Lumbroso S, Avances C, Siatka C, Sultan C** 2002 Potential action of IGF-1 and EGF on androgen receptor nuclear transfer and transactivation in normal and cancer human prostate cell lines. Mol Cell Endocrinol 198:105-114
41. **Huang D, Liu X, Plymate SR, Idowu M, Grimes M, Best AM, McKinney JL, Ware JL** 2004 Proteomic identification of 14-3-3 sigma as a common component of the androgen receptor and the epidermal growth factor receptor signaling pathways of the human prostate epithelial cell line M12. Oncogene. 23:6881-6889
42. **Kirchhoff C, Araki Y, Huhtaniemi I, Matusik RJ, Osterhoff C, Poutanen M, Samalecos A, Sipila P, Suzuki K, Orgebin-Crist MC** 2004 Immortalization by large T-antigen of the adult epididymal duct epithelium. Mol Cell Endocrinol 216:83-94
43. **Tabuchi Y, Toyama Y, Toshimori K, Komiyama M, Mori C, Kondo T** 2005 Functional characterization of a conditionally immortalized mouse epididymis caput epithelial cell line MEPC5 using temperature-sensitive simian virus 40 large T-antigen. Biochem Biophys Res Commun 329:812-823

44. **Leheup BP, Grignon G** 1993 Immunohistochemical localization of insulin-like growth factor I (IGF-I) in the rat epididymis. J Androl 14:159-163

45. **Baker J, Hardy MP, Zhou J, Bondy C, Lupu F, Bellve AR, Efstratiadis A** 1996 Effects of an Igf1 gene null mutation on mouse reproduction. Mol Endocrinol 10:903-918

46. **Antich M, Fabian E, Sarquella J, Bassas L** 1995 Effect of testicular damage induced by cryptorchidism on insulin-like growth factor I receptors in rat Sertoli cells.

    J Reprod Fertil 104:267-275

47. **Jones JI, Clemmons DR** 1995 Insulin-like growth factors and their binding proteins: biological actions. Endocrine Rev 16:3-34

48. **Kirby JL, Yang L, Labus JC, Hinton BT** 2003 Characterization of fibroblast growth factor receptors expressed in principal cells in the initial segment of the rat epididymis. Biol Reprod 68:2314-2321

49. **Desai KV, Flanders KC, Kondaiah P** 1998 Expression of transforming growth factor-beta isoforms in the rat male accessory sex organs and epididymis. Cell Tissue Res 294:271-277

50. **Desai KV, Kondaiah P** 2000 Androgen ablation results in differential regulation of transforming growth factor-beta isoforms in rat male accessory sex organs and epididymis. J Mol Endocrinol 24:253-260

51. **Tanji N, Tsuji M, Terada N, Takeuchi M, Cunha GR** 1994 Inhibitory effects of transforming growth factor-beta 1 on androgen-induced development of neonatal mouse seminal vesicles in vitro. Endocrinology 134:1155-1162

52. **Timme TL, Truong LD, Merz VW, Krebs T, Kadmon D, Flanders KC, Park SH, Thompson TC** 1994 Mesenchymal-epithelial interactions and transforming growth factor-beta expression during mouse prostate morphogenesis. Endocrinology 134:1039-1045

53. **Kyprianou N, Isaacs JT** 1989 Expression of transforming growth factor-beta in the rat ventral prostate during castration-induced programmed cell death. Mol Endocrinol 3:1515-1522

54. **Zhang QY, Qiu SD, Ge L** 2004 Studies on expression and location of VEGF protein in rat testis and epididymis. Shi Yan Sheng Wu Xue Bao 37:1-8

55. **Ergun S, Luttmer W, Fiedler W, Holstein AF** 1998 Functional expression and localization of vascular endothelial growth factor and its receptors in the human epididymis. Biol Reprod 58:160-168

56. **Gnessi L, Fabbri A, Spera** 1997 Gonadal peptides as mediators of development and functional control of the testis: an integrated system with hormones and local environment. Endocr Rev 18:541-609

57. **Nicander L, Osman DI, Ploen L, Bugge HP, Kvisgaard KN** 1983 Early effects of efferent ductile ligation on the proximal segment of the rat epididymis. Int J Androl 6: 91-102

58. **Turner TT, Riley TA** 1999 p53 independent, region-specific epithelial apoptosis is induced in the rat epididymis by deprivation of luminal factors. Mol Reprod Dev 53:188-197

59. **Hinton BT, Lan ZJ, Rudolph DB, Labus JC, Lye RJ** 1998 Testicular regulation of epididymal gene expression. J Reprod Fertil suppl 53:47-57

60. **Turner KJ, McIntyre BS, Phillips SL, Barlow NJ, Bowman CJ, Foster PM** 2003 Altered gene expression during rat Wolffian duct development in response to in utero exposure to the antiandrogen linuron. Toxicol Sci 74:114-128

61. **Hanawa H, Abe S, Hayashi M, Yoshida T, Yoshida K, Shiono T, Fuse K, Ito M, Tachikawa H, Kashimura T, Okura Y, Kato K, Kodama M, Maruyama S, Yamamoto T, Aizawa Y** 2002 Time course of gene expression in rat experimental autoimmune myocarditis. Clin Sci (Lond) 103:623-632

62. **Gomez R, Simon C, Remohi J, Pellicer A** 2003 Administration of moderate and high doses of gonadotropins to female rats increases ovarian vascular endothelial growth factor (VEGF) and VEGF receptor-2 expression that is associated to vascular hyperpermeability. Biol Reprod 68:2164-2171

63. **Cupp AS, Kim G, Skinner MK** 1999 Expression and action of transforming growth factor beta (TGFbeta1, TGFbeta2, and TGFbeta3) during embryonic rat testis development. Biol Reprod 60:1304-1313

Figures and Legends

Figure 1. Inhibition of rat nuclear (A) and microsomal (B) 5α-reductase activity *in vitro* by different concentrations of FK143 (FK). For nuclear (C) and microsomal (D) 5α-reductase, slope replots (squares) were obtained from $K_{m(app)}/V_{max(app)}$ vs [FK] and intercept replots (triangles) were obtained from $1/V_{max(app)}$ vs [FK].

Figure 1

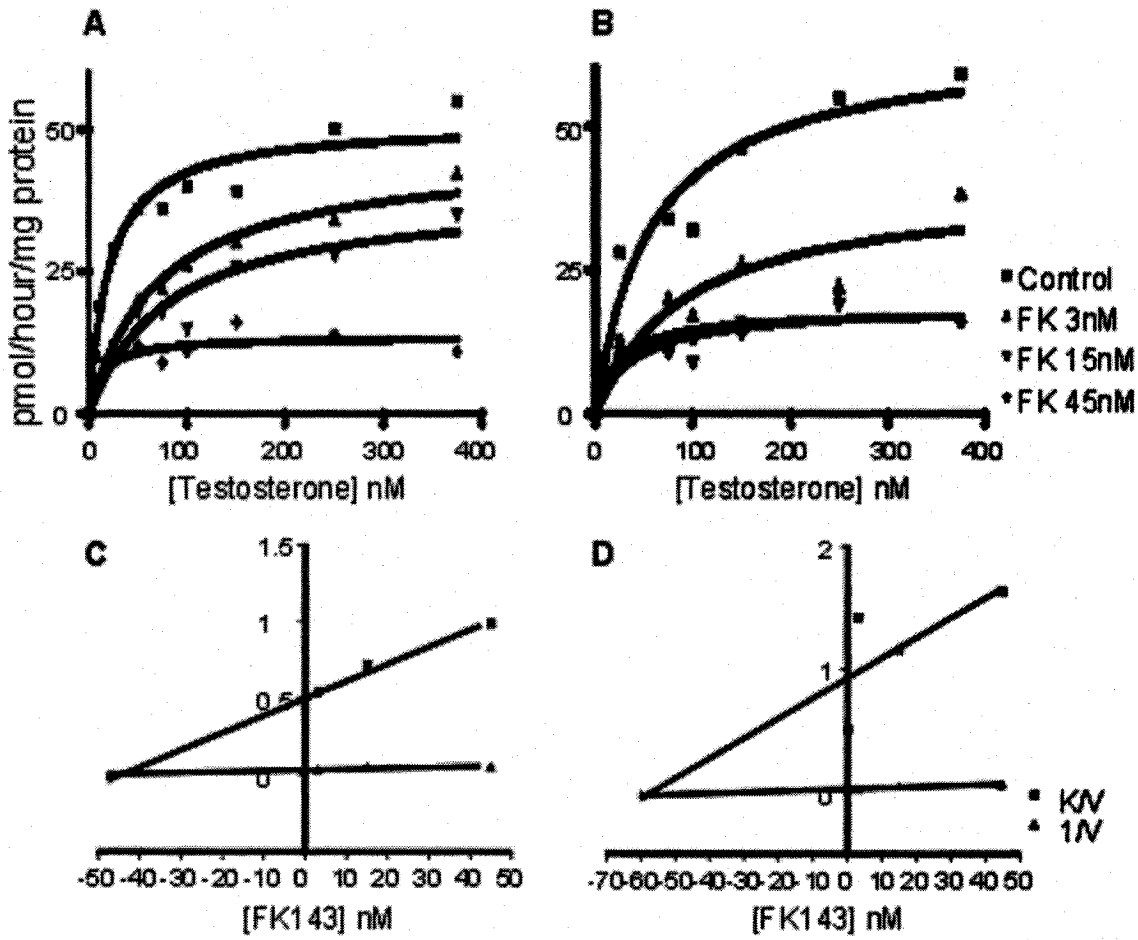

A
B
pmol/hour/mg protein
50
25
0
0
100
200
300
400
[Testosterone] nM
Control
FK 3nM
FK 15nM
FK 45nM
C
D
1.5
1
0.5
2
-50 -40 -30 -20 -10 0 10 20 30 40 50
-70 -60 -50 -40 -30 -20 -10 0 10 20 30 40 50
[FK143] nM
KV
1V

Figure 2. Quantitative real-time RT-PCR analysis of 5α-reductase type 1 (5aR-1), 5α-reductase type 2 (5aR-2) and androgen receptor (AR) mRNAs in the initial segment (IS), caput (CA), corpus (CO) and cauda (CD) epididymides. All gene expression values are expressed relative to the levels of cyclophilin mRNA and are represented as means +/- SEM. RNA extracted from epididymides of vehicle (black bars), PNU15776 (striped bars) and FK143 (white bars) treated rats (n=5/group) was used to analyze the effect of 5α-reductase inhibitor treatment on mRNA expression profiles. Asterisks (*) indicate significant differences between the vehicle-treated and inhibitor-treated groups ($p<0.05$).

Figure 2

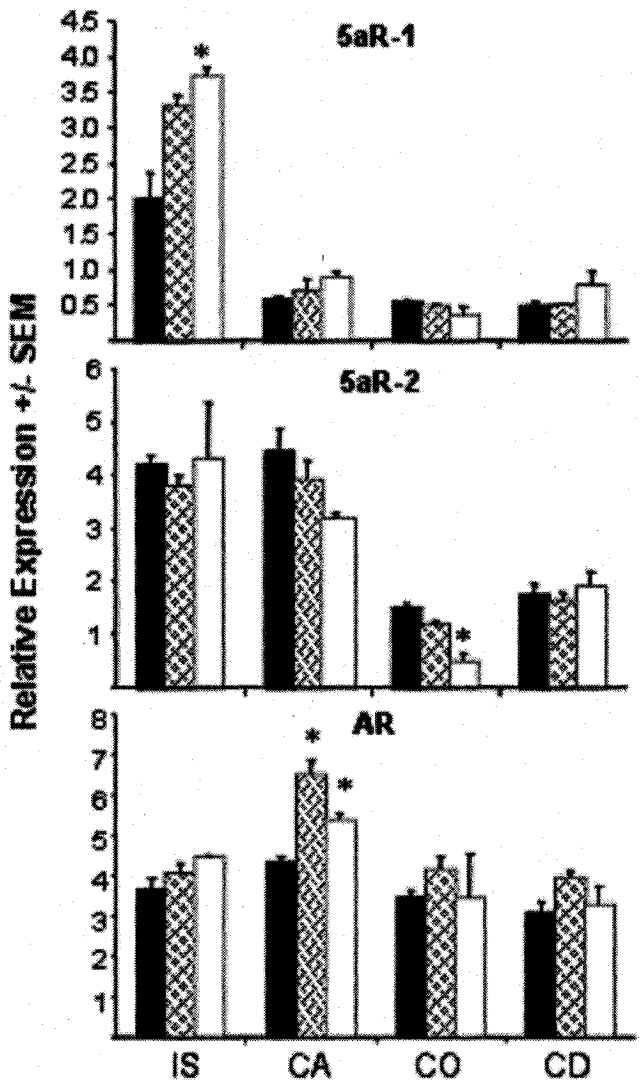


Figure 3. Quantitative real-time RT-PCR analysis of insulin-like growth factor-I (IGF-1), insulin-like growth factor-I receptor (IGF-1R), insulin-like growth factor-binding protein-5 (IGFBP-5) and insulin-like growth factor-binding protein-6 (IGFBP-6) mRNAs in the initial segment (IS), caput (CA), corpus (CO) and cauda (CD) epididymides. All gene expression values are expressed relative to the levels of cyclophilin mRNA and are represented as means +/- SEM. RNA extracted from epididymides of vehicle (black bars), PNU15776 (striped bars) and FK143 (white bars) treated rats (n=5/group) was used to analyze the effect of 5α-reductase inhibitor treatment on mRNA expression profiles. Asterisks (*) indicate significant differences between the vehicle-treated and inhibitor-treated groups ($p<0.05$).

Figure 3

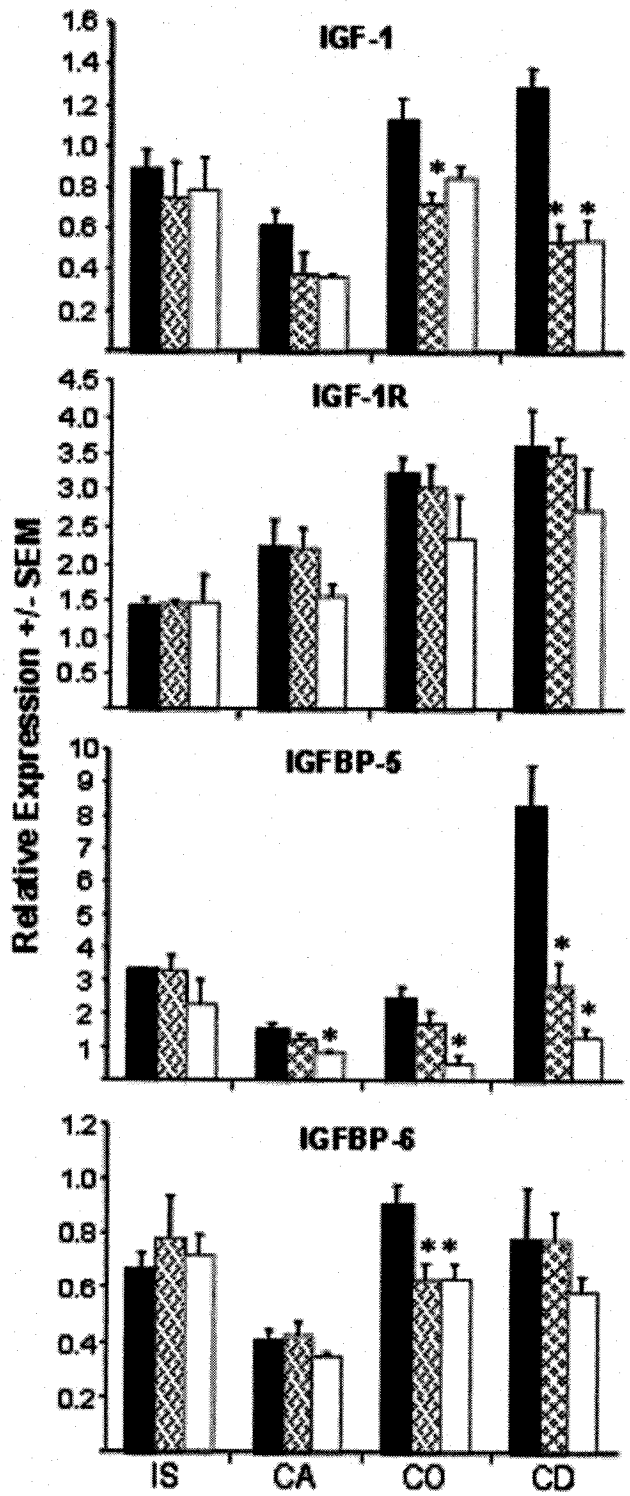

IGF-1
IGF-1R
IGFBP-5
IGFBP-6
Relative Expression +/- SEM
IS
CA
CO
CD

Figure 4. Quantitative real-time RT-PCR analysis of fibroblast growth factor-10 (FGF-10) and fibroblast growth factor receptor-2 (FGFR-2) mRNAs in the initial segment (IS), caput (CA), corpus (CO) and cauda (CD) epididymides. All gene expression values are expressed relative to the levels of cyclophilin mRNA and are represented as means +/- SEM. RNA extracted from epididymides of vehicle (black bars), PNU15776 (striped bars) and FK143 (white bars) treated rats (n=5/group) was used to analyze the effect of 5α-reductase inhibitor treatment on mRNA expression profiles. Asterisks (*) indicate significant differences between the vehicle-treated and inhibitor-treated groups ($p<0.05$).

Figure 4

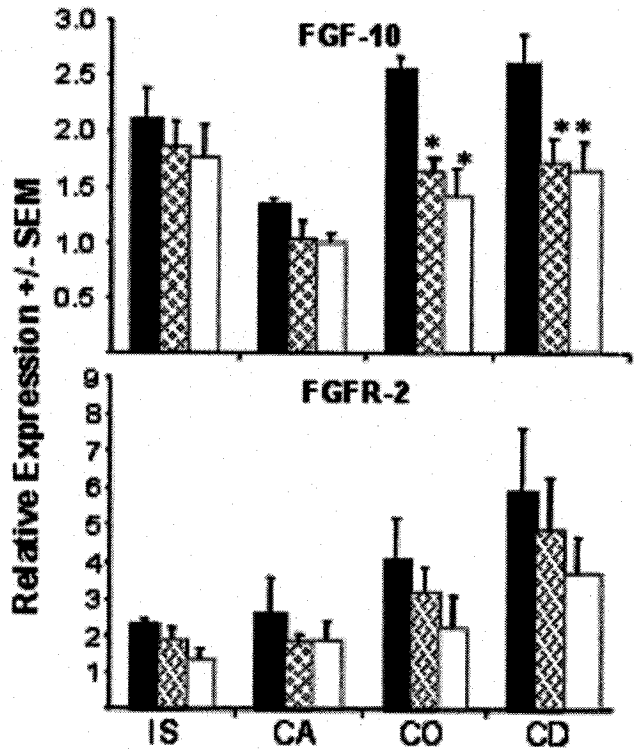

FGF-10
FGFR-2
Relative Expression +/- SEM
3.0
2.5
2.0
1.5
1.0
0.5
9
8
7
6
5
4
3
2
1
IS
CA
CO
CD

Figure 5. Quantitative real-time RT-PCR analysis of transforming growth factor-β1 (TGF-b1) and transforming growth factor-β1 receptor (TGF-b1R) mRNAs in the initial segment (IS), caput (CA), corpus (CO) and cauda (CD) epididymides. All gene expression values are expressed relative to the levels of cyclophilin mRNA and are represented as means +/- SEM. RNA extracted from epididymides of vehicle (black bars), PNU15776 (striped bars) and FK143 (white bars) treated rats (n=5/group) was used to analyze the effect of 5α-reductase inhibitor treatment on mRNA expression profiles. Asterisks (*) indicate significant differences between the vehicle-treated and inhibitor-treated groups ($p<0.05$).

Figure 5

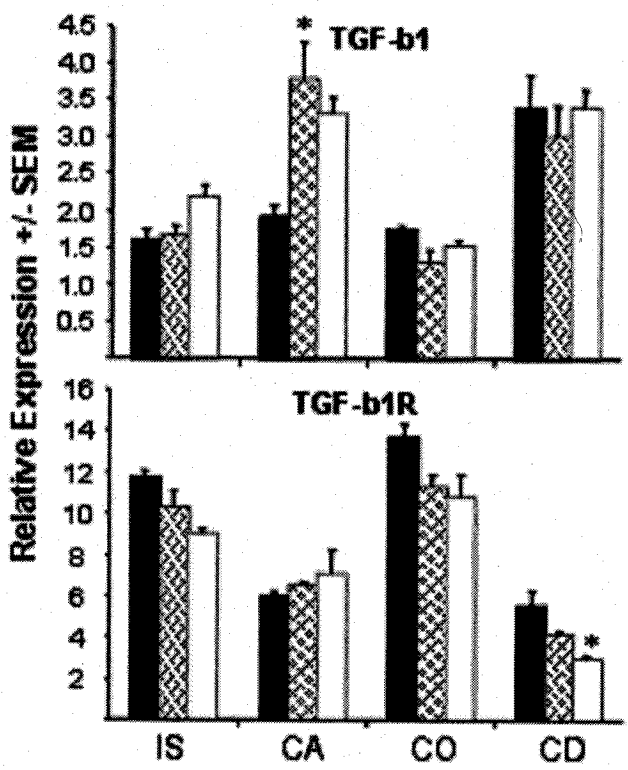
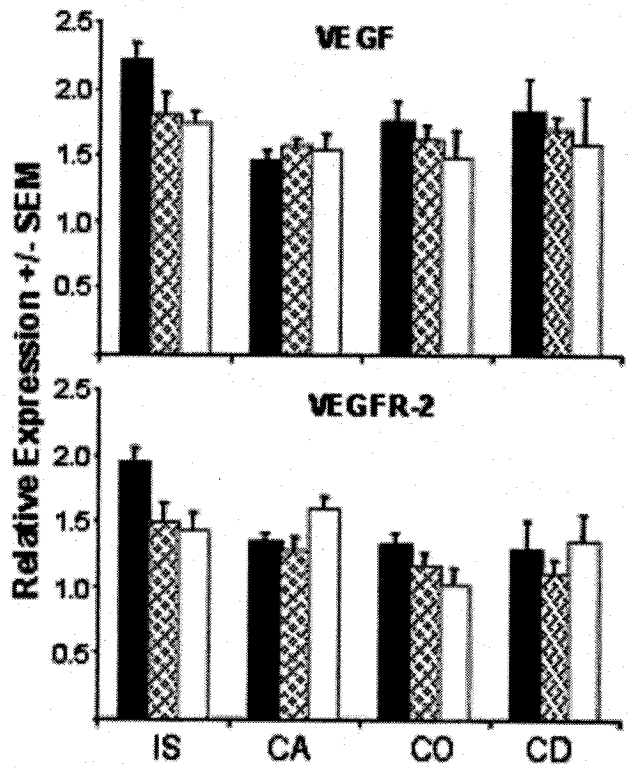

TGF-b1
TGF-b1R
Relative Expression +/- SEM
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
16
14
12
10
8
6
4
2
*
*
IS
CA
CO
CD

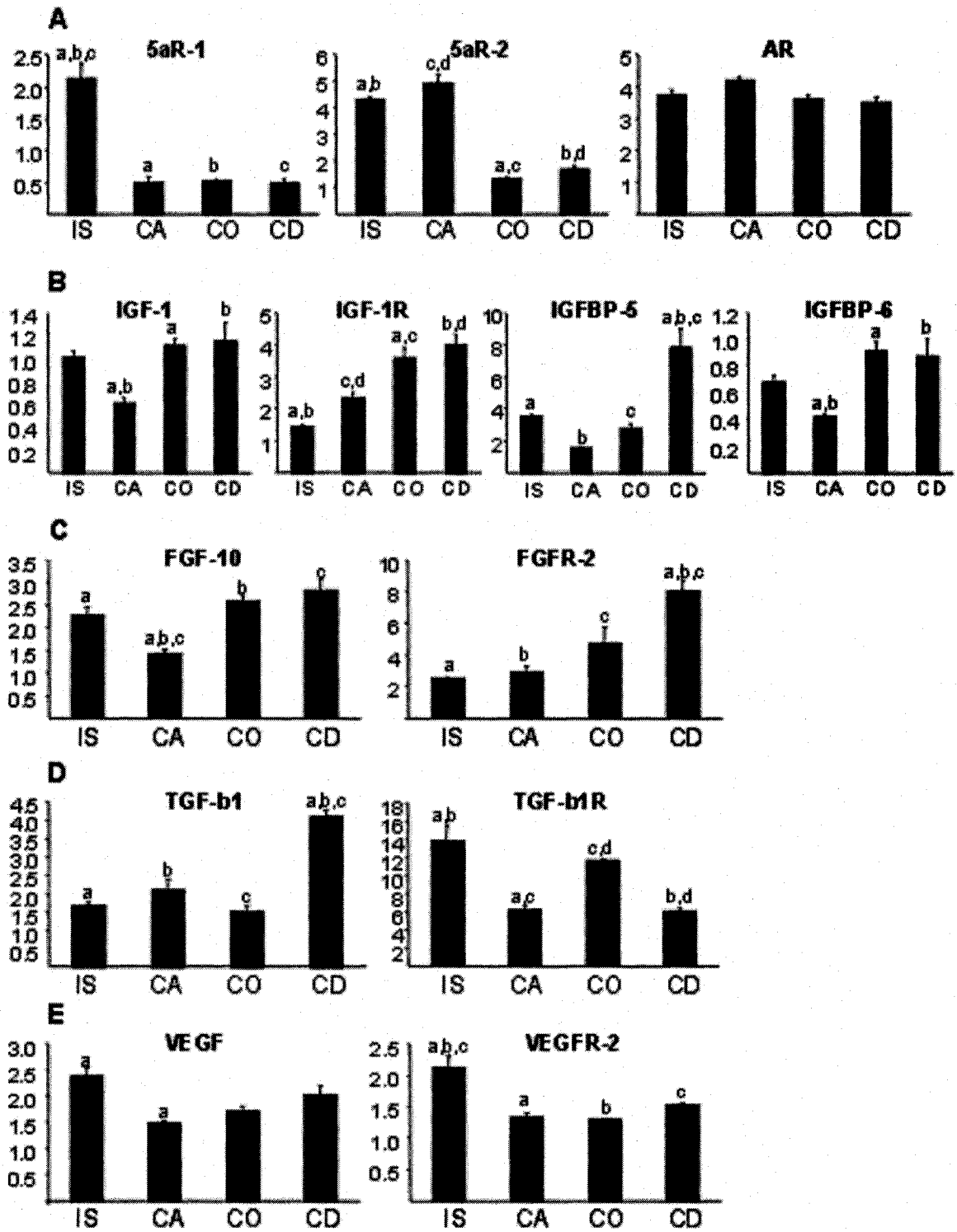
Figure 6. Quantitative real-time RT-PCR analysis of vascular endothelial growth factor (VEGF) and vascular endothelial growth factor receptor-2 (VEGFR-2) mRNAs in the initial segment (IS), caput (CA), corpus (CO) and cauda (CD) epididymides. All gene expression values are expressed relative to the levels of cyclophilin mRNA and are represented as means +/- SEM. RNA extracted from epididymides of vehicle (black bars), PNU15776 (striped bars) and FK143 (white bars) treated rats (n=5/group) was used to analyze the effect of 5α-reductase inhibitor treatment on mRNA expression profiles. Asterisks (*) indicate significant differences between the vehicle-treated and inhibitor-treated groups ($p<0.05$).

Figure 6



Supplemental Figure 1. Quantitative real-time RT-PCR analysis of steady-state gene expression in the initial segment (IS), caput (CA), corpus (CO) and cauda (CD) epididymides. A. 5α-Reductase type 1 (5aR-1), 5α-reductase type 2 (5aR-2) and androgen receptor (AR) expression profiles. B. Insulin-like growth factor-I (IGF-1), insulin-like growth factor-I receptor (IGF-1R), insulin-like growth factor-binding protein-5 (IGFBP-5) and insulin-like growth factor-binding protein-6 (IGFBP-6) expression profiles. C. Fibroblast growth factor-10 (FGF-10) and fibroblast growth factor receptor-2 (FGFR-2) expression profiles. D.Transforming growth factor-β1 (TGF-b1) and transforming growth factor-β1 receptor (TGF-b1R) expression profiles. E. Vascular endothelial growth factor (VEGF) and vascular endothelial growth factor receptor-2 (VEGFR-2) expression profiles. Paired superscripts (a, b, c, d) indicate significantly different values ($p<0.05$).

Supplemental Figure 1



Tables and Legends

Table 1. Details of primers used in quantitative real-time RT-PCR experiments.

NA: Not Applicable.

Accession numbers are from the Genbank database.

Gene	Accession No.	Forward primer	Reverse primer	Reference
5αR- 1	NM_017070	CGTCCTGCTGGCTATGTTTC	GAAGGCCAAGACAAAGGTGA	NA
5αR- 2	NM_022711	GGACCCTGATCCTGTGCTTA	ACACCACAAAGGAAGGCAAC	NA
AR	NM_012502	CTAGCGCGTGCCTTCCTTTACA	CCCACCTGCGGGAAGCT	60
IGF-1	NM_178866	CACAGGCTATGGCTCCAGCAT	TCTCCAGCCTCCTCAGATCACA	60
IGF-1R	NM_052807	CGCTGTGTGGACCGGGATTT	GCATGCACTCGCCATCGTG	60
IGFBP-5	NM_012817	GTGACCGCAAAGGATTCTACAAGA	GGCAGCTTCATCCCATACTTGTC	60
IGFBP-6	NM_013104	ACCTCGGCTGCCCCTATAAGG	TCAGTCTGGAGCTGCTGCAGTAC	60
FGF-10	NM_012951	GCTGCTGTTGCTGCTTCTTGTTG	GCTGCTGTTGCTGCTTCTTGTTG	60
FGFR-2	XM_341940	TTCCCCAGATTACCTGGAGATAGC	TCGTGGTCTTCATTCGGCAA	60
TGF-β1	NM_021578	GTTCTTCAATACGTCAGACATTCG	CATTATCTTTGCTGTCACAAGAGC	61
TGF-β1R	NM_012775	AATGTTACGCCATGAAAATATCCT	TACAACAGGTTCCATTTTTCTTCA	61
VEGF	NM_031836	CAGCTATTGCCGTCCAATTGA	CCAGGGCTTCATCATTGCA	62
VEGFR-2	NM_013062	TCAGAGACACTGAGCATGGAA	GTTTTCAGCTCTTCTGAGGCAA	62
Cyclophilin	NM_008907	ACACGCCATAATGGCACTGG	ATTTGCCATGGACAAGATGCC	63

Supplemental Table 1. Previously reported genes that showed a minimum 2-fold change in expression uniquely in one epididymal segment following PNU157706 treatment (reported in 12).

[1] The fold-changes expression values following PNU157706 treatment (PNU) were obtained from a previous study (12) and are included for comparison purposes.

[2] The fold-change expression values following FK143 (FK) treatment were obtained in the present study.

Accession numbers (Acc. Numbers) are for the Genbank database.

	Fold-change in expression		Acc.
	PNU[1]	FK[2]	Number
Initial Segment			
neurofibromatosis 2 (NF-2)	↓3.1	↓3.2	U61772
retinoblastoma protein (RBpp105)	↓2.1	↓3.2	D25233
ras-GTPase-activating protein (p120GAP)	↓2.0	↓3.6	L13151
Crk-associated substrate (Cas)	↑2.5	-	D29766
fatty acid amide hydrolase	↑3.8	↑2.5	U72497
corticosteroid 11β-dehydrogenase 1	↑4.0	↑4.9	J05107
very long chain acyl-CoA dehydrogenase	↑3.0	↑4.0	D30647
5-lipoxygenase	↑2.7	-	J03960
atrial natriuretic peptide clearance receptor	↓3.4	↓3.5	L27339
chloride channel protein 7	↑3.2	↑4.1	Z67744
Caput epididymidis			
cAMP-dependent protein kinase type II-beta regulatory chain	↓3.6	↓4.8	M12492
Ehk 3	↑7.5	-	U21954
neural thrombospondin 1-like protein (NELL1)	↑3.7	↑5.0	U48246
cathepsin B	↓3.7	↓5.4	X82396
short chain acyl-CoA dehydrogenase	↑4.2	↑3.5	J05030
Corpus epididymidis			
carbonic anhydrase 4	↓4.5	↓5.3	S68245
ras-related protein RAB13	↓2.3	↓5.7	M83678
ephrin B1	↓3.4	↓4.3	U07560
Janus tyrosine-protein kinase 1 (JAK1)	↓2.0	↓1.0	AJ000556
Cauda epididymidis			
RAB-related GTP-binding protein	↓2.8	↓1.1	M94043

muscle/brain cAMP-dependent protein kinase inhibitor	↓2.8	↑1.2	L02615
phosphatidylinositol 4-kinase	↓2.5	↓3.4	D83538
14-3-3 protein gamma subtype	↓3.8	↓3.5	S55305
ras-related protein RAB4B	↓2.4	-	X78605
phospholipase C gamma 2	↓3.3	↓4.1	J05155
phospholipase C delta 1	↓3.2	↓4.3	M20637
phospholipase C gamma 1	↓2.9	↓4.3	J03806
ATPase, hydrogen-potassium, alpha 2a subunit	↓5.0	↓3.9	M90398
sodium/potassium-transporting ATPase alpha 2 subunit	↓2.6	↓1.1	M14512
Band 3	↓7.3	↓5.0	J05167

Supplemental Table 2. Genes that showed a minimum 2-fold change in expression uniquely in one epididymal segment following FK143 treatment.

[1]The fold-change expression values following FK143 (FK) treatment were obtained in the present study.

[2]The fold-changes expression values following PNU157706 treatment (PNU) were obtained from a previous study (12) and are included for comparison purposes.

Accession numbers (Acc. Numbers) are for the Genbank database. Functional classifications are according to BD Bioscience (Clontech).

	Fold-change in expression		Acc.	Functional
	FK[1]	PNU[2]	Number	Classification
Initial Segment				
Decreased expression				
glucose-regulated 78-kDa protein	2.7	1.4	M14050	Chaperones & Heat Shock Proteins
Increased expression				
Prohibitin	2.9	1.5	M61219	Intracellular Adaptors & Receptor-Associated Proteins
neural visinin-like Ca^{2+}-binding protein NVP-3	2.8	1.1	D13126	Calcium-Binding Proteins
adenylyl cyclase type VIII			L26986	Adenylate/Guanylate Cyclases & Diesterases
Cytoplasmic β-actin	2.3	1.1	V01217	Cytoskeleton & Motility Proteins
Vasopressin V2 receptor	2.3	1.2	Z11932	Hormone/G Protein-Coupled Receptors
dual-specificity MAPKK2	2.2	1.1	D14592	Intracellular Kinase Network Members
Casein kinase II	2.2	1.1	L15619	Intracellular Kinase Network Members
Elongation factor 2	2.2	1.0	K03502	Translation Factors
Aminopeptidase B	2.1	1.1	U61696	Metalloproteinases
Macrophage migration inhibitory factor	2.1	1.5	U62326	Growth Factors, Cytokines & Chemokines
Cathepsin L	2.0	1.5	Y00697	Cysteine Proteases
Cofilin	2.0	1.3	X62908	Other Cytoskeleton & Motility Proteins
Caput epididymidis				
Decreased expression				
Gastric inhibitory polypeptide precursor	4.5	1.8	L08831	Other Extracellular Communication Proteins
Sodium channel SCNB2	5.4	1.9	U37026	Voltage-Gated Ion Channels
Potassium channel Kir6.2	4.2	1.8	X97041	Voltage-Gated Ion Channels
C-type natriuretic peptide precursor	4.0	1.9	D90219	Hormones
insulin receptor-related receptor-α	3.0	1.3	M90661	Intracellular Kinase Network Members
rab12, ras related GTPase	2.9	1.5	M83676	G Proteins; Other Trafficking & Targeting Proteins

Gax, growth-arrest-specific protein	2.8	1.1	Z17223	Other Transcription Proteins; Telomere-Associated Proteins
cytochrome c oxidase, subunit IV	2.6	1.3	X14209	Energy Metabolism
CD2, membrane glycoprotein	2.5	1.2	X05111	Cell-Cell Adhesion Receptors; Cell Surface Antigens
vacuolar ATP synth. 16-kDa proteolipid subunit	2.3	1.4	D10874	ATPase Transporters
erbB4, proto-oncogene	2.3	1.6	U52531	Growth Factor/Chemokine/Protein Kinase Receptors
creatine kinase	2.1	1.2	X59737	Energy/Nucleotide Metabolism
apurinic/apyrimidinic endonuclease	2.1	1.3	D44495	DNA Damage Repair Proteins & Ligases
Corpus epididymidis				
Decreased Expression				
Ca^{++}-dependent phospholipase A2 precursor	3.1	1.6	U03763	Phospholipases & Phosphoinositol Kinases
DNA-binding protein inhibitor ID3	2.4	1.9	D10864	Transcription Activators & Repressors
Monocarboxylate transporter MCT1	2.2	1.8	D63834	Symporters & Antiporters
Pancreatic secretory trypsin inhibitor I precursor (PSTI-I)	2.1	1.8	M27882	Protease Inhibitors
G1/S-specific cyclin D2	2.0	1.8	D16308	Cyclins; Oncogenes & Tumor Suppressors
Increased expression				
Ral A; GTP-binding protein	3.5	1.3	L19698	G Proteins
angiotensin converting enzyme	3.1	1.1	U03734	Metalloproteinases
tissue inhibitor of metalloproteinase-2	2.9	1.3	L31884	Protease Inhibitors
syntaxin 3	2.6	1.2	L20820	Trafficking & Targeting Proteins
14-3-3 protein theta	2.4	1.1	D17614	Kinase Activators & Inhibitors
GTP-binding protein; G-alpha-i2	2.4	1.0	M17528	G Proteins
Cauda epididymidis				
Decreased expression				
ATPase, transitional endoplasmic reticulum	2.6	1.4	U11760	Immune System Proteins
proteasome component C8	2.3	1.9	M58593	Proteosomal Proteins
SYNAPTOTAGMIN XI	2.2	1.4	AF000423	Exocytosis/Calcium-Binding

				Proteins
heterogeneous nuclear ribonucleoprotein K	2.1	1.5	D17711	RNA Processing, Turnover & Transport Proteins
Increased expression				
Plectin	2.6	1.3	X59601	Cytoskeleton & Motility Proteins
tissue carboxypeptidase inhibitor	2.1	1.1	U40260	Protease Inhibitors

CHAPTER 5

DISCUSSION

The androgen dependence of the male reproductive system has been established for decades; experimental manipulation or alteration of androgen status has been an important methodological approach for studying this complex system. In the present thesis, we have employed such an approach to exclusively study epididymal physiology. Importantly, however, we updated the methodology to better reflect our current understanding of the androgen-dependence of the epididymis, namely that DHT is the predominant androgen acting in this tissue (1-6) and furthermore, that both isozymes of 5α-reductase, the enzyme catalyzing the local conversion of testosterone to DHT, are present in the epididymis (7). Thus, the androgen manipulation approach used throughout this thesis was inhibition of DHT formation, which was facilitated by the timely development of dual 5α-reductase inhibitors (PNU157706, FK143) (8, 9). This chapter will discuss the advantages of our novel experimental approach and the significance of some of the key findings presented in this thesis. Special emphasis will be placed on future research directions that will not only serve to expand our current findings, but also to increase knowledge of epididymal physiology in general that will lay the basis for new therapeutic approaches targeted at the epididymis.

5α-Reductase Inhibition as an Approach to Study Epididymal Physiology

The importance of DHT in the epididymis was deduced decades ago from a combination of *in vitro* studies of 5α-reductase enzyme activity and direct *in vivo* measurements of steroid levels in this organ (1-6). Since then, however,

very few studies have attempted to show direct biochemical and functional consequences of decreased DHT in the epididymis. Rather, much of our current knowledge regarding androgen regulation of epididymal structure and functions was derived from total androgen ablation or withdrawal experiments with or without subsequent androgen replacement (reviewed in 10). Androgen ablation was achieved predominantly by bilateral orchidectomy (removal of the testes) and to a lesser extent by hypophysectomy (removal of the pituitary) which is a much more severe form of hormone withdrawal.

Structurally, orchidectomy causes a time dependent decrease in rat epididymis weight that reaches approximately 70% by 4 weeks post-orchidectomy; the decreased weight is associated with the loss of sperm and fluid input from the testis as well as with cell shrinkage and apoptotic cell death (11-13). Regressive changes occur predominantly in principal cells indicating that this cell type is particularly sensitive to androgens, while other epithelial cell types appear unaffected by orchidectomy (14).

Functionally, orchidectomy and hypophysectomy have demonstrated that the acquisition of sperm motility and fertilizing ability are dependent on androgens (15-17). Furthermore, using these kinds of techniques, important epithelial processes such as the transport and/or secretion of ions and small organic molecules, metabolic functions and protein and lipid secretion, processing and degradation have been shown to be dependent on androgens (reviewed in 10). At the molecular level, androgen ablation techniques have revealed the influence of androgens on the expression of specific genes within the different regions of

the epididymis including the 5α-reductase isozymes, gamma-glutamyl transpeptidases, carbonic anhydrase isoforms and various metallothioneins to name a few (7, 18-20). Recently, gene array technology has enabled a larger scale analysis of the effects of orchidectomy on epididymal gene expression; this study identified novel orchidectomy-responsive genes and it permitted the visualization of global trends in gene expression in the epididymis post-orchidectomy, including transient upregulation of gene expression which had not previously been described (21).

While total androgen ablation techniques have been vital for determining the roles of androgens in the epididymis and other reproductive organs, it has become clear that even more insight into androgen action stands to be gained by differentiating between the roles of the two major androgens, testosterone and DHT. Even though both androgens act via the same receptor, several lines of evidence indicate that they have unique roles. Their differential roles during development are perhaps the best characterized; testosterone is responsible for the normal development of the Wolffian-duct structures, the pubertal and post pubertal increase in muscle mass and the development of male sex drive (libido) while DHT ensures the development of the prostate, urethra, penis and scrotum and sexual maturation at puberty (22, 23).

Additionally, there is growing evidence that testosterone and DHT carry out unique roles in adult tissues. For example, the unique roles of androgens in the prostate have been particularly well studied because of their prominent role in two hugely prevalent pathologies affecting this tissue, namely benign prostatic

hyperplasia and prostate cancer. In fact, the effects of orchidectomy and 5α-reductase inhibitor treatment on prostate physiology have been extensively characterized (reviewed in 24-28).

Particularly relevant to this thesis are examples where T and DHT have been shown to elicit different gene expression responses in this tissue. Nearly 15 years ago, Rittmaster et al. first demonstrated the differential effects of castration and 5α-reductase inhibitor treatment on androgen-regulated gene expression in the rat prostate (29). More recently, using similar experimental protocols (castration and 5α-reductase inhibition) in combination with differential display PCR, Avila et al. identified several unique gene expression responses in the rat prostate that clearly suggested that some genes are regulated in different ways by testosterone and DHT in a manner that cannot simply be attributed to the differential affinity of these two hormones for the androgen receptor (30). Lastly, it is well known that the prostate regresses in orchidectomized rats and subsequent administration of exogenous testosterone stimulates prostatic regrowth (24-26). Dadras et al. showed that treatment with finasteride during the testosterone-stimulated prostatic regrowth in castrated rats, which prevents the conversion of testosterone to DHT by 5α-reductase type 2, alters the expression patterns of certain androgen-response genes (31).

These studies indicate the important role of androgen status in the regulation of gene expression in the prostate. Indeed, the prostate is the only tissue where concrete efforts have been made towards discerning the roles of testosterone and DHT. This thesis represents the first comprehensive attempt to

assess the specific roles of DHT in the epididymis, at both a molecular level (gene expression) as well as a functional level, through the inhibition of 5α-reductase. Additionally, this is the first set of studies specifically targeting the epididymis that employ dual 5α-reductase inhibitors. The use of dual inhibitors is an important feature of our experimental approach due to the fact that both 5α-reductase isozymes are now known to exist in the epididymis (7).

Our findings support the continued use of dual 5α-reductase inhibition as an experimental approach to study epididymal physiology. In fact, there are several significant advantages to this approach in comparison to other androgen-manipulation techniques. Firstly, when examining a specific tissue, it is obviously desirable to use a technique that targets this tissue, in our case the epididymis, while having limited effects on other tissues and systems. Clearly, the more pervasive the effects of any treatment, the more difficult it is to distinguish between the direct and indirect effects of that treatment. Due to the prevalence of the androgen receptor throughout the body and the well-established roles for testosterone in multiple organ systems, removal of the primary site of testosterone biosynthesis (the testes) by orchidectomy will consequently have a broad spectrum of physiological effects that are not limited to the reproductive system, much less the epididymis. In rats, for example, the circulating androgen concentration decreases to less than 10% of intact control levels by 2 hours following orchidectomy (32), such that all androgen target tissues, whether dependent on testosterone, DHT or both, are rapidly affected. Hypophysectomy, being a more severe form of hormone withdrawal, has even more extensive

effects than orchidectomy (33). Comparatively, inhibiting the formation of DHT by inhibiting 5α-reductase has less wide-ranging effects since testosterone (and other hormones) levels are essentially maintained at normal levels, forming the basis for a more specific approach with less confounding effects on other tissues.

Importantly, the testis itself does not appear to be affected by 5α-reductase inhibitor treatment (27, 34-36). This is a second key advantage of this experimental approach. Spermatogenesis in the testis and sperm maturation in the epididymis are intimately linked androgen dependent phenomena, consequently it is very difficult to distinguish between true effects/responses that originate in the epididymis and those that manifest from abnormal testicular functions. Deciphering the unique roles of the testis and epididymis in sperm function has implicit consequences for understanding the origins of male infertility. In this regard, dual 5α-reductase inhibition provides an intriguing method to study epididymal sperm-related functions separate from testicular sperm-related functions. Furthermore, with the emphasis in male contraceptive research on elucidating post-testicular targets for contraceptives, the need to uncover mechanisms of epididymal sperm maturation is even more apparent.

Finally, one last advantage of 5α-reductase inhibition in comparison to orchidectomy is that the testis obviously remains intact such that the input of new sperm into the lumen of the epididymis, as well as blood flow to the tissue, also remain intact. The continuous introduction of sperm enables detailed analyses of sperm maturation dynamics in the epididymis, while maintenance of blood supply to the tissue is integral to its structure and functions.

The establishment of dual 5α-reductase inhibition as a useful and advantageous approach to study the DHT dependence of the epididymis and the initial *in vitro* and *in vivo* characterization of two different inhibitor compounds was an important contribution of the present thesis; this has opened the door to many possible avenues of investigation.

It would have been desirable to measure the exact levels of testosterone and DHT present in each epididymal segment following 5α-reductase inhibitor treatment; however, this type of analysis was not truly feasible at the time because of the limited amount of inhibitor compound made available to us for our studies, the amount of animal tissues (especially the smaller epididymal segments) required for accurate hormone measurements and the lack of validated protocols for the measurement of DHT in rat epididymides. Furthermore, based on previous studies, it is generally accepted that 5α-reductase inhibitor treatment decreases DHT levels very significantly in serum and target tissues with limited effects on testosterone levels (27, 28, 34, 36). Therefore, the measurement of androgen levels was not considered a priority. Rather, in the present thesis, we used the approach of dual 5α-reductase inhibition and sought to answer three main questions: 1) What are the consequences of DHT depletion on epididymal gene expression? 2) What are the consequences of DHT depletion on epididymal sperm functions and can they be correlated to changes in gene expression? 3) How does DHT mediate/regulate differential epididymal gene expression and functions? The next sections serve to highlight some of our significant findings and place them in the context of

current epididymal research. Furthermore, suggestions for future research initiatives will be presented.

DHT and Epididymal Gene Expression

The development and advancement of gene array technology has presented tremendous opportunities for large-scale gene expression analyses in all areas of research. The availability of this technology was a decisive factor in determining our first objective to elucidate the effects of dual 5α-reductase inhibition on epididymal gene expression. To substantiate and strengthen the reliability of our gene array experiments, our studies incorporated many factors that were unprecedented in gene array experiments at the time this thesis was initiated, including the use of numerous replicates, stringent analysis criteria, dose-response expression profiling to analyze the effects of drug treatment (chapter 2) and additional analyses using a second drug of the same class (introduced in chapter 4).

While gene arrays have previously been used to examine gene expression in the epididymis (21, 37-39), to my knowledge, the study described in chapter 2 was the first report of the effects of treatment with a dual 5α-reductase inhibitor on global gene expression in this tissue and as such contributes greatly to our understanding of DHT-dependent gene expression in the epididymis. The corroborative gene array findings presented in chapter 4 complement this initial study. Following 5α-reductase inhibitor treatment, the expression of numerous genes increased and/or decreased in the epididymis, usually in a highly segment-

specific manner, reflecting the importance of DHT and the complexity of coordinated gene expression throughout the tissue that is likely fundamental to its multiple functions.

Among the many genes showing changes in expression following inhibitor treatment, one grouping I found particularly interesting were those involved in protein and lipid processing. Protein and lipid processing events are required for the direct modification of sperm surface components, a major feature of epididymal sperm maturation (40-43). Interestingly, the development of contraceptive vaccines that uniquely target sperm-specific antigens represents a widely researched approach for the regulation of male fertility (reviewed in 44). The viability of this approach was exemplified in a recent study by O'Rand et al. who found that a high percentage (78%) of male nonhuman primates immunized with Eppin, a testis/epididymis-specific protein, became infertile and the majority recovered fertility when immunization was stopped (45). In light of this study as well as others, it is certainly plausible that the DHT-directed processing of epididymal sperm-specific membrane components could provide unique epididymal targets for immunocontraception. Indeed, given that 5α-reductase inhibition affected the expression of several protein and lipid processing enzymes in a highly segment-specific manner in the epididymis, it is tempting to speculate that DHT regulates the availability of these enzymes to ensure their activity only when and where it is needed during the course of epididymal sperm maturation. As such, it would be interesting to find out whether the protein and lipid processing enzymes identified in chapter 2 act on sperm surface molecules and if

so, to then determine whether their reactions yield antigens that are unique to sperm cells since the therapeutic value of an antigen as an immunocontraceptive target is contingent upon specificity to germ cells.

The large-scale nature of the analyses that are achievable with gene array technology generated a vast amount of information that conceivably points toward the most novel avenues for future research. While I singled out one group of genes that I found particularly intriguing as candidates for further investigation, following up on any of the genes or gene families identified in chapter 2 would further our understanding of epididymal physiology. A next logical step would be to investigate whether changes in gene expression following dual 5α-reductase inhibitor treatment are paralleled by changes at the protein level using immunohistochemistry, provided that reliable antibodies are available for the proteins of interest. Immunohistochemistry would be preferable to Western blotting because the precise cellular localization of the proteins can be determined. Since unique functions of the various epididymal cell types have been and continue to be elucidated (46), identifying the cell type or types from which a specific protein originates can give additional insight into its potential role in the tissue.

The results presented in chapter 3 certainly suggest that the effects of 5α-reductase inhibition on epididymal gene expression result in deficits in sperm function, however, direct links between any of the affected genes identified by gene arrays and specific functional consequences remain to be determined. As such, the true value of gene and protein analyses would be borne out to the

fullest extent by determining whether the actual functions of specific proteins are affected by 5α-reductase inhibitor treatment as well. For example, given that a number of the genes identified in chapter 2 are enzymes involved in protein and lipid processing and metabolism, functional follow-up studies would naturally entail direct assays of enzymatic activity. Similarly, the expression of several oxidative stress-related genes were affected by 5α-reductase inhibitor treatment therefore, assessing the capacity of the epididymal epithelium to respond to oxidative stress following inhibitor treatment is another possible follow-up experiment. A student in our lab is using treatment with buthionine sulfoximine, a chemical that induces oxidative stress by inhibiting the rate limiting enzyme in glutathione synthesis, to assess the capacity of the epididymis to handle oxidative stress in the context of aging (47). It would be feasible to employ this same methodology to assess the capacity of the epididymis to handle oxidative stress following 5α-reductase inhibitor treatment as well.

Lastly, in regards to follow-up studies, epididymal intraluminal ion concentrations and pH represent other functional endpoints that could be measured following 5α-reductase inhibitor treatment, given that the expression of genes involved in fluid and ion transport and luminal acidification were among those identified in chapter 2. In this case, learning and perfecting delicate micropuncture techniques (48, 49) would be required to collect uncontaminated samples of intraluminal fluid from the different epididymal segments following 5α-reductase inhibitor treatment. Once accomplished, micropuncture techniques could also be used for the microanalysis of other luminal components, such as

proteins known to be secreted by the epididymal epithelium, to determine if they are affected by 5α-reductase inhibitor treatment and hence under the control of DHT.

DHT and Epididymal Sperm Function

The nature of the gene transcripts that were altered following 5α-reductase inhibitor treatment suggested their potential involvement in key epididymal epithelial processes that contribute to the formation of an optimal luminal microenvironment required for sperm maturation. These results prompted us to next determine whether sperm maturation was indeed compromised following 5α-reductase inhibitor treatment. Therefore, we focused specifically on analyzing different facets of sperm maturation in the epididymis and found that 5α-reductase inhibitor treatment had effects on sperm morphology, motility and fertilizing ability (chapter 3). It is reasonable to conclude that the cumulative effects of 5α-reductase inhibitor treatment on gene expression resulted in the compromised sperm functions described in chapter 3, which reaffirms the value of following-up on the different functionally relevant gene families identified by gene array in order to elucidate the missing links between epididymal gene expression and sperm functions.

In chapter 3, a standard mating study demonstrated that 5α-reductase inhibitor treatment resulted in subfertility; however, using this traditional method, the exact origin or cause of the subfertility could not be pinpointed. Due to the general lack of effect of 5α-reductase inhibitor treatment on the testis (27, 34-36)

it is highly unlikely that the subfertility arises from effects on this tissue. In contrast, there are well-documented effects of 5α-reductase inhibitor treatment on the seminal vesicles (50, 51), thus it is reasonable to postulate that deficiencies in seminal plug formation, one of the main functions of seminal vesicle secretions (52), may be contributing to the subfertility. I would venture to speculate however, that if the effects of 5α-reductase inhibitor treatment on the seminal vesicles were to subsequently affect fertility, it would be an "all or nothing" effect rather than the variable effects we observed in the current study, mainly because of the dramatic and highly consistent treatment effects on this tissue that are apparent in our studies and numerous others. It is also tempting to speculate that the variability in the fertility observed in the current study is due to the sheer number of epididymal sperm that must be consistently affected in order to achieve complete infertility. In rats for example, a greater than 90% decrease in the number of epididymal sperm is required to achieve complete infertility (53).

Clearly, more in depth studies are required to pinpoint the exact nature of the subfertility described in chapter 3. Essentially, to achieve fertilization, sperm must be able to reach, recognize, contact and penetrate the egg, while withstanding exposure to the unfamiliar environment of the female reproductive tract. It is plausible to think that decreased progressive sperm motility, as is observed in the study described in chapter 3, may compromise the ability of sperm to travel through the female reproductive tract and reach the egg. This could be assessed by flushing out, fixing and examining the contents of the uterus and oviducts after mating to reveal the nature and number of sperm that

reached these sites within the female tract. In fact, this approach was used to determine the cause of infertility in *c-ros* tyrosine kinase receptor knockout male mice (54). These mice have a unique epididymal phenotype in that they completely lack the initial segment. Despite normal testicular sperm output, epididymal sperm from these animals have slightly lower velocity and head movement parameters (approximately 20% lower than wildtype mice) and display various forms of flagellar angulation predominantly in the distal regions of the epididymis (55). Examination of the uterine and oviductal contents of female mice mated with *c-ros* knockouts showed that sperm were unable to reach the oviduct as a result of their bent tails forming an entangled sperm mass and their compromised flagellar vigor (54).

In addition to facilitating sperm migration to the site of fertilization, sperm motility is also believed to be required for penetration of the surface coats that surround the egg. In particular, hyperactivated motility, described as a type of vigorous non-linear motion that mammalian sperm exhibit as they progress through the female oviduct, has been implicated in this process (56). Studies have suggested that hyperactivated sperm motility provides increased vigor and thrust that facilitates sperm penetration of the surrounding cumulus cells and the zona pellucida, a glycoprotein matrix that envelopes the plasma membrane of the egg (57-60).

Computer-assisted sperm analysis (CASA) has proven to be a valuable tool for studying epididymal sperm motility patterns, and this type of analysis has progressed to become a regular component of sperm functional studies due in

large part to the work of Perreault et al, who tested and defined the CASA criteria for optimal and objective measurements of epididymal sperm motility (61, 62). Standard CASA measures are made 10-30 minutes after sperm are collected in a medium that is not conducive to capacitation thereby making measures of hyperactivation impossible. Recently however, Perreault's group addressed this problem and established novel criteria for detecting and monitoring hyperactivation of rat sperm motility using CASA that was facilitated by advances in CASA computer and optical systems (63). Now that measurements of rat sperm hyperactivation are possible, it would be interesting to determine whether 5α-reductase inhibitor treatment has an effect on this functional end-point as it does on other motility parameters.

Lastly, the ability of sperm to undergo the acrosome reaction, also a requirement for egg recognition and penetration (56), can be assessed following 5α-reductase inhibitor treatment using staining methods (i.e. chlortetracycline fluorescence staining) (64). The acrosome reaction is highly dependent on complex sperm membrane dynamics therefore it would be a particularly interesting functional endpoint to measure given that 5α-reductase inhibitor treatment affects the expression of numerous genes that potentially interact with and modify the sperm plasma membrane (chapters 2 and 4).

DHT Signaling Mechanisms in the Epididymis

The epididymis is not a homogeneous tissue; rather, it displays highly regionalized tissue organization, function and gene expression. Due to its

complexity, deciphering the regulatory mechanisms governing the epididymis is a challenging objective to tackle. Our experiments clearly demonstrated that DHT has an important role in regulating this tissue, and it does so in a highly segment-specific manner. What remained unclear was the identity of segment-specific signaling/regulatory molecules and pathways involved in mediating the effects of DHT on epididymal gene expression and function, which formed the basis of our third and final objective. We touched upon this matter using gene array technology (chapters 2 and 4) and identified transcription factors and other signaling molecules with altered gene expression following 5α-reductase inhibitor treatment. For the most part however, the genes identified in this way were isolated signaling molecules (i.e. not from the same pathway) such that specific conclusions regarding the involvement of signaling pathways in epididymal regulation were difficult to make. We therefore wanted to approach our third objective in a more comprehensive manner by focusing our analyses on specific signaling systems using the novel and sensitive technique of quantitative real-time RT-PCR.

As we found out, DHT mediated regulation of the epididymis extends to the level of the enzymes that control its production (5α-reductases) as well as the receptor that mediates its action (androgen receptor). The differential effect of 5α-reductase inhibitor treatment on the expression of the two 5α-reductase isozymes in the epididymis was particularly interesting. This finding was consistent with what has previously been reported regarding the regulation of 5α-reductase expression in other tissues (65-67) and strongly suggests that the two

isozymes carry out unique roles despite the common reaction they catalyze. Whether or not this is the case has not yet been proven; the development of reliable type-specific inhibitors provides one approach that would help resolve this issue.

Alternatively, a more in depth examination of the phenotypes of the type specific 5α-reductase knockout mice developed by Mahendroo et al. might also give insight into the unique roles of each isozyme (68). While it has been reported that there is no effect on reproduction in type 1 5α-reductase knockout male mice and the type 2 5α-reductase knockout males exhibit a mild virilization defect, thorough examinations and/or descriptions of many specific tissue phenotypes, including the epididymis, have not yet been reported. Unfortunately, despite the existence of 5α-reductase knockout animals, they have not been made generally available such that these types of analyses can be undertaken. It should be noted that Mahendroo et al. concluded from their studies that testosterone alone is required for differentiation of the male mouse urogenital tract and that the synthesis of DHT serves largely as a signal amplification mechanism in this animal model (68). This is in contrast to the requirements for both testosterone and DHT in the proper development of the reproductive system in men. Furthermore, studies in male rats using pharmacological inhibitors also support a two-androgen model of sexual differentiation (34, 69, 70), indicating that the rat may be a more suitable animal model for understanding the roles of DHT within the male reproductive system.

We showed that the expression of the androgen receptor is affected in a segment-specific manner by 5α-reductase inhibitor treatment *in vivo*. It would also be interesting to assess the effects of treatment on androgen receptor function *in vitro*, for example by examining androgen receptor transactivation of reporter gene constructs containing functional androgen response elements (AREs) in established cell culture systems such as the LnCAP prostate cancer cell line (71). Additionally, using similar *in vitro* reporter gene assays, it would be possible to test whether ligand-bound androgen receptor binds directly to the promoter regions of genes identified in chapters 2 and 4 that changed (increased or decreased) in expression following 5α-reductase inhibitor treatment. These studies would have the potential of elucidating those genes that are direct targets of androgen action, reveal novel AREs, distinguish between AREs that lead to the induction or repression of gene expression as well as differentiate between the effects of testosterone and DHT-bound androgen receptor complexes on gene expression.

Determining the roles of growth factors and growth factor signaling in the development, regulation and pathogenesis of the reproductive system, particularly of the prostate, has become an intriguing and widespread area of research in the field of reproduction. Using quantitative real-time RT-PCR, we were able to analyze the longitudinal expression patterns of several growth factor family members in the epididymis and determine whether these patterns were regulated by DHT. While the results presented in chapter 4 suggest that DHT regulates diverse epididymal processes by activating different growth factor

signaling pathways, substantiating the activation of these pathways presents a challenging task for several reasons, not the least being the inherent difficulty in measuring the transient changes in gene expression and phosphorylation status that occur when a signaling cascade is activated.

Generally, in vitro cell-culture systems are better suited for signaling analyses. For example, studies using prostate cell lines have provided evidence to support androgen receptor cross talk with growth factor signaling pathways resulting in both enhanced androgen responses and receptor ligand-independent activation and modulation of androgen receptor transactivation (72-75). Given these findings and our current gene expression results, analogous studies of epididymal androgen and growth factor signaling interactions are certainly warranted. Until recently, however, studies of this type were not truly feasible due to the lack of viable epididymal cell culture systems. Fortunately, with the recent emergence of several different epididymal cell lines (reviewed in 76), new tools now exist that could enable more in depth studies of androgen and growth factor signaling in the epididymis. A study by Sipila et al. is a promising example of the viability of these cell lines for the analysis of signaling systems in the epididymis (77); in this particular study, inhibitors of specific signal transduction pathways were used to elucidate the epididymal regulation of PEA3, a member of the family of Ets transcription factors that is highly expressed in the adult epididymis (78).

In addition to evaluating signaling mechanisms, epididymal cell lines have numerous potential applications for studying epididymal genes and proteins and

their regulation by various endogenous and/or exogenous factors. It would certainly be interesting to determine the cellular consequences of different androgen treatments. In collaboration with another student, I have initiated ongoing studies in our lab to characterize the androgen-dependence of gene expression in available cell lines; using the same gene array technology employed throughout this thesis, we are assessing the effects of androgen status on gene expression in the mouse proximal caput cell line (79). Importantly, we have the benefit of our large-scale epididymal gene expression data with which to make comparisons in order to determine how representative the cell line is of the actual *in vivo* situation. Furthermore, these initial studies were designed to differentiate between the effects of testosterone and DHT to ultimately assess the suitability of this *in vitro* system for studying androgen action in the epididymis.

Finally, despite the numerous potential applications of the newly emerging epididymal cell lines and the insight into epididymal physiology that stands to be gained, it needs to be recognized that cell lines are only representative of one isolated epididymal segment. As such, cell lines could never truly represent the complex regionalized diversity of the epididymis as a whole, a hallmark feature that has made the epididymis such an interesting and unique tissue to study in the first place.

Final Conclusions

A novel androgen manipulation approach, dual 5α-reductase inhibitor treatment, was designed and implemented in this thesis to specifically study the

roles of DHT in the epididymis. This approach revealed novel roles for DHT in the segment-specific expression of numerous genes implicated in important epididymal epithelial cell processes, in maintaining key features and function of epididymal sperm and in androgen and growth factor signaling mechanisms in the epididymis. Collectively, the studies presented in this thesis contribute greatly to our understanding of androgen action in the epididymis and lay the groundwork for many novel investigations of epididymal physiology. From a clinical standpoint, a greater understanding of epididymal sperm maturation would enable more informed diagnoses of the underlying epididymal causes of male infertility and indicate potential treatments for this pathology. Furthermore, the epididymis represents an extremely promising target for male contraceptive approaches that are highly specific with few to no side effects, especially when compared to testis-based or hormonal contraceptive approaches that are disadvantaged by delayed onset of effectiveness and reversibility, accompanying androgen-dependent side effects and documented ethnic variations in efficiency (80, 81).

References

1. Turner TT, Jones CE, Howards SS, Ewing LL, Zegeye B, Gunsalus GL. 1984 On the androgen microenvironment of maturing spermatozoa. Endocrinology. 115:1925-1932.
2. Gloyna RE, Wilson JD. 1969 A comparative study of the conversion of testosterone to 17-beta-hydroxy-5-alpha-androstan-3-one (Dihydrotestosterone) by prostate and epididymis. J Clin Endocrinol Metab. 29:970-977.
3. Robaire B, Ewing LL, Zirkin BR, Irby DC. 1977 Steroid delta4-5alpha-reductase and 3alpha-hydroxysteroid dehydrogenase in the rat epididymis. Endocrinology. 101:1379-1390.
4. Monsalve A, Blaquier JA. 1977 Partial characterization of epididymal 5 alpha reductase in the rat. Steroids. 30:41-51.
5. Tindall DJ, French FS, Nayfeh SN. 1972 Androgen uptake and binding in rat epididymal nuclei, in vivo. Biochem Biophys Res Commun. 49:1391-1397.
6. Orgebin-Crist MC, Davies J. 1974 Functional and morphological effects of hypophysectomy and androgen replacement in the rabbit epididymis. Cell Tissue Res. 148:183-201.
7. Viger RS, Robaire B. 1996 The mRNAs for the steroid 5 alpha-reductase isozymes, types 1 and 2, are differentially regulated in the rat epididymis. J Androl. 17:27-34.

8. di Salle E, Giudici D, Radice A, Zaccheo T, Ornati G, Nesi M, Panzeri A, Delos S, Martin PM. 1998 PNU 157706, a novel dual type I and II 5alpha-reductase inhibitor. J Steroid Biochem Mol Biol. 64:179-186.
9. Hirosumi J, Nakayama O, Chida N, Inami M, Fagan T, Sawada K, Shigematsu S, Kojo H, Notsu Y, Okuhara M. 1995 FK143, a novel nonsteroidal inhibitor of steroid 5 alpha-reductase: (2) In vivo effects on rat and dog prostates. J Steroid Biochem Mol Biol. 52:365-373.
10. Ezer N, Roabire B. 2002 Androgenic Regulation of the Structure and Functions of the Epididymis. In: Robaire B, Hinton BT (eds), The Epididymis: From Molecules to Clinical Practice. New York: Kluwer Academic/Plenum Press Publishers. pp 297-316.
11. Brooks DE. 1977 The androgenic control of the composition of the rat epididymis determined by efferent duct ligation or castration. J Reprod Fertil. 49:383-385.
12. Robaire B, Ewing LL, Zirkin BR, Irby DC. 1977 Steroid d4-5a-reductase and 3a-hydroxysteroid dehydrogenase in the rat epididymis. Endocrinology. 101:29-40.
13. Fan X, Robaire B. 1998 Orchidectomy induces a wave of apoptotic cell death in the epididymis. Endocrinology. 139:2128-2136.
14. Moore HD, Bedford JM. 1979 Short-term effects of androgen withdrawal on the structure of different epithelial cells in the rat epididymis. Anat Rec. 193:293-311.

15. Benoit MJ. 1926 Recherches anatomiques, cytologiques et histophysiologiques sur les voies excrétrices du testicule, chez les mammifères. Arch d'Anat d'histo et d'embryo. 5:173-412.
16. White WE. 1932 The effect of hypophysectomy on the survival of spermatozoa in the male rat. Anat Rec. 54:253-273.
17. Dyson AL, Orgebin-Crist MC. 1973 Effect of hypophysectomy, castration and androgen replacement upon the fertilizing ability of rat epididymal spermatozoa. Endocrinology. 93:391-402.
18. Palladino MA, Hinton BT. 1994 Expression of multiple gamma-glutamyl transpeptidase messenger ribonucleic acid transcripts in the adult rat epididymis is differentially regulated by androgens and testicular factors in a region-specific manner. Endocrinology. 135:1146-1156.
19. Kaunisto K, Fleming RE, Kneer J, Sly WS, Rajaniemi H. 1999 Regional expression and androgen regulation of carbonic anhydrase IV and II in the adult rat epididymis. Biol Reprod. 61:1521-1526.
20. Cyr DG, Dufresne J, Pillet S, Alfieri TJ, Hermo L. 2001 Expression and regulation of metallothioneins in the rat epididymis. J Androl 22:124-135.
21. Ezer N, Robaire B. 2003 Gene expression is differentially regulated in the epididymis after orchidectomy. Endocrinology. 144:975-988.
22. George FW, Wilson JD. 1994 Sex determination and differentiation. In: Knobil E, Neill JD (eds), The Physiology of Reproduction, Second Edition. New York: Raven Press, Inc. pp 13-28.

23. Siiteri PK, Wilson JD. 1973 Testosterone formation and metabolism during sexual differentiation in the human embryo. J Clin Endocrinol Metab. 38:113-125.

24. Sandberg AA. 1980 Endocrine control and physiology of the prostate. Prostate. 1:169-184.

25. Aumuller G. 1983 Morphologic and endocrine aspects of prostatic function. Prostate. 4:195-214.

26. Sinowatz F, Amselgruber W, Plendl J, Kolle S, Neumuller C, Boos G. 1995 Effects of hormones on the prostate in adult and aging men and animals. Microsc Res Tech. 30:282-292.

27. Steers WD. 2001 5alpha-reductase activity in the prostate. Urology. 58(6 Suppl 1):17-24.

28. Bartsch G, Rittmaster RS, Klocker H. 2002 Dihydrotestosterone and the concept of 5alpha-reductase inhibition in human benign prostatic hyperplasia. World J Urol. 19:413-425.

29. Rittmaster RS, Magor KE, Manning AP, Norman RW, Lazier CB. 1991 Differential effect of 5 alpha-reductase inhibition and castration on androgen-regulated gene expression in rat prostate. Mol Endocrinol. 5:1023-1029.

30. Avila DM, Fuqua SA, George FW, McPhaul MJ. 1998 Identification of genes expressed in the rat prostate that are modulated differently by castration and Finasteride treatment. J Endocrinol. 1998 Dec;159(3):403-11.

31. Dadras SS, Cai X, Abasolo I, Wang Z. 2001 Inhibition of 5alpha-reductase in rat prostate reveals differential regulation of androgen-response gene expression by testosterone and dihydrotestosterone. Gene Expr. 9:183-194.

32. Isaacs JT. 1984 Antagonistic effect of androgen on prostatic cell death. Prostate. 5:545-557.

33. Engel FL, Kostyo JL. 1964 Metabolic actions of pituitary hormones. In: Pincus G, Thimann KV, Astwood EB (eds), The Hormones: Physiology, Chemistry and Applications. New York: Academic Press, pp 69–139.

34. George FW, Johnson L, Wilson JD. 1989 The effect of a 5 alpha-reductase inhibitor on androgen physiology in the immature male rat. Endocrinology. 125:2434-2438.

35. Overstreet JW, Fuh VL, Gould J, Howards SS, Lieber MM, Hellstrom W, Shapiro S, Carroll P, Corfman RS, Petrou S, Lewis R, Toth P, Shown T, Roy J, Jarow JP, Bonilla J, Jacobsen CA, Wang DZ, Kaufman KD. 1999 Chronic treatment with finasteride daily does not affect spermatogenesis or semen production in young men. J Urol. 162:1295-1300.

36. Rhoden EL, Gobbi D, Menti E, Rhoden C, Teloken C. 2002 Effects of the chronic use of finasteride on testicular weight and spermatogenesis in Wistar rats. BJU Int. 89:961-963.

37. Jervis KM, Robaire B. 2001 Dynamic changes in gene expression along the rat epididymis. Biol Reprod. 65:696-703.

38. Jervis KM, Robaire B. 2002 Changes in gene expression during aging in the Brown Norway rat epididymis. Exp Gerontol. 37:897-906.

39. Jervis KM, Robaire B. 2003 Effects of caloric restriction on gene expression along the epididymis of the Brown Norway rat during aging. Exp Gerontol. 38:549-560.

40. Turner TT. 1991 Spermatozoa are exposed to a complex microenvironment as they traverse the epididymis. Ann N Y Acad Sci. 637:364-383.

41. Dacheux JL, Dacheux F. 2002 Protein Secretion in the Epididymis. In: Robaire B, Hinton BT (eds), The Epididymis: From Molecules to Clinical Practice. New York: Kluwer Academic/Plenum Press Publishers. pp 151-168.

42. Hall JC, Hadley J, Doman T. 1991 Correlation between changes in rat sperm membrane lipids, protein, and the membrane physical state during epididymal maturation. J Androl. 12:76-87.

43. Jones R. 1998 Plasma membrane structure and remodelling during sperm maturation in the epididymis. J Reprod Fertil Suppl. 53:73-84.

44. McLaughlin EA, Holland MK, Aitken RJ. 2003 Contraceptive vaccines. Expert Opin Biol Ther. 2003 Aug;3:829-841.

45. O'rand MG, Widgren EE, Sivashanmugam P, Richardson RT, Hall SH, French FS, VandeVoort CA, Ramachandra SG, Ramesh V, Jagannadha Rao A. 2004 Reversible immunocontraception in male monkeys immunized with eppin. Science. 306:1189-1190.

46. Hermo L, Robaire B. 2002 Epididymal cell types and their functions. In: Robaire B, Hinton BT (eds), The Epididymis: From Molecules to Clinical Practice. New York: Kluwer Academic/Plenum Press Publishers. pp 81-102.

47. Zubkova EV, Robaire B. 2004 Effect of glutathione depletion on antioxidant enzymes in the epididymis, seminal vesicles, and liver and on spermatozoa motility in the aging brown Norway rat. Biol Reprod. 71:1002-1008.

48. Hinton BT. 1980 The epididymal microenvironment: a site of attack for a male contraceptive? Invest Urol. 18:1-10.

49. Levine N, Marsh DJ. 1971 Micropuncture studies of the electrochemical aspects of fluid and electrolyte transport in individual seminiferous tubules, the epididymis and the vas deferens in rats. J Physiol. 213:557-570.

50. Luttge WG, Jasper TW, Sheets CS, Gray HE. 1978 Inhibition of testosterone 5alpha-reductase activity and growth of accessory sex tissues in castrated male mice. J Reprod Fertil. 53:45-50.

51. Kao LW, Weisz J. 1979 Inhibition of steroid 5 alpha-reductase in vivo: effect on suppression of luteinizing hormone and stimulation of accessory sex organs by testosterone in the orchidectomized rat. J Endocrinol. 81:209-220.

52. Luke MC, Coffey DS. 1994 Male Sex Accessory Tissues Structure, Androgen Action, and Physiology. In: Knobil E, Neill JD (eds), The Physiology of Reproduction, 2nd edition. New York: Raven Press. pp 1478

53. Robaire B, Smith S, Hales BF. 1984 Suppression of spermatogenesis by testosterone in adult male rats: effect on fertility, pregnancy outcome and progeny. Biol Reprod. 31:221-230.

54. Yeung CH, Wagenfeld A, Nieschlag E, Cooper TG. 2000 The cause of infertility of male c-ros tyrosine kinase receptor knockout mice. Biol Reprod. 63:612-618.

55. Yeung CH, Sonnenberg-Riethmacher E, Cooper TG. 1999 Infertile spermatozoa of c-ros tyrosine kinase receptor knockout mice show flagellar angulation and maturational defects in cell volume regulatory mechanisms. Biol Reprod. 61:1062-1069.

56. Yanagimachi R. 1994 Mammalian Fertilization. In: Knobil E, Neill JD. (eds), The Physiology of Reproduction. New York: Raven Press, Ltd. pp 189-317.

57. Suarez SS, Katz DF, Owen DH, Andrew JB, Powell RL. 1991 Evidence for the function of hyperactivated motility in sperm. Biol Reprod. 44:375-381.

58. Suarez SS, Dai X. 1992 Hyperactivation enhances mouse sperm capacity for penetrating viscoelastic media. Biol Reprod. 46:686-691.

59. Pacey AA, Davies N, Warren MA, Barratt CL, Cooke ID. 1995 Hyperactivation may assist human spermatozoa to detach from intimate association with the endosalpinx. Hum Reprod. 10:2603-2609.

60. Stauss CR, Votta TJ, Suarez SS. 1995 Sperm motility hyperactivation facilitates penetration of the hamster zona pellucida. Biol Reprod. 53:1280-1285.

61. Slott VL, Suarez JD, Perreault SD. 1991 Rat sperm motility analysis: methodologic considerations. Reprod Toxicol. 5:449-458.

62. Slott VL, Suarez JD, Poss PM, Linder RE, Strader LF, Perreault SD. 1993 Optimization of the Hamilton-Thorn computerized sperm motility analysis system for use with rat spermatozoa in toxicological studies. Fundam Appl Toxicol. 21:298-307.

63. Cancel AM, Lobdell D, Mendola P, Perreault SD. 2000 Objective evaluation of hyperactivated motility in rat spermatozoa using computer-assisted sperm analysis. Hum Reprod. 15:1322-1328.

64. DasGupta S, Mills CL, Fraser LR. 1993 Ca(2+)-related changes in the capacitation state of human spermatozoa assessed by a chlortetracycline fluorescence assay. J Reprod Fertil. 99:135-143.

65. Torres JM, Ortega E. 2003 Differential regulation of steroid 5alpha-reductase isozymes expression by androgens in the adult rat brain. FASEB J. 17:1428-1433.

66. Torres JM, Gomez-Capilla JA, Ruiz E, Ortega E. 2003 Semiquantitative RT-PCR method coupled to capillary electrophoresis to study 5alpha-reductase mRNA isozymes in rat ventral prostate in different androgen status. Mol Cell Biochem. 250:125-130.

67. Torres JM, Ortega E. 2003 Precise quantitation of 5alpha-reductase type 1 mRNA by RT-PCR in rat liver and its positive regulation by testosterone and dihydrotestosterone. Biochem Biophys Res Commun 308:469-473.

68. Mahendroo MS, Cala KM, Hess DL, Russell DW. 2001 Unexpected virilization in male mice lacking steroid 5 alpha-reductase enzymes. Endocrinology. 142:4652-4662.

69. Anderson CA, Clark RL. 1990 External genitalia of the rat: normal development and the histogenesis of 5 alpha-reductase inhibitor-induced abnormalities. Teratology. 42:483-496.

70. Imperato-McGinley J, Binienda Z, Arthur A, Mininberg DT, Vaughan ED Jr, Quimby FW. 1985 The development of a male pseudohermaphroditic rat using an inhibitor of the enzyme 5 alpha-reductase. Endocrinology. 116:807-812.

71. Horoszewicz JS, Leong SS, Chu TM, Wajsman ZL, Friedman M, Papsidero L, Kim U, Chai LS, Kakati S, Arya SK, Sandberg AA. 1980 The LNCaP cell line--a new model for studies on human prostatic carcinoma. Prog Clin Biol Res. 37:115-132.

72. Culig Z, Hobisch A, Cronauer MV, Radmayr C, Trapman J, Hittmair A, Bartsch G, Klocker H. 1994 Androgen receptor activation in prostatic tumor cell lines by insulin-like growth factor-I, keratinocyte growth factor, and epidermal growth factor. Cancer Res. 54:5474-5478.

73. Culig Z, Hobisch A, Cronauer MV, Hittmair A, Radmayr C, Bartsch G, Klocker H. 1995 Activation of the androgen receptor by polypeptide growth factors and cellular regulators. World J Urol. 13:285-289.

74. Orio F Jr, Terouanne B, Georget V, Lumbroso S, Avances C, Siatka C, Sultan C. 2002 Potential action of IGF-1 and EGF on androgen receptor

nuclear transfer and transactivation in normal and cancer human prostate cell lines. Mol Cell Endocrinol. 198:105-114.

75. Huang D, Liu X, Plymate SR, Idowu M, Grimes M, Best AM, McKinney JL, Ware JL. 2004 Proteomic identification of 14-3-3 sigma as a common component of the androgen receptor and the epidermal growth factor receptor signaling pathways of the human prostate epithelial cell line M12. Oncogene. 23:6881-6889.

76. Kirchhoff C, Araki Y, Huhtaniemi I, Matusik RJ, Osterhoff C, Poutanen M, Samalecos A, Sipila P, Suzuki K, Orgebin-Crist MC. 2004 Immortalization by large T-antigen of the adult epididymal duct epithelium. Mol Cell Endocrinol. 216:83-94.

77. Sipila P, Shariatmadari R, Huhtaniemi IT, Poutanen M. 2004 Immortalization of epididymal epithelium in transgenic mice expressing simian virus 40 T antigen: characterization of cell lines and regulation of the polyoma enhancer activator 3. Endocrinology. 145:437-446.

78. Xin JH, Cowie A, Lachance P, Hassell JA. 1992 Molecular cloning and characterization of PEA3, a new member of the Ets oncogene family that is differentially expressed in mouse embryonic cells. Genes Dev. 6:481-496.

79. Araki Y, Suzuki K, Matusik RJ, Obinata M, Orgebin-Crist MC. 2002 Immortalized epididymal cell lines from transgenic mice overexpressing temperature-sensitive simian virus 40 large T-antigen gene. J Androl. 23:854-869.

80. Wang C, Swerdloff RS. 2004 Male hormonal contraception. Am J Obstet Gynecol. 190(4 Suppl):S60-68.

81. Kamischke A, Nieschlag E. 2004 Progress towards hormonal male contraception. Trends Pharmacol Sci. 25:49-57.

LIST OF ORIGINAL CONTRIBUTIONS

1. Treatment using novel dual 5α-reductase inhibitors was shown, for the first time, to be a viable and useful approach to study DHT action in the rat epididymis. Prior to these studies, there were very few reported analyses of the effects of 5α-reductase inhibitor treatment on the epididymis and none that used a dual inhibitor to maximally decrease DHT levels.

2. The effects of dual 5α-reductase inhibitor treatment on gene expression in the rat epididymis were determined using cDNA arrays containing 1178 cDNAs. It was demonstrated that treatment affected epididymal gene expression in a dose-dependent and highly segment-specific manner.

3. Gene expression in the initial segment responded uniquely to dual 5α-reductase inhibitor treatment in that a similar number of genes increased and decreased in expression, whereas in the other epididymal segments the predominant effect of treatment was decreased gene expression.

4. Insight was provided into the DHT regulation of genes potentially involved in important processes that contribute to the formation of the epididymal luminal microenvironment that is critical for proper sperm maturation; these processes included fatty acid and lipid metabolism, regulation of ion and fluid transport, luminal acidification, oxidative defense and protein processing and degradation.

5. Treatment with a dual 5α-reductase inhibitor had segment-specific effects on sperm motility. There was no effect of treatment on the motility of sperm from the caput epididymidis. Treatment decreased the percentages of motile and progressively sperm and altered the characteristic sperm motion parameters of sperm from the cauda region.

6. Phase-contrast and electron microscopy showed that dual 5α-reductase inhibitor treatment affected sperm morphological changes associated with epididymal sperm maturation, but did not affect sperm ultrastructural features that are established in the testis. Namely, treatment resulted in a higher proportion of sperm from the cauda epididymidis that retained their cytoplasmic droplet.

7. The effects of dual 5α-reductase inhibition on male fertility were determined. Females naturally mated with treated males had fewer successful pregnancies and a higher rate of preimplantation loss. The rate of postimplantation loss and progeny outcome were unaffected.

8. The epididymal expression profiles of both 5α-reductase isozymes and the androgen receptor were determined using the novel technique of quantitative real-time RT-PCR. These profiles were analogous to cDNA array and Northern blot analyses of gene expression.

9. Novel segment-specific expression profiles for IGF, FGF, TGF and VEGF family members were established in the epididymis using quantitative real-time RT-PCR.

10. Two different dual 5α-reductase inhibitors were shown to have analogous effects on epididymal gene expression.

11. Dual 5α-reductase inhibitor treatment had gene and segment-specific effects on the expression of genes involved in mediating androgen signaling in the epididymis. The expression of 5α-reductase type 1 and androgen receptor increased in the proximal epididymis following treatment.

12. Dual 5α-reductase inhibitor treatment had gene and segment-specific effects on the expression of growth factor signaling genes in the epididymis. The expression of IGF-1, IGFBP-5, IGFBP-6, and FGF-10 decreased in the distal epididymis following treatment.

APPENDIX

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

For office use only
Protocol #: 706
Investigator #: 365
Approval End Date: Jan 31, 2004
Facility Committee: MED

McGill University
Animal Use Protocol – Research

Title: Regulation of epididymal functions
(must match the title of the funding source application)

New Application: **Renewal of Protocol:** # 206 **Pilot:** **Category** *(see section 11)*: D

1. Investigator Data:

Principal Investigator: Bernard Robaire **Phone #:** 398-3630
Department: Pharmacology and Therapeutics **Fax#:** 398-7120
Address: 3655 Promenade Sir-William-Osler **Email:** brobaire@pharma.mcgill.ca

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

Name: Bernard Robaire **Work #:** 398-3630 **Emergency #:** 935-3828
Name: Trang Luu **Work #:** 398-6241 **Emergency #:** 731-6517

3. Funding Source:

External: X
Source (s) : CIHR
Peer Reviewed: YES X NO**
Status : Awarded Pending
Funding period: ~~ongoing~~ 4/1/02 - 3/31/07

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

For Office Use Only:

ACTION	✓	DATE
CCs	✓	Feb 9/03
DB(G)	✓	Nov 17/03
APPROVED		

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

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

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

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

Principal Investigator's signature:		**Date:** Feb 12, 2003

Approved by:

Chair, Facility Animal Care Committee:		**Date:** 12/2/2003
University Veterinarian:		**Date:** 2/20/03
Chair, Ethics Subcommittee (as per UACC policy):		**Date:** 2/20/03
Approved Animal Use	**Beginning:** Feb 1, 2003	**Ending:** Jan 31, 2004

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

McGill University Internal Radioisotope Permit

20050242

Permit Holder & Position	Building (Office)		Building (Lab)	Laboratory Classification		Date Issued
BERNARD DR. ROBAIRE, PROFESSOR	MCINTYRE BUILDING		MCINTYRE BUILDING	BASIC	**PLEASE POST**	2004/09/01
Department	Room Number(s)	Telephone	Room Number(s)	Telephone		Expiry Date
PHARMACOLOGY AND THERAPEUTICS	104	398-3630	115,116	398-6241		2005/08/31

PERSON(S) APPROVED TO WORK WITH RADIOISOTOPES

Name	Train.	Cond(s)	Class(es)	Radioisotope(s)
BERNARD ROBAIRE, PROFESSOR	Y	2, 4, 3	1	P-32, H-3, C-14, I-125
NATALI HENDERSON, PH.D. STUDENT	Y	2, 4	1	P-32, H-3, C-14, I-125
KATIA ZUBKOVA, MSC STUDENT	Y	2, 4	1	P-32, H-3, C-14, I-125
SHAYESTA SEENUNDUN, MSC STUDENT	Y	2, 4	1	P-32, H-3, C-14, I-125
TRANG LUU, TECHNICIAN	Y	2, 4	1	P-32, H-3, C-14, I-125
MAHSA HAMZEH, QUALIFYING STUDENT	Y	2, 4	1	H-3, P-32
FARIDA VAISHEVA, RESEARCH ASSISTANT	Y	2,4	1	H-3, P-32
LUDOVIC MORCOW, PH.D STUDENT	Y	2, 4	1	H-3, P-32
SOPHIE ANNE LAMOUR, GRADUATE STUDENT	Y	2, 4	1	P-32, H-3, c-14, I-125

GENERAL LICENCE CONDITIONS (OPEN AND/OR SEALED SOURCES)

- The permit must be posted with the CNSC safety poster in the permit holder's premises.
- Radioisotope handling shall be in accordance with the McGill Radiation Safety Policy Manual.
- The permit holder must ensure that all persons mandated to work with radioisotopes be properly trained in radiation safety prior to start of work.
- Radioactive work areas must be clearly identified with radiation warning signs.
- Smoking, eating, drinking, storage of foods or drink and the application of cosmetics and contact lenses are prohibited in areas where radioisotopes are used.
- All procedures involving radioactive materials should be carried out on spill trays or on benches lined with disposable absorbent material.
- Procedures that might produce airborne radioactive contamination should be carried out in a functioning fume hood.
- When hand or clothing contamination is possible, protective gloves and clothing must be worn.
- After handling radioactive material and especially before leaving the laboratory, personnel must ensure that all parts of their cloths are not contaminated.
- Purchase and disposal of radioisotopes must be kept electronically or documented in a log book.
- For disposal of radioactive waste, consult the McGill Radiation Safety Policy Manual and/or McGill WMP.
- Wipe tests must be performed and records be kept in a log book.
- The permit must reflect the exact conditions under which radioactive material is used. If changes must be made, contact the RSO at 398-1538.
- The device(s) containing the sealed source(s) must have a radiation symbol and an identification label bearing the name and telephone number of the permit holder.
- Leak tests must be performed on sealed sources equal to or greater than 50 MBq (1.35mCi).
- Extremity dosimeter (i.e. ring or wrist badges) must be worn if 50 MBq or more of P-32, Sr-89, Sr-90 & Y-90 are used.
- Workers using I-125 or I-131 on open bench (5 MBq), in a fume hood (50 MBq) or a vented glove box

Approved Unsealed Radioisotope(s) and Location(s)

Isotope	Possession Limit	Stored	Handled
H-3	< 400 MBq (11 mCi)	116	114, 115
C-14	< 40 MBq (1.1 mCi)	116	114, 115
P-32	< 1 GBq (27 mCi)	117, 116	114, 116
S-35	< 400 MBq (11 mCi)	116	114, 115
I-125	< 400 MBq (11 mCi)	116/1329	114, 116/1329

Approved Sealed Radioisotope(s) and Location(s)

Permanently Housed Source(s)

Isotope	Activity	Stored	Handled
Ra-226	10 uCi (remov	114	114
H-3	196000 dpm	114	114
C-14	105900 dpm	114	114
RA-226	10uCi	114	114

Accessible Source(s)

Isotope	Activity	Stored	Handled
H-3	194,600 dpm	114	114
C-14	71,700 dpm	114	114

Personnel Conditions

1 .Must attend thyroid bioassays within 5 days of use if 50 MBq (1.35 mCi) of I-125 are manipulated in a fume hood.
2 .Must wear a whole-body film badge, if gamma, x-ray or high energy beta emitters are used.
3 .Must wear an extremity TLD dosimeter, if more than 50 MBz (1.35 mCi) of P-32, Sr-89, Sr-90 or Y-90 are used.
4 .Classified as Radiation User.
5 .Classified as Nuclear Energy Worker (NEW).
6 .Does not work with any radioisotopes but may be indirectly exposed.

Workload Classes

1 .Work load < 10MBq (270 uCi) of unsealed radioisotopes in open areas.
2 .Work load < 10MBq (270 uCi) of unsealed radioisotopes in a fume hood.
3 .Work load > 10MBq (270 uCi) of unsealed radioisotopes in open areas.
4 .Work load > 10 MBq (270 uCi) of unsealed radioisotopes in a fume hood.
5 .Work with sealed sources.
6 .Individual does not work with radioactive sources but normal working conditions involve presence in a room where radioactive material is used or stored.

Joseph Vincelli
RSO & Occupational Hygienist
McGill Environmental Health & Safety

Date 27/04/

For: Dr. Ian Butler, Chairperson
McGill University Laboratory Safety Committee
Associate Vice-Principal (Research)

Health Canada / Santé Canada

Health Products and Food Branch / Direction générale des produits de santé et des aliments

20 May 2005

Natali Anne Henderson
Pharmacology & Therapeutics
McIntyre Medical Sciences Building, Room 115
McGill University
3655 Promenade Sir William Osler
H3G 1Y6

Dear Miss Henderson,

As per your request, I hereby grant you permission to reprint, as part of your thesis, the two following manuscripts that I have co-authored:

1) Henderson NA, Cooke GM, Robaire B. Effects of PNU157706, a dual 5-alpha-reductase inhibitor, on gene expression in the rat epididymis. J Endocrinol 2004 May; 181(2):245-261

2) Henderson NA, Cooke GM, Robaire B. Segment-specific expression of signaling pathway genes in the rat epididymis and the effects of dual 5-alpha-reductase inhibitor treatment. Submitted for publication.

Yours sincerely,

Gerard M. Cooke, Ph.D.

Toxicology Research Division
Sir Frederick G. Banting Research Centre
2202D1 Tunney's Pasture
Ottawa, ON
K1A 0L2
Tel: (613) 957-0990
Fax: (613) 941-6959
E-mail: Gerard_Cooke@hc-sc.gc.ca

Proprietary Rights Notice for the Society for Endocrinology's online journals

1. The person using the Society for Endocrinology's online journals may view, reproduce or store copies of articles comprising the journals provided that the articles are used only for their personal, non-commercial use. Uses beyond that allowed by the "Fair Use" limitations (sections 107 and 108) of the US Copyright law require permission of the publisher.

2. For permissions to copy beyond that permitted by copyright law, contact the Reproduction Rights Organisation in your country (e.g. the Copyright Clearance Center (USA) or the Copyright Licensing Agency (UK)).

3. Any uses and or copies of these journals in whole or in part must include the customary bibliographic citation, including author attribution, date, article title, journal title, and the URL http://www.endocrinology-journals.org/ and MUST include a copy of the copyright notice.

4. Personal accounts and/or passwords may not be shared.

Proprietary Rights Notice for Biol Reprod Online