Androgen Action in the Maintenance of Epithelial Cell Integrity in the Rat Epididymis

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ABSTRACT

Androgens are responsible for maintaining epididymal structure and functions. However, little is known about how androgen action is mediated and the identity of mechanisms underlying the restoration of epididymal cell integrity after androgen deprivation. It was therefore the goal of this thesis to determine the initial and sequential role of androgen action in altering cellular architecture and function in an androgen-deprived condition.

The first objective was to elucidate the morphological changes in the regressed rat epididymal cell epithelium before and after hormone replacement. Using morphometric analysis and antibodies to cell proliferation markers, we determined changes in epithelial cell height and lumen diameter, as well as in the number of nuclei labeled, respectively, in different regions and at various time points after testosterone replacement in the regressed epididymis. We concluded that testosterone induces an increase in the number of new cells and re-expansion of existing cells in the regressed epididymis.

The second objective was to determine the sequence of gene activation or suppression that occurs in an androgen deprived tissue upon re-administration of the two active metabolites of testosterone, dihydrotestosterone and estradiol by using Affymetrix Rat Genome Microarray chips. Interestingly, there are few genes that were regulated by estradiol, while many were affected by dihydrotestosterone. Using Pathway Assist software, we identified the early response pathway activated by dihydrotestosterone. Epidermal growth factor (EGF) and insulin-like growth factor

(IGF1) appear to play an important role in the pathway due to their function in regulation and expression of many other genes.

Lastly, we established the intracellular signaling pathway that may play a central role in mediating androgen action in restoring epithelial cell integrity in the epididymis. Involvement of two potential pathways activated by dihydrotestosterone, MAPK/ERK and AKT, in the proximal caput (PC-1) epididymal cell line was investigated. Using specific inhibitors for each pathway and an androgen receptor antagonist, we assessed the involvement of the androgen receptor in these pathways. IGF1 and EGF receptors were found to be the important mediators of MAPK/ERK pathway activations.

Collectively, the results obtained from these studies provide a greater understanding of the mechanisms of androgen action in the epididymis.

RÉSUMÉ

Les androgènes sont responsables du maintien de la structure et des fonctions de l'épididyme. Cependant, nous en savons très peu sur la manière dont les androgènes agissent et l'identité des méchanismes sous-jacents à la restauration de l'intégrité cellulaire épididymale après privation des androgènes. Le but de cette thèse a donc été de déterminer le rôle initial et séquentiel de l'action des androgènes dans l'alteration de la structure architecturale et de la fonction en condition de privation des androgènes.

Le premier objectif a été d'élucider les changements morphologiques dans l'épithélium cellulaire épididymal dans le rat régressé avant et après remplacement de l'hormone. En utilisant une analyse morphométrique et des anticorps pour marquer la prolifération cellulaire, nous avons respectivement déterminé les changements dans la hauteur des cellules épithéliales et du diamètre de la lumière ainsi que le nombre de noyaux marqués dans différentes régions et à différents moments après remplacement de la testostérone (T) dans l'épididyme régressé. Nous avons conclu que la T induit une augmentation du nombre de nouvelles cellules et une ré-expansion des cellules existantes dans l'épididyme régressé.

Le second objectif a été de determiner la sequence d'activation ou de suppresion des gènes qui arrivaient dans un tissu privé d'androgènes après ré-administration des deux metabolites actifs de la T, dihydrotestostérone (DHT) et l'estradiol (E2) en utilisant des micropuces Affymetrix Rat Genome. Peu de gènes ont été régulés par l'E2, alors que beaucoup ont été affectés par la DHT. En utilisant, le programme Pathway Assist, nous avons identifé le chemin de réponse

activé tôt par la DHT. Le facteur EGF (Epidermal growth factor) et le facteur IGF1 (Insulin-like growth factor) semblent jouer un rôle important dans le chemin dû à leur fonction dans la régulation et l'expression de plusieurs gènes.

Finalement, nous avons établi le chemin de signalisation intracellulaire qui pourrait jouer un rôle central dans la médiation de l'action des androgènes pour restaurer l'intégrité cellulaire épithéliale dans l'épididyme. L'implication de deux chemins potentiels activés par la DHT, MAPK/ERK et AKT, dans la lignée cellulaire épididymale PC-1 a été exploré. En utilisant des inhibiteurs spécifiques pour chaque chemin et un antagoniste du récepteur à androgènes (AR), nous avons évalué la participatation d'AR dans ces chemins. Les récepteurs IGF1 et EGF ont été identifés comme des médiateurs importants de l'activation du chemin MAPK/ERK.

Ensemble, les résultats obtenus de ces etudes apportent une meilleure comprehension des mechanisms d'action des androgens dans l'épididyme.

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PREFACE

Thesis Format

This is a manuscript-based thesis, which conforms to sections I.C. of the "Thesis Preparation and Submission Guidelines" of the Faculty of Graduate Studies and Research of McGill University. This thesis is composed of five chapters. Chapter one is a general introduction; it includes a comprehensive review of the epididymis, androgens, and androgen regulation in the epididymis. This chapter concludes with a rational for the studies presented in this thesis. Chapter two to four are data chapters followed by connecting text to ensure continuity of the thesis. Chapter two was published in Journal of Andrology: Hamzeh M, Robaire B (2009) Effect of testosterone on epithelial cell proliferation in the regressed rat epididymis. J Androl 30:200-212. The copyright agreement of the respective publisher by the American Society of Andrology (http://www.andrologysociety.com) allows this manuscript to be included in this thesis provided it is for non-commercial use. Chapters three and four have been submitted for publication. Chapter five contains a discussion of the overall results and includes ideas for future studies; it is followed by a list of original contributions. References are included at the end of each chapter. The ethics certificates for work on animal subjects and for the use of radioactive material and the proprietary right notice for the Journal of Andrology are submitted separately.

Contribution of Authors

All the experiments and analysis described in this thesis were completed by the candidate with the exception of the microarray experiment described in chapter three, which was done at the DNA Microarray Centre at McGill University, Genome Quebec Innovation Centre by Yannik Fortin.

CHAPTER 1

Introduction

1. The Epididymis

The epididymis (derived from the Greek words meaning adjacent to the testis) is a major component of the male excurrent duct system present in all mammals (1). In the late 20th century, advanced technology in research allowed scientists to resolve many unknowns with respect to epididymal characteristics, concerning the role of this tissue in sperm storage, maturation and the development of sperm potential to fertilize an oocyte (2-4). Since then, the number of studies to understand the epididymal physiology has dramatically increased. The following part of the introduction will focus on the structure, functions, cell culture, genomic and proteomic profile of the epididymis.

1.1. Structure

1.1.1. Gross Anatomy

Epididymides occur in pairs, one attached to the posterior side of each testis and they are connected to the testis via the efferent ducts. The mammalian epididymis is a single, long and highly convoluted tubule comprised of three major compartments: the lumen that contains spermatozoa and fluid, the epithelial cells, and the inter-tubular compartment composed of connective tissue occupied by smooth muscles, blood vessels, nerves, and lymphatics (5;6). The epithelial cells consist of different cell types that are discussed in the next section. The epididymis is generally divided into four distinct regions based on structural and functional parameters: the initial segments, caput (head), corpus (body), and cauda (tail) (Fig. 1a) (5-7). It is suggested that the proximal part of the epididymis is derived from the

mesonephric tubules, while the rest of the epididymis is derived from the mesonephric or Wolffian duct (8;9). The microenvironment of each region is unique and specialized with respect to different proteins, ions, and organic solutes secreted and absorbed by epithelial cells of related region (1).

1.1.2. Cell types of the Epithelium

There are six types of epithelial cells in the epididymis: principal, basal, clear, narrow, halo, and apical cells; their presence and proportion are region-specific along the epididymis (Fig. 1b) (6;10).

Principal cells are the prominent cell types and are particularly sensitive to the presence of androgens. These columnar cells appear along the entire tissue with approximately 65-80% of the total epithelial cell population but show structural and functional differences in each region (6;11). Principal cells play an active role in the synthesis of a large number of proteins, secretion and transportation of small organic molecules, as well as endocytosing proteins found in the lumen of the epididymis (6;11-13). These result in creating different compositions of the luminal fluid along the tubule which is crucial for sperm maturation, as discussed in the next section.

The second most abundant cell types, basal cells, appear throughout the epididymis. Flat and elongated in shape, they adhere to the basement membrane with processes that extend along the basement membrane and cover a large proportion of the circumference of the duct (6;11). They have a role in cellular detoxification and regulation of electrolyte and water transport (14;15).

Clear cells are large cells that are present in the caput, corpus, and cauda epithelium of the epididymis. They have endocytic properties and can actively take up materials from the lumen (6;11). A part of these materials comprises cytoplasmic droplets that are released by spermatozoa as they transit through the epididymis (16). They also mediate luminal acidification; the key proteins for this process localized selectively in these cells are H^+-adenosine triphosphatase (vacuolated [V]-ATPase) in their apical region, carbonic anhydrase II, and soluble adenylate cyclase (17-19).

Narrow cells are narrower than principal cells with a thin process of cytoplasm that reaches the basement membrane and are only found within the epithelium of the initial segment (20). They are involved in endocytosis, secretion of H^+ ions into the lumen, and region-specific expression of proteins such as glutathione-S-transferases and lysosomal enzymes (19;20).

Halo cells are small cells with a clear cytoplasm located at the base of the epithelium throughout the epididymis (6). It has been suggested that halo cells are the primary immune cells in the epididymis; these cells consist of helper T lymphocytes (CD4+), cytotoxic T lymphocytes (CD8+), and monocytes (21;22).

Apical cells are similar to narrow cells as they are only found in the epithelium of the initial segment, but they differ from narrow cells in their morphological appearance, relative distribution, and expression of proteins. They have apically located spherical nuclei and do not contact the basement membrane (23). Aside the observation that there are many proteolytic enzymes in these cells and that they

have the ability to endosytosis substances from the lumen, little is known about their specific functions (20).

Figure 1. Schematic representation of the epididymis and tubule compartments.

a) The epididymis is divided into four major regions: initial segments, caput, corpus, and cauda. Spermatozoa produced in the testis enter the initial segment through the efferent duct. They traverse along the duct and remain in the cauda epididymidis until ejaculation.

b) The three compartments of the epididymis as well as the main cell types of the tissue are illustrated. Adapted from reference (7).

Figure 1a)

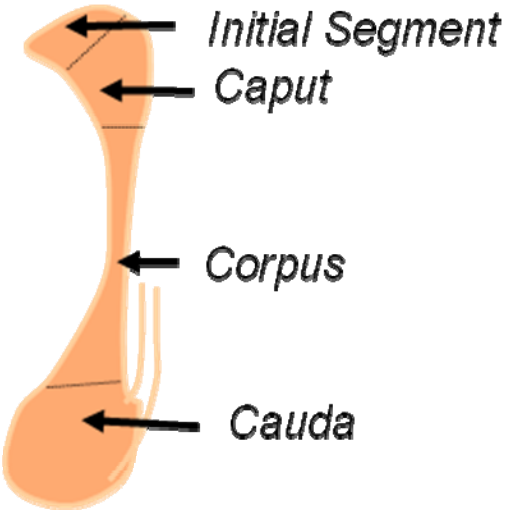
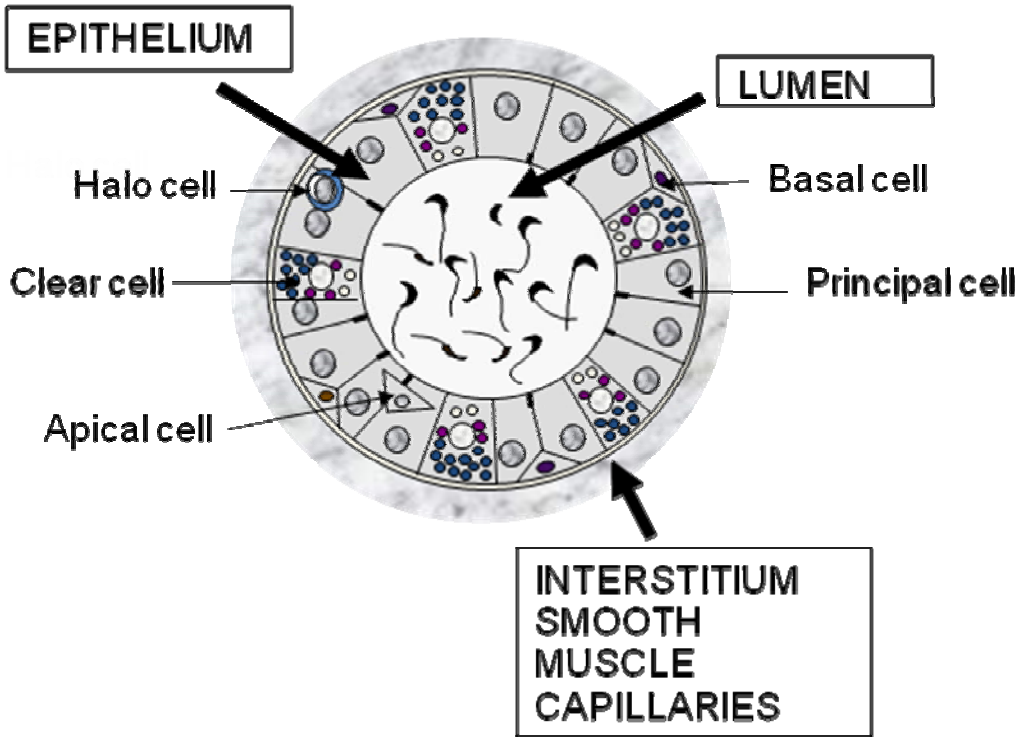


Figure 1b)



1.1.3. Epididymal Differences between Mammalian Species

The regional characteristics of the epididymal epithelium, the luminal microenvironment, and changes in sperm maturation seem quite similar in all the mammals studied; however, there are some differences between the species. The length of epididymis is species-specific; it can measure 1 m in mice (24), 3 m in rats (25), 3 to 6 m in humans (26), and up to 80 m in horses (27). Besides that, the sperm transit time through the duct varies according to species; it takes 11 days in the rat and 5 days in humans (6). Although the epididymis is generally divided into four regions, it can be subdivided into more than four regions depending on the species and depending upon the type of study. While some research groups have identified 10 segments in the mouse epididymis (28), 19 in the rat (29), and 9 in the boar (30) epididymis based on their gene expression pattern, others have proposed 9 distinct segments in the mouse epididymis (31) and 7 in the human epididymis (32) based on their protein profile pattern. It has also been divided into 6 intra-regional segments surrounded by connective tissue septa in the mouse epididymis and 13 in the rat epididymis (33). In my thesis I will use the most common scheme of the epididymis with four regions.

1.2. Functions

The four main functions of the epididymis are sperm transport, final maturation, storage, and protection of sperm from harmful agents (7).

1.2.1. Sperm Transport

Released spermatozoa from the testis are passed through the efferent ducts to the epididymis by testicular fluid and the beat of the ciliated cells of the efferent ducts. However, in the epididymis, they are subjected to the drastically reducing fluid flow because of the massive fluid uptake taking place in the efferent ducts and the initial segment, consequently they transport against an increasing hydrostatic pressure from testis to cauda region, and the epithelium is lined by immotile stereocilia. The mechanism responsible for transporting the contents has been attributed to the frequent muscular contractions of the smooth muscle surrounding the epididymal tubule (6). Neuronal regulation is also important for epididymal contractility. The cauda region particularly has prominent adrenergic innervation with an increase in the thickness of smooth muscle (34;35). Contractility of the epididymal tubule is also influenced by several factors, both hormonal (e.g., testosterone, estrogen) (36;37) and neuronal (e.g., oxytocin, vasopressin) (38-40). Prostaglandins, adrenergic and cholinergic drugs, and temperature also affect the frequency and amplitude of contractions in the epididymal tubule (7).

1.2.2. Sperm Maturation

Sperm are functionally matured and acquire fertilizing ability as they pass through the epididymis (41). The exact region in the epididymis where sperm first gain fertilizing potential varies among species, but generally they become fertile when they reach the distal corpus and proximal cauda (42;43). The percentage of motile spermatozoa is also increased by maturation processes in the cauda region; caput epididymal sperm can swim in a circular pattern, while cauda epididymal sperm can move vigorously and in a straight path (7). Other maturational changes are the migration of the cytoplasmic droplet along the sperm flagellum during epididymal transit, until it is finally shed in the distal region of the epididymis. There are also changes in the sperm nuclear chromatin, which include the formation of stabilizing disulfide bonds between sperm nuclear protamines. Furthermore, the sperm plasma membrane undergoes extensive remodeling of the lipid, protein, and glycoprotein composition; these changes are the result of proteolytic processing and the action of glycolytic enzymes (44-46). Collectively, all changes occur to produce mature sperm with fertilization ability by the time they leave the epididymis.

1.2.3. Sperm Storage

The cauda epididymidis is a major site for sperm storage. In most mammalian species, 50-80% of spermatozoa in the excurrent ducts are present in this region and approximately 50% of them are available for ejaculation; although, in mammals, they can be stored in cauda epididymidis for more than 30 days and retain their function (7). In most mammalian species, sperm stay in an immotile state until

ejaculation; it is thought the maintenance of an acidic pH in the cauda luminal fluid of several species and the presence of immobilin in the rat and hamster play a role in their immobilization during storage. Immobilin, a high molecular weight, mucus-like protein, is secreted by principal cells in the proximal region of the epididymis and is selectively endocytosed by clear cells in the distal cauda epididymidis (47;48). This protein is also thought to protect sperm from shearing during ejaculation (49).

1.2.4. Sperm Protection

The blood-epididymal barrier, formed by the apical tight junctions between adjacent principal cells, creates a protective specialized microenvironment that is important for sperm maturation. In addition, extensive defense mechanisms have been developed in the epididymis to protect sperm from harmful substances such as reactive oxygen species (ROS) (7). The latter is produced by sperm and is required for chromatin condensation and capacitation; however, spermatozoa are very susceptible to lipid peroxidation by ROS because of high content of polyunsaturated fatty acids present in sperm membranes. Lipid peroxidation of sperm membranes has been associated with midpiece defects, decreased motility and intracellular ATP levels, and impaired capacity for fertilization (50-53). The epididymis has numerous antioxidant defense mechanisms; these include the synthesis and secretion of specific proteins such as defensins and defensin-like molecules (7;54), antioxidant and conjugating enzymes, and other antioxidant compounds such as glutathione, taurine, and zinc (55). The major antioxidant enzymes are glutathione peroxidases (Gpx) (56-58), Gamma-glutamyl transpeptidase (GGT) (59-62), indoleamine

dioxygenase (63), and superoxide dismutase (64;65). Expression and activity of different glutathione S-transferases (GST) have been also established in the epididymis (66-70).

1.3. Organ and Cell Culture of the Epididymis

In vitro cell culture systems provide a useful tool for understanding the cellular and molecular mechanisms involved in this tissue. The epididymis, a tissue with complexity of several functions carried out in various regions and cell types, has been investigated in isolation. Over the past few years, many attempts were made to culture epididymal tubules as both organ and primary cell culture for different regions of the epididymis of several species, and more recently, immortalized cell lines have been developed as well (6).

1.3.1. Organ Culture

The use of organ culture to study this tissue has several advantages. For instance, the endocrine environment of the tissue can be precisely defined and easily manipulated, the tissue retains its hormone responsiveness, and anatomic relationships between various cell types and the histological architecture are preserved. Two main methods, static and continuous flow methods, have been used in various species to study epididymal function and sperm maturation. The dependency of this tissue on androgen, more specifically on dihyrotestosterone (DHT) for sperm maturation (71) has been demonstrated; the action of DHT is mediated via the synthesis of RNA and proteins (72), many of them are potentially important for the maturation of sperm (73-77). These cultures, however, can only retain their structural features and characteristics for a few days.

1.3.2. Primary Cell Culture

Using primary cell culture makes it possible to obtain more information with respect to the hormonal regulation and functions of a particular cell type under defined conditions. Such primary cultures have been generated from a number of species, including mice (78), rats (79-81), hamsters (82), rams (83), dogs (84), bulls (85), boars (86), and humans (87;88). These cells are initially round with spherical appearance upon isolation, but they flatten under culture conditions. Some of their morphological features are maintained, e.g., cell polarity, presence of microvilli, Golgi apparatus, prominent rough and smooth endoplasmic reticula, lipid droplets, and multivesicular bodies (81;89). They also maintain some of their functional characteristics including expression of epididymal genes (84), protein secretion (78;87;90), and testosterone metabolism (91). In addition, co-culture studies of sperm with these cells have shown an increase in sperm survival and motility (92;93). Despite demonstration of their usefulness, primary cell cultures have some limitations for the study of epididymal functions. They divide very slowly and can dedifferentiate after a few passages; they have a short life span (81;94). In addition, they are often contaminated with other cell types including fibroblasts.

1.3.3. Immortalized Cell Line

Because of limitations described above for using the organ and primary cell culture, immortalized cell lines of the epididymal epithelium have been developed. Until recently, the molecular events mediating hormone-responsive gene expression was studied in cervical HeLa and prostatic PC-3 cells and extrapolated to the epididymis (95). However, the results may not reflect the same events observed in the normal epididymis. Since then, several stable epididymal epithelial cell lines have been established; these include IMCE canine, (PC1, DC1, DC2, and DC3) mouse caput, A and B2 mouse caput, mE mouse caput, MEPC5 mouse caput, and RCE rat caput epididymis. The majority of epididymal cell lines were generated by transfecting primary cultures of epithelial cells with a plasmid containing a viral oncogene such as simian virus 40 large T-antigen (SV40LT) *in vitro*.

In 1991, the first cell line derived from human fetal epididymis was generated, but the cells lost their epididymis-specific appearance after a few passages (96). Ten years later, an immortalized canine epididymis cell line derived from the whole adult epididymis of mongrels dogs was generated, which was the first cell line from a differentiated, adult epididymal epithelium. These cells maintain the expression of androgen receptor mRNA and protein, and some molecular markers expressed along the epididymis. However, androgen induction of the epididymal marker genes studied was not achieved and may reflect a partial loss of the differentiated phenotype of these cells (97).

Consequently, two other cell lines were generated including MEPC5 and RCE derived from the mouse (98) and rat (99) caput epididymidis, respectively. MEPC5

was established by infecting primary cultured cells with a temperature-sensitive SV40LT which results in cells growth at a permissive temperature of 33^0C (98). These cells retain some characteristics of differentiated epididymis epithelial cells and express a number of epididymal caput-expressed genes such as androgen receptor, retinoic acid receptor α, polyoma enhancer activator 3, and sulfated glycoprotein-2. The RCE cell line is the only rat epididymal cell line established to date (99). They display many characteristics of epithelial principal cells *in vivo* and express many tight (occluding and claudin) and gap junctional (connexin) proteins. This cell line, however, is not androgen responsive and is not completely polarized in terms of distribution of cellular organelles during growth on collagen-coated plates. In addition, RCE cells are not a pure population of caput principal cells; some cells share features similar to principal cells of the intermediated zone and clear cells.

Immortalization of epididymal epithelial cells by using the SV40LT plasmid has also been achieved *in vivo* using transgenic techniques. To prevent growth of aggressive tumor that would kill the transgenic mouse, two strategies were applied.

First, a temperature-sensitive mutant of the oncogene (tsSV40LT) was introduced into mice. Thus, cells immortalization is only achieved when cultured at the permissive temperature (100). Cell lines generated by using this technique are PC-1 from proximal caput, and Dc-1, DC-2, and DC-3 from distal caput derived from four sequential segments of the mouse caput epididymidis (101). These pure populations of epithelial cells can be maintained for more than 1 year with a similar polarity to principal cells *in vivo*; they express cytokeratine, a marker of epithelial cells, as well as a number of principal cell markers that are regulated by androgen

such as epididymal retinoic acid-binding protein (E-RABP). In addition, they express androgen receptor and are androgen responsive.

The second strategy used in transgenic mice was done by generating immortalized epididymal epithelium by expressing SV40LT under a 5.0-kb mouse glutathione peroxidase 5 promoter (GPX5-Tag1) (102). Eighteen epithelial cell lines, named mE-Cap, were generated (103). These cell lines retain several features specific to epididymal epithelium, such as expression of mouse epididymal 1 (ME1), mouse epididymal protein 9 (MEP9), androgen receptor (AR), and polyoma enhancer activator 3 (PEA3) that is one of the transcription factors responsible for epididymal gene expression. However, unlike the PC-1 cell line, the level of AR is markedly reduced in mE-Cap cell lines.

Lastly, the cell lines A and B2 were derived from primary cultures of the mouse caput epididymidis (104). They are spontaneously immortalized cell lines and selected for their ability to proliferate and maintain their characteristics for a long period. Although they adopt some of the characteristic features of principal cells *in vivo* and express several epididymal genes, they are not responsive to androgen.

Such immortalized epithelial cell lines constitute valuable tools for studying the cellular and molecular events in this tissue, despite the fact that they lose part of their differentiated state, but they maintain some of the characteristics of differentiated principal cells and are transfectable.

1.4. Genomic and Proteomic Profile in the Epididymis

Underlying many functions of the epididymal epithelium is the controlled expression of genes and proteins. Differential expression of proteins and mRNA along the epididymis is a hallmark of this tissue; numerous studies have been focused on these matters. Several of their highlights are described in this section.

1.4.1. Gene Expression

Gene array technology has provided a powerful method for large-scale gene expression analysis along the epididymis, thereby giving greater insight into the understanding of epididymal functions. In addition, other approaches also have been used to identify individual gene expression such as Northern blots, reverse transcriptase-polymerase chain reaction (RT-PCR), and *in situ* hybridization. Nearly all of the genes identified have characteristic longitudinal expression profiles in the epididymis of all species examined. Region-specific differences in gene expression indicate different functions of each region which is important for the process of sperm maturation (28;30;105-108). It is likely that the differential response of the regions to androgen withdrawal, aging, and stress reflects discrete regulatory events. Regional gene expression data for the rat and mouse epididymis are available online at the following websites:
www.ttuhsc.edu/cbb/faculty/cornwall/default.asp (109) and
http://mrg.genetics.washington.edu. (29)

The proximal part of the epididymis is very active in protein synthesis and secretion compared to the distal region, and many genes encode secretary proteins

that are highly expressed in the proximal part (6). These genes include growth factors (e.g., fibroblast growth factors), tumor suppressors (e.g., brain-expressed myelocytomatosis oncogene), antioxidant enzymes (e.g., γ-glutamyl transpeptidase), and proteases (e.g., matrix degrading metalloprotease matrilysin), protease inhibitors (e.g., cyctatin 12), endopeptidase (e.g., cathepsin H). Others encode intracellular proteins, including transcription factors, signaling molecules and kinases (110). PEA3 is one of the transcription factors that is highly expressed in the initial segments, and it acts as both an activator and inhibitor of Gpx5 and GGT-IV (111-113); PEA3 knockout male mice are infertile (114). Another transcription factor, CCAAT/enhancer-binding protein β (C/ebpβ) is expressed in the proximal regions of the mouse epididymis and regulates the expression of Cres (115). The protein tyrosine kinase receptor, c-Ros is highly expressed in the initial segment of the epididymis; knockout of c-Ros in male mice results in the failure of the initial segment to develop and differentiate, thereby leading to immotile sperm(116). The proto-oncogene A-raf kinase involved in signaling cascade and in transmiting signals from cell surface receptors to transcription factors in the nucleus to regulate gene expression is highly expressed in the initial segments and proximal caput (117).

Some genes, such as epididymis-specific lipocalin genes, are expressed differentially along the epididymis; six lipocalin genes have been identified. Lipocalin 5 (Lcn5 or retinoic acid-binding protein), is expressed from mid caput to cauda (118), is a transcription factor that regulates epididymis-specific genes in which Forkhead box A2 (Foxa2) interacts with androgen receptor and binds to Lcn5 promoter and eventual gene expression (119). The others are known to be important for sperm

maturation, including cysteine-rich secretory protein (CRISP) 1 (108;120;121) and 4 (122), cystatin-related epididymal spermatogenic (Cres) genes (123). A number of ubiquitous lysosomal enzymes are highly expressed in the epididymis such as β-galactosidase, β-hexosaminidase, α-mannosidase. Finally, several expressed peptides may act as paracrine signaling molecules, including neuropeptide Y, proenkephalin, and nerve growth factor (NGF) (110).

In addition to being region-specific, the gene expression profile varies by cell type. While the majority of identified genes are expressed in the principal cells, a few genes have been only localized to the other cell types. There are discussed in greater detail in the next section.

1.4.2. Proteomic Profile

In conjunction with advances in genomic technology, proteomic studies were also carried out by matrix-assisted laser desorption/ionization (MALDI) imaging mass spectrometry (IMS) on tissue sections and laser capture microdissection epididymal cells, and two-dimensional (2D) gel electrophoresis and mass spectrometry. In addition, other approaches also have been used to identify individual protein expression such as immunohistochemistry (IHC) and Western blots. Elucidation of proteins expression and localization contributes greatly for better understanding of the specific links between epididymal gene transcription, translation and function.

Using different aspects of IMS technology, over 400 proteins have been monitored in the mouse epididymis; more than 50 of these proteins display

regionalized epididymal expression or secretion patterns (31). Among these proteins are retinoic acid binding protein (ERABP), glutathione peroxidase (GPX5), and cysteine rich secretory protein-1 (CRISP-1).

Epididymal proteomes and secretomes of different mammals (e.g., rat (124), stallion (125), boar (126), platypus (127), and human (32)) have been established by 2D gel and mass spectrometry.

Unlike in most of the mammalian species, human epididymal proteins are not highly regionalized and appeared in different regions, reflecting the different pattern of sperm maturation and storage between species. Some of these proteins act as secretory proteins (e.g., clusterin, the most abundant protein, and CRISP), lipid binding proteins (e.g., phosphatidylethanolamine-binding protein, and NCP2/CTP/HE1), ion binding proteins (e.g., lactotransferrin, and calmadulin), proteins binding small hydrophobic molecules (e.g., prostaglandin D2 synthetase), antioxidant enzymes (glutamyltransferase, glutathione S-transferase P, thioredoxin peroxidase, and superoxide dismutase), protease and protease inhibitors (e.g., cystatin, and cathepsin D), enzymes (lactate dehydrogenase, malate dehydrogenase, carbonic anhydrase, triose phosphate isomerase, and epididymal SPAM1), HE3, E12, and actin. Except for some major proteins that corresponded to serum components such as albumin, transferrin and α-1-antitrypsin, almost all of the proteins present in the luminal fluid are synthesized by the epithelium (32;128;129).

Using immunohistochemistry methods, several epididymal cell type-specific proteins have been identified. Vacuolar H^+ ATPase, located in the apical part of narrow and clear cells (19;130;131), is required to maintain an acidic luminal pH.

$CD4^+$ and $CD8^+$ leukocytes are expressed in the halo cells in all regions of the epididymis (22). Sulfated glycoprotein-2 (clusterin) is localized in the principal cells along the epididymis (132). Aquaporin 5 and 9 are expressed in the apical membrane of the principal cells in the corpus and cauda regions (133). An antioxidant enzyme, glutathione S-transferase pi, is localized in principal and basal cells of the initial segment and caput and in basal cell of the corpus and cauda epididymidis (134).

2. Regulation of the Epididymis

Several factors regulate the structure and function of epididymis. The most important ones are hormones and non-hormonal factors that originate from the testis. They are described in the following section.

2.1. Hormones

2.1.1. Steroid Hormones

Androgens are the major regulators of epididymal structure and functions (135-137). The main androgen responsible for the maintenance of epididymal cell integrity is dihydrotestosterone (DHT), the active metabolite of testosterone (138-141). 5α-reductases types 1 and 2 are enzymes responsible for the conversion of testosterone to DHT; the activity of type 1 is higher in the proximal than the distal parts of the epididymis, whereas type 2 is expressed uniformly throughout the epididymis, suggesting that conversion of incoming testosterone to DHT takes place mostly in the initial segment (142;143). Androgens and their regulation will be focused on in more depth in section 3.

While it is the main regulator, testosterone is not the only hormone involved in epididymal regulation. Another hormone that acts in regulation of epididymal processes is estradiol. The enzyme cytochrome P450 aromatase irreversibly catalyzes the conversion of testosterone to estradiol (144). In the epididymis, aromatization could occur either in the luminal or the epithelial compartments, particularly in the proximal regions (7). In addition, the presence of cytochrome P450 aromatase in epididymal spermatozoa has been established in several species,

including rat (145), mouse (146), bear (147), and rooster (148). The aromatase enzyme is localized to cytoplasmic droplets in the sperm tail, exhibiting a decreasing expression as sperm traverse the epididymis. Subsequent studies have detected aromatase mRNA, protein, and enzyme activity in epithelial cells, indicating the ability of epididymal principal cells to synthesize estradiol from testosterone in many species such as rat (149), monkey (150), and human (151). In addition, the other sources of estradiol in the adult male reproductive system are both Leydig cells and germ cells in the testis of adults that can actively synthesize estradiol (146;152); the estradiol concentration in caput epididymis fluid can be as high as 250pg/ml, which is higher than in female serum blood (153). Both the absence of aromatase and an excess of estrogen result in male infertility; excess of estradiol induces an increase in androgen binding protein mRNA and mediates negative regulation of proteins involved in nuclear chromatin condensation during spermatogenesis (154;155).

Two estrogen receptors (ERα and ERβ) have been characterized within the epididymis. ERα is localized in narrow, apical and some basal cells of the initial segment, in principal and clear cells of the caput region, and in clear cells of the cauda epididymidis in mice and rats. In contrast, ERβ is detected throughout the entire epididymis but is most abundant in the corpus and cauda regions (7;156).

While these two receptors exhibit 95% identity in the DNA binding domain, less than 55% homology have been detected in their ligand binding domain, and that indicates their functional differences (157;158). Gene knockout studies were used to determine the functional role of estradiol in the efferent ducts and epididymis. ERα knockout mice are infertile because of the inability of efferent duct and the initial

segment of the epididymis to re-absorb the large volume of fluid secreted by the testis, which in turn results in swollen efferent ducts, testicular atrophy, and impaired spermatogenesis (159-161). This re-absorption process is therefore believed to be regulated by estradiol and is proposed to be mediated by the cystic fibrosis transmembrane conductance regulator (CFTR) receptor (162). In contrast, ERβ knockout mice are fertile, having a normal testis and epididymis (163). Therefore, the role of ERβ in the epididymis awaits further investigation. A recent study has identified the expression of few genes regulated by estradiol in the mouse caput epididymidis following orchidectomy; however, the concentration of estradiol used is far greater than that found under normal physiological conditions (164).

2.1.2. Non-steroid Hormones and Vitamins

Oxytocin, a neurohypophysial hormone made in the hypothalamus, plays important roles in the epididymis. The oxytocin receptor has been found to be expressed in this tissue in many species such as human, macaque monkey, and ram. Depending on the species examined, it is localized to peritubular cells as well as to principal and basal cells of the epididymal epithelium in a region-specific manner (40;165-167). The functions of oxytocin and its receptor have been identified by both *in vivo* and *in vitro* studies (7); they promote contractility of the duct, which in turn promotes sperm transport through the epididymis (168). They also promote the stimulation of 5α-reductase activity in the initial segments of the epididymis that results in the formation of DHT (169). Although the mechanism of stimulation has not yet been identified, it has been suggested to be mediated through tyrosine kinase activation and enzyme phosphorylation (170).

Retinoids are highly potent molecules with pleiotropic action in a wide range of biological processes during development and in the adult (171). They most likely play a regulatory role in epididymal function. Several retinoids were identified in the epididymis, including retinol (vitamin A), retinyl ester, all-*trans* retinoic acid, and 9-*cis*-retinoic acid; the concentration of first two retinoids decreased from the caput to the cauda regions, and an inverse gradient has been shown for the concentration of *trans*- and *cis*- retinoic acids in the epididymis. The binding of two nuclear retinoic acid receptors, RARs/RXRs, to retinoic response elements modulates gene expression that is responsible for their biological effects. RARα is localized throughout the epididymis with a high level of immunostaining in the initial segment,

proximal caput, and distal cauda epididymidis (7;172). In addition, the cellular retinol binding protein (CRBP) I and II, and cellular retinoic acid-binding protein (CRABP) I and II are localized in the epididymal epithelial cells of the rat and mouse in a region-specific manner. The retinoic acid receptors and the retinoic acid carrier protein E-RABP are responsible for the retinoid signaling pathway in the epididymis, and are synthesized and secreted into the lumen from the distal caput to the cauda epididymidis. To elucidate the role of retinoids in the epididymis, gene knockout studies have been done. Expression of a dominant negative mutant of RARα induced loss of organization of the columnar epithelium lining the cauda epididymidis, and its transformation by to a squamous metaplasia resulting in blockage or rupture of the duct with inflammation and ultimately, infertility (173;174). The RARα/γ double-null mutants have also shown severe dysplasia or complete agenesis of the epididymis (175).

Vitamin D receptors have been identified in the epididymis. This tissue was found to take up 25(OH)D_3 and metabolize it into two metabolites (176). Interestingly, one of these metabolites, 24,25$(OH)_2$D, was shown to be at higher levels in the epididymis, particularly in the cauda region, than in any other tissue studied. Metabolites of vitamin D are involved in the regulation of phosphorus secretion into the epididymal lumen (6).

Vitamin E plays important roles in the maintenance of spermatid viability and in the functional and structural appearance of the epididymal epithelial cells (177).

2.2. Testicular Factors

The regulation of the epididymal structure and functions, in particular the initial segment, is also dependent on factors coming from the testis directly to the epididymal lumen, other than androgens directly to the epididymal lumen. This type of paracrine regulation has been coined as “lumicrine” since this mode of regulation occurs in a duct/tubal system(178). Such regulation has been studied by efferent duct ligation (EDL), and orchidectomy followed by androgen replacement. EDL is a method commonly used to differentiate between the testicular delivery of androgens and non-anderogenic factors to the epididymis. Dependency of the initial segment of the epididymis on testicular factors is reflected by the structure and gene expression of epithelial cells (179-181). Efferent duct ligation induces apoptosis in epithelial principal cells, within 12 hours in the proximal region of the initial segment (182;183). The wave of apoptosis is completed after 3 days. Apoptosis is not detected significantly in any other region of the epididymis during 15 days after EDL, at which point the epithelial cell height and tubule diameter of the initial segment are decreased to about half of control values, the sparsely granulated endoplasmic reticulum has disappeared, and the golgi apparatus seems to be relatively inactive (181). Further investigation demonstrated that epithelial apoptosis in the initial segment is independent of p53, a key apoptotic pathway molecule in many tissues and conditions (183).

Epithelial gene expression of the initial segment of the epididymis is also dependent on testicular factors. These genes include proenkephalin (184), cystatin-related epididymal specific (CRES) (123), 5α-reductase type 1 (185), γ-glutamyl

transpeptidase IV (GGT) (61), polyoma enhancer activator 3 (PEA3) (113), gluthatione peroxidase 5 (Gpx5) (57;186) and A-raf (187). In addition, androgen-binding protein (ABP), a protein that regulates functions of the proximal regions of the epididymis, is dependent on nonsteroidal testicular factors. It is synthesized by Sertoli cells in the testis and secreted into the seminiferous tubule, of which 80% enters the epididymal lumen (7). ABP is also synthesized and secreted by epididymal principal cells along the epididymis, and there is evidence that this protein may be under the control of a testicular factor (188). ABP after binding androgen is taken up by the principal cells of the initial segment and caput regions, where it can regulate epididymal nuclear 5α-reductase.This mechanism is important to provide a high concentration of dihydrotesosterone, which is required to mediate androgen action in the epididymis (7). In addition, luminal fluid ABP is speculated to regulate the bioavailibity of androgens in the extracellular space of the epididymis and protect androgens from metabolism. It has also been suggested that sex-hormone binding globulin, an ABP analogue, stimulates protein synthesis and secretion in the caput epididymis in the presence of ABP (179).

Basic fibroblast growth factor (bFGF) is another testicular factor that interacts with its cognate receptor on the apical cell of initial segment and regulates GGT IV activity via signaling transduction cascades, e.g., the ras-raf-MAPK pathway and PEA3 activation (179;189;190). Several genes including steroid 5α-reductase type 1 expressed in the rat initial segment contain the PEA3/Ets binding motif, and most of them are regulated by testicular factors (191).

The presence of spermatozoa is another factor that might regulate initial segment function. Some evidence indicates that testicular spermatozoa can carry ligands and transport them to different epididymal regions. Many molecules are known to bind to spermatozoa such as growth factor receptors; they then dissociate from the sperm surface upon entering the initial segment and become available to stimulate their cognate receptors on the apical surface of the epididymis (7).

Recently, microarray analysis of gene expression was also used to determine the effect of testicular factor deprivation by EDL. This type of analysis allowed examination of the transcriptome-wide gene expression of each region of the epididymis. Regional gene expression data for the rat epididymis are available at the Mammalian Reproductive Genetics websites: http://mrg.genetics.washington.edu (192). Consistent with the results found previously, a largest number of affected genes was observed in the proximal region of the epididymis. Prostaglandin D_2 synthase (Ptgds), CD52 antigen (Cd52), and glutathione peroxidase 3 (Gpx3) are example of genes that are up-regulated in at least one region, and cystatin 8 (Cst8; also known as CRES), defensin β1 (Defb1), and 5α-reductase 1 (Srd5a1) are example of genes that are down-regulated in all regions of the epididymis after EDL (192).

2.3. Growth Factors

Growth factors are a group of polypeptides that induce a variety of cell activities such as cell division and differentiation in a paracrine or endocrine manner. Several studies have suggested that they may play an important role in the epididymis (193). While most of studies were focused more on basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and epidermal growth factor (EGF), the role of these growth factors and others is not fully understood in the epididymis.

FGFs are a large family of growth factors, possessing various biological functions. Similar to other growth factor receptors, FGFRs are membrane proteins with extracellular ligand binding domains and intracellular tyrosine kinase domains that activate signaling cascades. The Fgf and several different Fgf receptors transcripts are differentially expressed along the mouse epididymis, and all four types of FGFR are localized in the principal cells of the initial segments in the rat. Thus a lumicrine mode of action for FGF has been suggested (189). The role of bFGF on the GGT activity has been established after testicular factors deprivation in the epididymal lumen (190).

VEFG was characterized as a potent mitogen specific for vascular endothelial cells. The expression of Vegf and its receptors has been detected in the mouse and human epididymis (194;195). Several biological effects of VEGF via activation of its receptor have been explored; these include an increased number of capillaries, opened tight junctions, and intracellular vesicles. Interestingly, over expression of VEGF in the testis and epididymis of transgenic mice causes infertility (196). It is

believed that VEGF is secreted by Sertoli cells and reaches the epididymis via the lumicrine route. Alternatively, it may regulate the activity of epididymal epithelium, in particular the initial segment in the mouse (193).

EGF, mainly produced by the submaxillar gland, has a potential role in the male reproductive system. Lack of this growth factor leads to a marked decrease in the number of epididymal sperm cells (197). The EGF receptor is present in both the testis and epididymis. It is immunolocalized intensely in the cytoplasm of principal cells in the caput, and only apical cytoplasm of principal cells in the corpus and cauda epididymis (198). These findings suggest the possibility that EGF plays a meaningful role in the regulation of the epididymis.

Insulin-like growth factor (IGF) is structurally related to proinsulin, and is regulated by IGF binding proteins. It is localized in the rat epididymal epithelial cells (199). Null mutations for this growth factor result in decreased level of androgen, a poorly developed epididymis, reduced spermatogenesis and organ size, and infertility (200). IGF-1, therefore, is important for maintaining epididymal integrity.

It has also been suggested that some growth factors secreted by the proximal epididymis may regulate the more distal epididymal regions. These growth factors involved in paracrine mechanism of regulation include nerve growth factor (NGF), platelet-derived growth factor (DPGF), and transforming growth factor-β (TGF-β) (197); the precise role of these growth factors and their regulation in the epididymis needs to be further investigated.

3. Androgens

Androgens are nonaromatized C19 steroids that mediate a wide range of developmental and physiological responses in the reproductive and non-reproductive systems (201). In males, androgen is responsible for sexual differentiation in *utero* and for pubertal changes; it also plays a role in maintaining libido, spermatogenesis, muscle mass, bone mineral density, and erythropoiesis. Reproductive functions of androgens are mediated through coordination of diverse physiological processes ranging from brain functions to specific cell functions in the target tissue (202). In male, the main androgens are testosterone and its more potent metabolite, dihydrotestosterone (DHT). The following section focuses on regulation of androgen synthesis and secretion, mechanisms of androgen action, and androgen regulation in the epididymis.

3.1. Regulation of Androgen Synthesis and Secretion

3.1.1. Hypothalamus-Pituitary-Testis Axis

The gonadotropins, LH and FSH control steroidogenesis and spermatogenesis respectively, in the testis (203). They are produced and secreted by the gonadotropic cells of the anterior pituitary in response to the hypothalamic gonadotropin-releasing hormone (GnRH). Both androgen synthesis and gamete maturation are regulated by the hypothalamus and pituitary via a negative feedback mechanism (204;205). Testosterone also inhibits the secretion of both GnRH and gonadotropins. Similar to testosterone, estrogen produced by Leydig cells also reduces LH stimulation of testosterone biosynthesis; both androgen and estrogen receptors are localized to the hypothalamus and the pituitary gland (203). Binding of LH to the G-protein coupled membrane receptors on testicular Leydig cells stimulates adenylate cylase activity and an increase in cyclic adenosine monophosphate (cAMP) formation which subsequently leads to protein kinase activation, resulting in steroid hormone formation(206). LH and cAMP regulate steroidogenesis in an acute (minutes) or chronic (hours) manner (207).

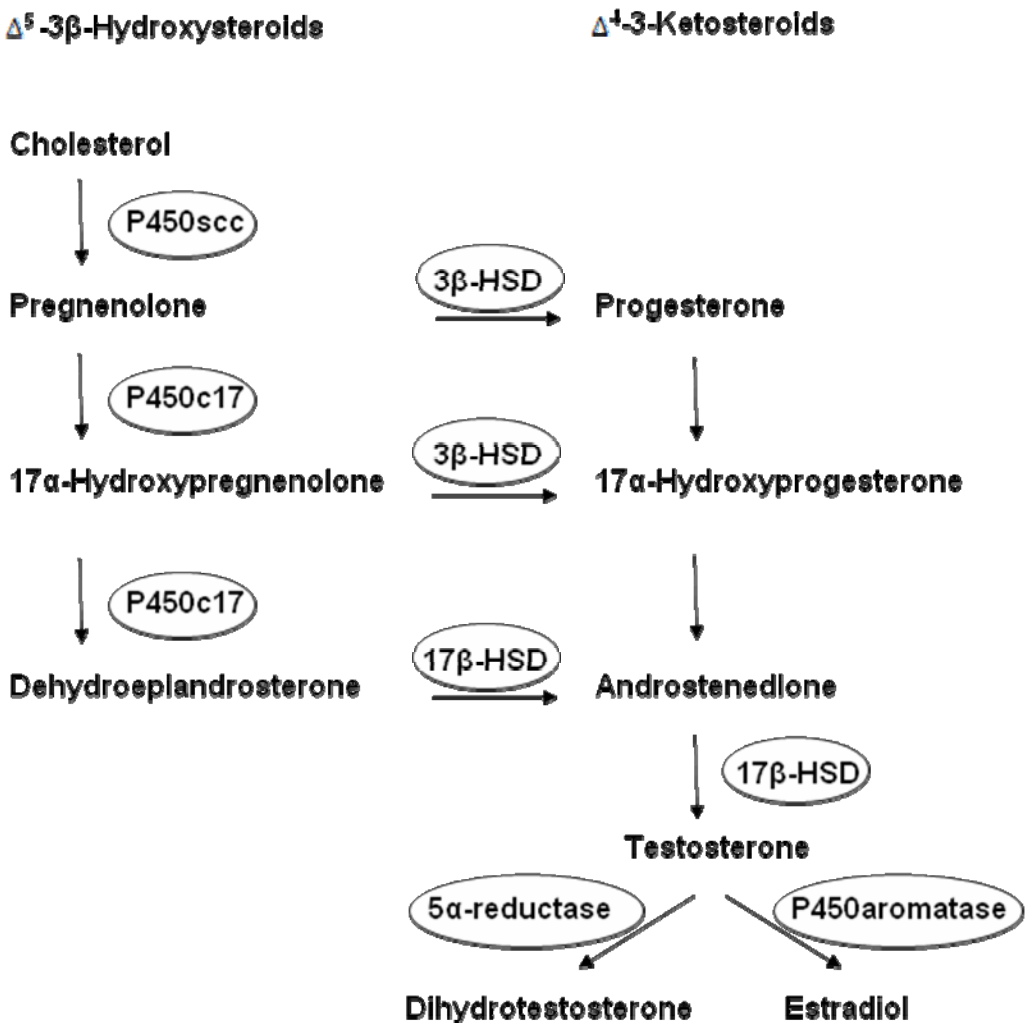
3.1.2. Steroidogenesis in Leydig Cells

While androgens are produced predominantly by Leydig cells of the testis, adrenal glands also produce androgens at much lower levels (208). Adult Leydig cell testosterone production depends on the pulsatile secretion of LH by the pituitary gland (209). A cascade of events is initiated by binding of LH to specific receptors on the Leydig cell plasma membrane which results in increased cAMP formation and cAMP-dependent phosphorylation of proteins through protein kinase A (PKA) (210). In response to LH, cAMP stimulates the transport of cholesterol to the inner mitochondrial membrane where it is metabolized into pregnenolone by the cytochrome P450 enzyme CYP11A. Pregnenolone moves to the smooth endoplasmic reticulum where it is subsequently converted to progesterone, androstenedione, and is finally metabolized to testosterone by type 3 of 17β-hydroxysteroid dehydrogenase (17β-HSD3), an enzyme that is exclusively expressed in Leydig cells (211-213). Consequently, androgen production of the adrenal glands is limited to dehydroepiandrosterone (DHEA) and androstenedione (Fig. 2).

Figure 2. Testosterone biosynthetic pathways from cholesterol

Testosterone is synthesized from cholesterol in Leydig cells through the Δ^5-3β-Hydroxysteroids or Δ^4-3-Ketosteroids pathway. Cholesterol is transported to the inner mitochondrial membrane and is metabolized to pregnenolone by the cytochrome P450cholesterol side chain cleavage (P450scc). The latter is metabolized to testosterone by enzymes located in the smooth endoplasmic reticulum: 3 β hydroxysteroid dehydrogenase (3β-HSD), cytochrome P450 17α-hydroxylase/17,20 lyase, and 17β hydroxysteroid dehydrogenase (17β-HSD). Adapted from references (211;212).

Figure 2


Δ5 -3β-Hydroxysteroids
Δ4-3-Ketosteroids
Cholesterol
P450scc
Pregnenolone
3β-HSD
Progesterone
P450c17
17α-Hydroxypregnenolone
3β-HSD
17α-Hydroxyprogesterone
P450c17
Dehydroepiandrosterone
17β-HSD
Androstenedione
17β-HSD
Testosterone
5α-reductase
P450aromatase
Dihydrotestosterone
Estradiol

3.1.3. Secretion, Transport, and Metabolism

After synthesis, lipophilic steroids immediately diffuse through the cell membrane into the circulation down the concentration gradient (214). Testosterone is highly bound to plasma proteins, and only 1-3% of total testosterone circulates freely in the blood. The vast majority of protein-bound testosterone is bound to albumin with low affinity or to a carrier protein called sex hormone-binding globulin (SHBG) with high affinity (206). SHBG is an analogue of androgen binding protein with a single steroid-binding site; both are products of a single gene and they differ only in the types of oligosaccharide associated with them (215). The free testosterone which exists upon rapid dissociation of albumin-bound testosterone, diffuses from the intravascular space into the interstitium and then to target cells where it can bind receptors to exert biological effects (206). Testosterone is converted to other active steroid hormones, principally, either dihydrotestosterone (5α-DHT) by 5α-reductases in the presence of NADPH or estradiol (17β-E2) by aromatase. Both reactions are irreversible and rate-limiting (216;217). These steroids have different receptors, thereby different biological activities are revealed in their respective target tissues.

Testosterone can also be metabolized in the blood to inactive 17-ketosteroids called androsterone and etiocholanolone. Both of these metabolites are excreted mainly as glucuronide conjugates or to a lesser extent as sulfate conjugates (208).

3.2. Mechanisms of Androgen Action

The traditional mode of androgen action is via its cognate nuclear receptors. The androgen receptor is expressed in reproductive tissues, as well as in many non-reproductive tissues. The mechanisms by which AR exerts different actions in diverse tissues can be attributed to several factors including, the tissue-specificity of the co-regulators involved, the DNA response element-specificity of target genes, and the expression of metabolizing enzymes and their relative levels (201). For instance, muscles do not have significant amounts of 5α-reductase; therefore testosterone acts directly on the AR without converting to DHT. In addition to the classical action of androgens, increasing evidence suggests that similar to estrogen and progesterone, androgens can also exert rapid non-genomic effects (218-220). As with other steroid hormones, the non-genomic actions of androgens are widely assumed to be mediated by cell surface membrane receptors; however, in some cases the androgen receptor itself is not involved in the mechanism (221). In the following section, the androgen receptor and its mechanisms of action are explored in more details.

3.2.1. Androgen Receptor

The androgen receptor is a member of the nuclear receptor superfamily that functions as ligand-dependent transcription factors. Structurally, it is composed of a variable amino-terminal transactivation domain, a highly conserved DNA-binding domain (DBD), a hinge domain with a nuclear localization signal (NLS), and a C-terminal ligand-binding domain (LBD) (222-224). In mammalian tissues, a single gene for the AR has been identified; in humans, the gene consists of 8 exons, and multiple transcripts are produced with variable lengths of a polymorphic region of CAG repeats in exon 1(225;226). The length of the repeat is inversely correlated with the transactivation activity of AR (227). A number of clinical disorders of the AR are associated with an increase in size of a polymorphic tandem CAG repeat. The functional significance of AR in male sex differentiation has been demonstrated in several cases of germ line AR mutations which cause androgen insensitivity syndrome, producing phenotypic females. AR mutations can cause a diverse range of diseases such as testicular feminization mutation (Tfm) syndrome, prostate cancer, and Kennedy's disease (228-231). In addition to men, the Tfm has also been described in other species such as rats (232), mice (233), and dogs (234). The human AR amino acid sequence is very similar to that of the rat with identical sequences in the DNA- and ligand-binding domains (235). In mammals, the AR is mainly expressed in androgen target tissues including, skeletal muscle, bone, skin, brain, liver, and cardiovascular system, as well as in male reproductive tissues (201).

In the epididymis, the AR is ubiquitously expressed throughout all regions of the tissue and in all species examined such as rat (155), mouse (236), rabbit (237), goat (238), stallion (239), monkey (240), and human (241). Depending on the species studied, AR is immunolocalized to all epididymal regions and epithelial cells with different intensity, except the halo cells because of their origin; intense staining is observed in the principal cells of the human tissue with a declining concentration from the caput to the cauda epididymidis (7). The androgen receptor is a key transcription factor mediating androgen-induced signaling. Expression of androgen responsive genes is regulated by the AR, upon binding as homodimers to androgen response elements (AREs) located within the promoter regions of target genes. Most AREs contain a consensus sequence composed of two 6-base assymetrical elements separated by three spacer nucleotides; 5'-AGAACAnnnTGTTCT-3' (242).

Male AR knockout (ARKO) mice show a feminized appearance of the external genitalia; they have small inguinal testes with spermatogenesis arrested at pachytene spermatocytes. All of the seminal vesicles, vas deferens, epididymis, and prostate are absent in ARKO males (243-245). They have very low serum testosterone levels, high LH serum levels, and normal serum estrogen levels. The androgen receptor, therefore, is essential for the development of male reproductive tissues and completion of spermatogenesis, but not for its initiation.

3.2.2. Genomic Androgen Action

Similar to the other steroid receptors, unbound AR is mainly located in the cytoplasm and is associated with heat shock proteins. In target tissues, testosterone either acts directly through the AR or is reduced to dihydrotestosterone (DHT) by 5-α reductase, before binding to AR. DHT is more a potent AR ligand than testosterone; it binds to the AR with a 2-3 fold higher affinity and 5 fold slower dissociation rate than testosterone (246). Upon binding of testosterone or DHT, the androgen receptor undergoes a series of conformational changes. These include the dissociation of heat shock proteins from the AR, and its dimerization and phosphorylation. The complex is proposed to be detected by import receptors such as importin-α and importin-β which bind to AR nuclear localization signal (NLS) and is translocated to the nucleus (247;248). In the nucleus, the DNA-binding domain region of the receptor binds to the androgen response elements (AREs) located in the promoter, enhancer or repressor regions of target genes. Several co-regulators of transcription such as co-activators and co-repressors, and transcription factors associated with RNA polymerase II, are recruited for the transactivation of AR-regulated gene expression (249). All of these processes are initiated by the ligand-induced conformational changes in the ligand-binding domain. The nature of AR bound-ligands determines the stability of AR-DNA complexes and, ultimately, the amount of transcription initiated. Therefore, differences in ligand binding can potentially result in differences in ARE function and subsequent transcription production. This effect is evident where gene expression has been found to be regulated differently in response to testosterone and DHT in the rat prostate and

may be due to the lower affinity of the AR for testosterone (250;251). Subsequently, non-ligand-bound AR is shuttled back to the cytoplasm and recycled for further ligand binding, or is degraded by the proteasome (252).

3.2.3. Non-genomic Androgen Action

In addition to its well-characterized genomic action, the non-genomic mode of action of androgen has also been reported in many cells such as oocytes (253), skeletal muscle cells (254), osteoblasts (255;256), glioma cells (257), prostate cancer cells (258;259), and Sertoli cells (260;261). The non-genomic action of the AR is characterized by rapid onset varying from seconds to minutes, and is also insensitive to inhibitors of transcription and translation; this indicates the absence of a direct involvement of nuclear receptors (220). These non-genomic effects can be mediated 1) by a non-classical AR that is possibly associated with the plasma membrane and some extend through a classical intracellular-AR, 2) in the absence of an AR by direct binding to a specific-binding site of the target molecule, or 3) via changes in membrane fluidity, as hydrophobic androgen can interact with the polar head of membrane phospholipids to influence membrane fluidity (Fig. 3) (262;263). The initiation of second messenger cascades in the non-genomic pathway may ultimately modulate the transcriptional activity of AR or other transcription factors and has been implicated in a number of cellular effects, including gap junction communication, cellular adhesion, intracellular calcium-hemostasis, aortic relaxation, and neuronal plasticity (262;264).

Androgens act through several pathways and they are connected to each other. The localization of the AR to the plasma membrane is not well characterized. However, the AR has been found to be associated with caveolin, an integral membrane protein that serves as a scaffolding protein for many signaling molecules, including phosphatidylinositol-3 (PI-3K), Ras, and Src (265;266); AR localization to caveolin is ligand dependent. The majority of serum androgens bind glycoproteins such as sex hormone-binding globulin (SHBG) and its analogue, androgen-binding protein (ABP). They are multifunctional proteins that act to regulate the androgen responses and the bioavailability of free androgen to target cells (267-269). SHBG is located on membranes of hormone-responsive cells such as those present in the epididymis, testis, prostate, skeletal muscle, and neurons (270-272). Therefore, the AR transmits its signals via G_s-protein coupled SHBG-receptors (SHBG-R) through the modulation of adenylate-cyclase with cAMP synthesis and PKA activation. For activation of this pathway, SHBG must first bind to the SHBG-R before binding to the androgen, and SHBGs bound to steroid are not able to interact with the SHBG-R (273;274). In the prostate, SHBG/SHBG-R complexes activated by estradiol can cross-talk with the AR, and are able to activate AR, even in the absence of androgen (267). Activated PKA stimulates the expression of the AR-regulated genes such as prostate-specific antigen (PSA) (275;276). The SHBG/SHBG-R complex acts as part of a steroid signaling pathway independent of the classical intracellular steroid receptors.

The MAPK pathway is another important route mediated by G-protein in response to hormone. The extracellular signal-regulated protein kinases (Erk-1/2)

are the only members of the MAPK family activated by androgen via the AR and G-protein coupled receptor (256;277;278). The androgen-AR complex interacts with the Src-homology 3 (SH3) domain of Src kinase, resulting in c-Src kinase activity (279;280) and subsequently leads to activation of Ras, which is able to activate other protein kinase cascades in the pathway. Once Ras is activated, it recruits and binds Raf-1, a serine-threonine kinase. Raf-1 is activated when it associates with the membrane, and subsequently, activates the MAPK-Erk-kinase (MEK), and Erk-1/2 (277). Alternatively, androgen activates L-type calcium channels via interacting with a membrane associated AR (mAR) through an inhibitory G-protein (281;282). This increase in intracellular calcium activates PKC, and via calmodulin (CAM) activates the PKA and MAPK pathways. In addition, upon binding of the androgen to mAR, G-protein (G_q) activity is modulated, which leads to activation of phospholipase C (PLC). Synthesis of inositol-3-phosphate (IP_3) and diacylglycerol (DAG) is induced by PLC. Increases in IP_3 result in the release of intracellular calcium ion stores from the sarcoplasmic reticulum (SR), and consequently the activation of MEK/Erk pathway (283). These protein kinases play a key role in cell growth, differentiation, and function at both the transcriptional and post-transcriptional level by phosphorylating a range of proteins, including nuclear transcription factors, protein phosphatases, as well as hormones and growth factors. Androgen can also rapidly activate the phosphatidylinositol-3 kinase/Akt pathway in a ligand-binding independent manner (284;285). Activated PI-3K catalyzes the phosphorylation of PIP_2 to yield PIP_3, in response to many growth factors and cytokines. PIP_3 can

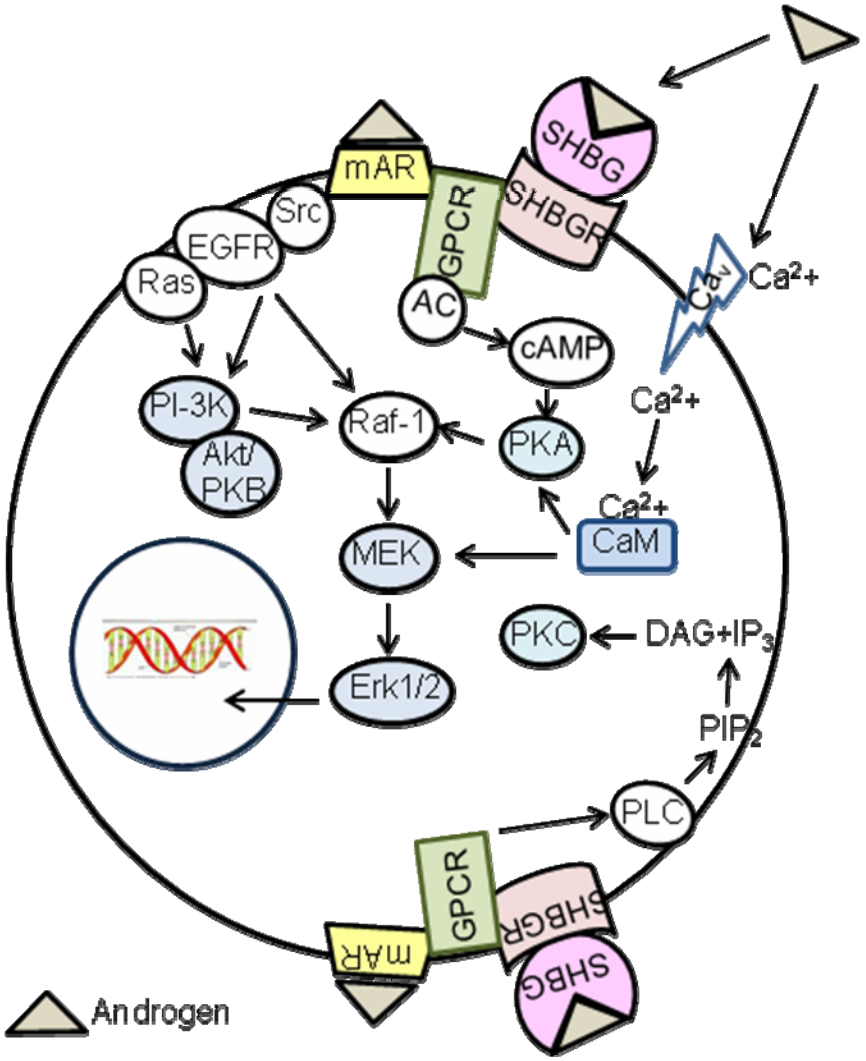
directly stimulate protein kinase B (PKB), also called Akt, which in turn induces other kinases to phosphorylate and activate PKB (285;286).

There is evidence that androgen may interact with ion-channels including the $GABA_A$ receptor, resulting in an increase in intracellular calcium, and consequently in membrane potential (283). Furthermore, androgen can change membrane flexibility by interacting with phospholipids in the membrane bilayer, subsequently altering the function of sodium/potassium or calcium ATPase (287-289). Despite the elucidation of all these pathways, the physiological relevance of non-genomic actions of androgen has not yet been determined and more extensive researche is still required.

Figure 3. Non-genomic, rapid action of androgen

Androgens mediate their rapid effects and stimulate second messenger cascades through several mechanisms. SHBG: sex hormone-binding globulin; SHBGR: SHBG receptor; mAR: membrane associated androgen receptor; CaM: calmodulin; cAMP: cyclic adenosine-3,5-monophosphate; GPCR: G protein-coupled receptor; AC: adenyl cyclase; PKA: protein kinase A; PKC: protein kinase C; PKB: protein kinase B; PI3K: phosphatidylinositol-3-kinase; Ca_v: voltage-gated Ca^{2+}-channel; Src: steroid receptor coactivator; IP_3: inositol-1,4,5-triphosphate; DAG: diacylglycerol; EGFR: epidermal growth factor receptor; MEK: mitogen-activated protein/extracellular signal-regulated kinase; Erk: extracellular signal-regulated protein kinase; PLC: phospholipase C. Adapted from reference (221).

Figure 3


mAR
SHBG
SHBGR
GPCR
Src
EGFR
Ras
AC
cAMP
Ca2+
Ca2+
PI-3K
Akt/
PKB
Raf-1
PKA
Ca2+
CaM
MEK
PKC
DAG+IP3
Erk1/2
PIP2
PLC
GPCR
SHBGR
mAR
SHBG
Androgen

3.3. Androgen Regulation of the Epididymis

The epididymis is a highly androgen-dependent tissue; sources of androgen in the epididimis are androgens that are directly input by the adjacent testis, and circulating androgens supplied by the vasculature. The level of androgen in the testicular fluid entering the epididymis is approximately 10 times higher than circulating androgen level. Testosterone bound to ABP is taken up by principal cells of the epididymis where it is reduced to DHT (7;290).

The role of DHT in mediating androgen action in the epididymis has been established from lines of evidence obtained *in vitro* and *in vivo*. Firstly, DHT was found to be the active androgen present in epididymal nuclei after injection of radiolabeled testosterone (291). Also, *in vitro,* epididymal cells can synthesize DHT from testosterone (140;292), which has been demonstrated to be more potent than testosterone in maintaining epididymal functions, a result consistent with the *in vivo* system (71;138).

Androgen regulation of epididymal structure and function has been established by either androgen deprivation via GnRH antagonist treatment and bilateral orchidectomy (the removal of both testes after the efferent duct and testicular blood vessels are ligated), or treatment with AR antagonists to inhibit androgen action. Orchidectomy, followed by androgen replacement, is the most commonly used method to ascertain the role of androgen in the tissue because it results not only in the loss of androgens but also of testicular factors (290). In this section several key effects of androgen on the structure, and gene and protein expression of the epididymis will be discussed.

3.3.1. Structure of the Epididymis

The most apparent consequence of orchidectomy is the decrease in reproductive tissue weight. In the rat epididymis, it induces epididymal weight to decrease to 25% of the control over two weeks and by a further 5% in the subsequent two weeks (290). This weight loss is due to the removal of spermatozoa and luminal content, as well as the cytoplasmic shrinkage along the epididymis. Unlike with other androgen-dependent male reproductive tissues, testosterone replacement, even at supraphysiological levels, cannot restore the epididymal weight to control levels, but maintains it to approximately half of that of the control, indicating that nearly 50% of epididymal weight is attributable to spermatozoa and luminal fluid bathing them (140). Orchidectomy also induces a decrease in luminal diameter and epithelial cell height and the thickening of the lamina densa of the basement membrane (293). Total epididymal protein, RNA, and DNA content are reduced following orchidectomy but DNA concentration is increased; this increase occurs as a result of decline in cell volume. Principal cells are particularly sensitive to the presence of androgen. Morphological changes seen in an androgen-deprived state of these cells include a striking loss of apical microvilli from their surface, lysosome accumulation, vacuolization, disappearance of endoplasmic reticulum and vesicles from the cell apex, and increased endocytosis (294;295). Epididymal androgen receptor and 5α-reductase activity are also decreased in this state. (290).

In addition, androgen withdrawal by orchidectomy induces a wave of apoptotic cell death along the rat epididymis (182). The affected cells are primarily principal cells; apoptosis is seen in the initial segment of epididymal epithelium 18

hours after orchidectomy and after 2, 4, and 5 days in caput, corpus, and cauda, respectively, and disappear after 1 week, apparently due to the gradual removal of testicular contents from the lumen. The percentage of the apoptosis-positive tubules is higher in the proximal regions than in the corpus and cauda epididymidis. Testosterone replacement at physiological levels can reverse aggressive changes in the caput, corpus, and cauda epididymidis after orchidectomy, but not in the initial segment. As discussed earlier in section 2.2, the initial segment region is dependent on both testosterone and testicular factors.

Unlike other sex accessory tissues such as the prostate, the epididymis has a low mitotic index (296), and androgens have no effect on the mitotic rate in the adult epididymis (297). This effect may be due to the anti-proliferative signals that originate from the transcription factor B-myc, which is highly expressed in the epididymal epithelium, and its expression is dependent on both androgens and testicular factors (298).

3.3.2. Epididymal Gene Expression and Proteome

In addition to epididymal morphology, androgens also regulate the expression of many genes and synthesis of numerous proteins synthesized in this tissue, which are consequently important for epididymal functions. Ion transport, intermediary metabolism, activity of certain enzymes, and synthesis and secretion of a number of proteins that are thought to be essential for sperm maturation, are regulated directly or indirectly by androgens (6). Therefore, as it is expected, orchidectomy results in an altered expression of many genes. Interestingly, only few of these genes contain functional androgen response elements (AREs) in their promoter region, including glutathione peroxidase (Gpx5) (299;300), lipocalin 5 (95), reproductive homobox5 (301), and Crisp-1 (302). In addition to them, many different genes have been identified as being androgen responsive in the epididymis. Androgen receptor (303), glutathione peroxidase 3 (Gpx3) (304), carbonic anhydrase 2 (Car2), carbonic anhydrase 4 (Car4) (305) are examples of such genes. The expression of others is dependent on both androgens and testicular factors, and these do not return to the original level upon androgen replacement after orchidectomy; some of these genes include proenkephalin, cyctatin-related epididymal specific, 5α-reductase 1, γ-glutamyl transpeptidase (GGT) (179), EP17 (306), a disintegrin and metalopeptidase domain 28 (Adam28), and solute carrier organic anion transporter family member 4C1 (Slco4c1) (307). Moreover, certain genes such as GGT differentially respond to androgen in the different regions of the tissue, suggesting that region-specific AR coregulators, or a combination of AR coregulators with transcription factors can mediate androgen regulation of epididymal genes (308).

Using microarray technology, genome-wide expression profiling of androgen regulation of genes expressed in the epididymis has been undertaken (164;307;309;310). While some of the genes expressed are regulated by androgen along all the tissue, others are region-specific. The epididymal retinoic acid-binding protein (Erabp) (310;311), a disintegrin and metalloprotease 7 (Adam7) (310;312), thiopurine methyltransferase (Tpmt) (313), and glutathione S-transferase mu (Gstm2) (314) are up-regulated, and insulin growth factor binding protein 5 (Igfbp5) and secreted phosphoprotein-1 (Spp-1) (310) are down-regulated in all regions of the epididymis in orchidectomized mice treated with androgen. Highlights of genes regulated by androgen in a region specific manner include ros1 proto-oncogene (Ros1) and lipocalin 8 (Lcn8) in the initial segment, Gpx5, mannosidase 1 α (Man1α), angiotensin converting enzyme (Ace), glutamine synthetase (Glul), myo-inositol synthase A1 (Isyna1), aflatoxin B1 aldehyde reductase (Afar), and amino acid transporter N2 (Aatn2) in the caput region, and Car2, Car4, glycine dehydrogenase (Gldc), Myomesin 2 (Myom2), and protein tyrosine phosphatase receptor type 0 (Ptpro0) in the corpus region, and Serpine-2 and prostaglandin-endopreoxide synthase 2 (Ptgs2) in the cauda epididymidis (310). In addition, the beta-defensins Defb41 and Defb42 genes were identified by *in silico* analysis; they are androgen-regulated genes with antimicrobial activity, mainly expressed in the proximal caput epididymidis (315).

Using two-dimentional gel electrophoresis followed by mass spectrometry, proteomic profiling of epididymal protein regulated by androgen during rat genital tract development has been described (316). Several proteins have been

characterized as cystoskeletal proteins (e.g., α-tubulin, β-actin, tropomyosin 5, and F-actin capping protein α-2), RNA binding proteins (e.g., hnRNP A2/B1/A3), transport proteins (e.g., transferrin, fibrinogen β/γ, and serum albumin precursor), chaperones (e.g., Hsp-47), and glycolytic enzymes (e.g., α-elonase, triosephosphate isomerase, and glyceraldehydes 3-phosphatedehyrogenase). In addition, RhoGDI1was identified as a protein that was regulated by androgen at the level of posttranslational modification; this protein along glycolytic enzymes induced by androgen, may be an important functional link between signaling pathways and cytoskeletal rearrangements in growth and development of the male internal genital tract.

Since all of these genes and proteins under the control of androgens are involved in processes for the formation of an optimal luminal microenvironment, any changes in their expression would be expected to have a serious impact on proper sperm maturation. Despite the fact that many data exist with respect to the role of androgens in the epididymis, still no concrete information is available pertaining to the sequential effect of androgens in maintaining the integrity of this tissue structurally and functionally.

4. Formulation of Project

Androgens play crucial roles in male reproductive and non-reproductive tissues. Understanding of the physiology of male reproductive system and the way androgens act has clinical implications in the development of new treatments for reproductive tissue cancers and age-related pathologies such as benign prostatic hyperplasia, in the management of alopecia and acne, treatment of male infertility, as well as in the design of effective male contraceptives. Among different male reproductive tissues, the epididymis has been found as an attractive target tissue, particularly, for elucidating underlying causes of male infertility or control of male fertility because it is not only dependent on androgens, but is also the site of production of functional sperm. It has a unique cellular and longitudinal organization and creates a highly specialized microenvironment in which sperm are gradually matured as they pass through its lumen. Despite these therapeutic enticements, much remains to be known about androgen action and mechanisms involved in mediating and regulating epididymal functions. It is therefore the overall goal of the present thesis to determine the role of androgen action in maintaining cellular architecture and function in the epididymis. To achieve this goal, three key aspects of androgen action are investigated in an androgen-deprived and regressed epididymis model after androgen replacement.

It is well known that androgen withdrawal induces a decrease in weight and a dramatic change in histology of the epididymis. Androgen replacement can partially restore epididymal weight; that weight gap is presumably due to the weight of spermatozoa and the luminal fluid bathing them and will not return after

orchidectomy. In addition, the mitotic rate in the adult epididymis is known to be very low. Therefore, chapter 2 of this thesis addressed how the sequential effects of testosterone restore the epididymal architecture in each region of the regressed rat epididymis. In this study size of the tubule and the number of new cells were investigated.

Behind all of the structural changes induced by androgen replacement in an androgen-deprived tissue, there must be a cascade of events with respect to gene regulation. While the regulation of many genes by androgen has been described in the epididymis, the impact of androgen stimulation in the sequence of gene activation or suppression is still poorly understood. Chapter 3 of this thesis will examine the sequence of gene activation or suppression in the regressed rat epididymis upon re-administration of two active metabolites of testosterone. Several lines of evidence from the literature have confirmed that dihyrotestosterone (DHT) is the main androgen acting on the epididymis. Estradiol also plays an important role in the integrity of epididymal function. Thus, two metabolites of testosterone, DHT and estradiol (E2) were used for this study. In addition, as discussed above, several *in vivo* studies have confirmed that the proximal region of the epididymis is very active in protein synthesis and secretion. Hence, the initial segment and caput epididymidis were employed for this experiment. This was accomplished on a large-scale with Affymetrix Microarray technology using Rat Genome 230-2 Microarray chips, followed by using Pathway Assist Software to visualize known regulatory relationships between the genes obtained by array analysis.

As discussed above, the epididymis is composed of numerous cell types. Principal cells are the most abundant cells, and are particularly sensitive to androgen. With the advent of immortalized epididymal cells, it has become possible to understand the role of androgen action in a pure population of principal cells, by isolation at the cellular level. While the consequences of various androgenic conditions on gene regulation were investigated in the epididymal cell line, the rapid action of androgen at the cellular level has never been explored.

Therefore, chapter 4 examined the intracellular signaling pathway activated by DHT that may have an important role in mediating androgen action, in restoring epithelial cell integrity in the epididymis. Involvement of two potential pathways activated by DHT, MAPK/ERK and AKT, in the mouse proximal caput (PC-1) epididymidis cells was investigated. Using specific inhibitors for each pathway, activation of signaling cascades in response to hormone of the related pathway were assessed.

These studies provide a better understanding of androgen action in the maintenance of epididymal cell integrity.

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CHAPTER 2

Effect of Testosterone on Epithelial Cell Proliferation in the Regressed Rat Epididymis

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Abstract

It is well established that testosterone plays a crucial role in maintaining the integrity of epididymal structure and function. However, the role of testosterone in restoring the cellular architecture of the regressed epididymis is not well known. The present study was undertaken to test the hypothesis that testosterone triggers the regressed epididymis by re-expanding existing cells and inducing cell proliferation. Testosterone-dependent epididymal morphology was evaluated in orchidectomized, regressed rats after initiation of treatment with testosterone. Besides that, the proliferative activity of epithelial cells in all regions of the epididymis of the orchidectomized, regressed rats was assessed at 1, 3, 7, and 28 days after testosterone replacement. Epithelial cell proliferation decreased after testosterone withdrawal and increased following testosterone administration. We found that BrdU-incorporation and PCNA expression increased significantly 3 days after testosterone replacement in all regions of the regressed epididymis except in the initial segment. The highest mitotic activity was seen in the corpus epididymidis at 3 days postimplantation. Using specific markers for each cell type, we found no significant changes in the proportion of each cell type compared to control. We observed labeled nuclei in all epithelial cell types in control; however, principal cells were the major cell types that responded to testosterone after regression. These observations demonstrate that the mammalian epididymis is not a static tissue without any significant cell renewal, both under control conditions and when androgen exposure is altered, thus providing new insight in the role of androgen in restoration and maintenance of the architecture of the epididymis.

Introduction

The mammalian epididymis, a single coiled tubule, is divided into 4 distinct regions based on structural and functional parameters: the initial segment, caput, corpus, and cauda (1;2). Maturation of spermatozoa, including the acquisition of motility and capacity to fertilize an oocyte (3;4), occurs during transit through the unique and dynamic microenvironment of the epididymal lumen and is supported by androgen dependent activities of the epididymal epithelium (3;5); the latter is comprised of 5 major cell types: principal, basal, clear, apical, and halo cells. Of these, the principal cells are the most abundant (6) and are particularly sensitive to the presence of androgens (7;8). Morphological changes seen after orchidectomy include a striking loss of apical microvilli, disappearance of endoplasmic reticulum and vesicles from the cell apex, lysosome accumulation, vacuolization, and increased endocytosis (7;9). Androgen deprivation by orchidectomy induces epididymal weight loss to 25% of control over 2 weeks, and a further 5% loss in the following 2 weeks; this weight loss is due to cytoplasmic shrinkage, removal of spermatozoa and the luminal fluid coming from the testis (10;11) and a wave of apoptosis along the epididymis (12). Testosterone replacement, even at suraphysiological levels, cannot restore the epididymal weight to control levels, but restores it to approximately 50-60% to that of control (13). Furthermore, all four segments of the epididymis do not respond similarly to exogenous testosterone administration; after orchidectomy, restoration of circulating testosterone levels appears sufficient to reverse regressive changes in the caput, corpus and cauda epididymidis but not in the initial segment. Decreases in tubule diameter, epithelial

cell height, degree of cytoplasmic differentiation, and maintenance of several proteins following efferent duct ligation demonstrate the dependency of the initial segment on testicular fluid for maintenance of normal structure and function (14;15).

Using tritiated thymidine autoradiographic studies, cell turnover in the epididymal epithelium of rodents has been investigated (16). In the epididymis of the adult rat and hamster, cell proliferation and DNA synthesis are extremely low; among all the cell types, principal cells have the highest proliferative activity (17-19). Principal and basal cells of young adult rats have relatively higher mitotic activity than those of fully mature animals (17). Furthermore, after androgen withdrawal, the incidence of cells undergoing apoptosis is very low, and these cells appear to be primarily principal cells; apoptosis is seen in the epithelium of the initial segment of the epididymis 18h after orchidectomy and after 2, 4, and 5 days in caput, corpus, and cauda, respectively, and becomes undetectable by 8 days post-orchidectomy (12).

The purpose of the present study was to investigate, in all regions of the epididymis, whether testosterone triggers an enhancement of the proliferative activity of the regressed epithelial cells over time, leading to re-establishment of the epithelial cell structure. First, we determined the morphological changes in the regressed epididymal epithelium after testosterone administration. Second, we assessed mitotic activity of epididymal cells by using two different mitotic markers, bromodeoxyuridine (BrdU) and proliferating nuclear antigen (PCNA) (20). BrdU, a halogenated derivative of thymidine, is incorporated into nuclei during the DNA synthetic phase of the cell cycle (21;22). PCNA is an auxiliary protein of DNA

polymerases δ and ε, an enzyme necessary for DNA synthesis (23-25); it is also involved in DNA repair. Using specific markers for each cell type, we identified whether the proportion of each cell type was changed upon re-administration of testosterone in the regressed epididymis. We found that principal cells were the major cell type showing mitotic activity in the regressed epididymis in the presence of testosterone and that testosterone triggers a restoration of the morphological changes induced by orchidectomy.

Matherials and Methods

Animals and Treatments

Male Sprague-Dawley rats (300-350 g), purchased from Charles River Laboratories Ltd (St. Constant, Quebec), were used for all experiments. Rats were housed at the McIntyre Animal Resources Centre, McGill University, on a 14 hours light, 10 hours dark schedule with food and water provided *ad libitum.* They were divided into 9 groups (n=4-5/group). One group served as sham control and the other 8 groups were orchidectomized through the scrotal route; both testes were removed after a ligature was placed around the efferent ducts and testicular blood vessels. Eight days after surgery, sufficient time to allow elimination of circulating testosterone and emptying of the epididymal duct (12), 4 groups of rats were implanted subdermally with empty polydimethylsiloxane (Silastic) rods while the other 4 groups received testosterone-filled rods. PDS (Dow Corning Medical Grade 0.078 mm ID, 0.125 mm OD) implants were prepared as previously described (26). Filled implants containing testosterone (3x6.2 cm; Steraloids Inc, Newport, Rhode

Island) released steroid at a rate of 24 µg/cm/day, to approximate concentrations found in rete testis fluid, *i.e*., 10-12 times greater than those of plasma (11;27). All animals were treated with one intraperitoneal injection of BrdU (100 mg/kg; Sigma, St Louis, Missouri) 2 hours prior to sacrifice. At this time after treatment, labeled cells were in the S or G2 phase of the cell cycle (28). Animals were perfused 1, 3, 7, and 28 days post implantation. The design of this study was approved by the McGill University Animal Care Committee (protocol No. 206).

Tissue Preparation

Male Sprague Dawley rats (n = 4-5) were anesthetized with an IP injection of anaesthetic cocktail of Vetalar (Ketamine HCL 115.4 mg/ml, Bioniche, Belleville, ON), Anased (Xylazine HCL 20 mg/ml, Novopharm, Toronto, ON), normal saline, and Atravet (Acepromazine Maleate 10 mg/ml; Ayerst, Montréal, Canada) 20:10:10:1. The epididymides of each rat were fixed with Bouin's solution via perfusion through the abdominal aorta for 10 minutes. After perfusion, epididymides were removed and cut along their long axis. The tissues were then immersed overnight in Bouin fixative, dehydrated, and embedded in paraffin. Sections (6 µm) were cut on a microtome and mounted on glass slides.

Morphometric Observations

Thirty-five to forty cross sections of each epididymal region (8-10 each from four separate rats) were measured to obtain mean values for tubule diameter and epithelial height. Microvilli were not included in measuring epithelial height.

Immunohistochemistry for PCNA and BrdU

Immunostaining was done as described previously (29). Mouse anti-PCNA (1:3000) and BrdU (1:500 [3µg/ml; Sigma]) affinity-purified monoclonal antibodies were used. Negative controls were processed in parallel, but without the primary antibodies. Using a light microscope, we analyzed the number of immunoreactive cells, as determined from the observation of 100 tubules from 3 cross sections of each epididymal region (n = 4-5).

Immunohistochemical Staining of Epididymal Cell Types

Sections were prepared and immunostained as described above, using a Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, California). Rabbit anti-GSTpi (GSTP1) antibody (1:250 [20 µg/ml]; MBL International, Woburn, Massachusetts) was used to identify basal cells; GSTP1 is localized in principal and basal cells of the initial segment and caput and in basal cells of the corpus and cauda epididymidis; its expression is independent of testicular fluid (30). Rabbit anti-V-ATPase B1/2 (vacuolar proton adenosinetriphosphatase, ATP6V1B1) polyclonal antibody (1:250 [0.8 µg/ml]; Santa Cruz Biotechnology, Inc, Santa Cruz, California) was used for clear cells; ATP6V1B1 pump were immunolocalized in the narrow cells and clear cells of the epididymis (31). Goat anti-CD4 polyclonal antibody (1:50 [4µg/ml]; Santa Cruz) was used for halo cells (32). Rabbit anti-clusterin (CLU) - alpha/beta polyclonal antibody (1:50 [4µg/ml]; Santa Cruz) was used for principal cells (33). For each rat (n = 4/ per group), 5 or 6 cross sections of corpus

epididymidis from control and 3 days postimplantation groups were stained and measured to obtain the proportion of each cell type.

Serum Testosterone Analysis

At the time of sacrifice, blood was collected and serum was obtained by centrifuging the blood for 2 minutes at 12,000 x g. Supernatants were collected and frozen at -20°C for further analysis. A commercially available testosterone ELISA kit (Research Diagnostics, Flanders, New Jersey) was used to measure the total serum testosterone concentration in each group, according to the manufacturer's instructions. Sensitivity of the assay was 0.1ng/ml, and the intraassay coefficient of variation was less than 10%. Serum testosterone values were 2.6 ± 0.8 ng/ml in sham control, undetectable in orchidectomized with empty implant, and 8.4 ± 2.7 ng/ml in the orchidectomized group with testosterone implant (n = 5/group).

Statistical Analysis

Statistical analysis was done using 1-way ANOVA followed by the multiple comparisons Dunnett's test, or unprotected t test. Data were expressed as means ± SEM. Values of $p \leq 0.05$ were regarded as statistically significant.

Results

Measurement of Tubular Diameter and Epithelial Cell Height

Tubular diameters of the regressed epididymides were decreased to 31%, 14%, 13%, and 43% of control in the initial segment, caput, corpus, and cauda

regions, respectively; these changes remained constant for orchidectomized animals with empty implants throughout the 28-day study period. After testosterone replacement, tubule diameters were significantly increased after 3 days in all segments except the initial segment and cauda; these showed a significant increase only by day 7 (Fig. 1). Orchidectomy resulted in a significant decrease in epithelial cell height in the initial segment and the caput regions, no change in the corpus and an increase in the cauda region; these trends were sustained throughout the 28-day study period except for the corpus region where a decrease was observed by the end of the study period (Fig. 2). The significant increase in cell height in the cauda region at all time points is presumably due to the removal of the fluid from the extensive lumen, thus causing expansion of epithelial cells. After testosterone replacement, the height of epithelial cells of the corpus region showed the most rapid and most extensive response; a significant increase was noted in that region at 3 days, while the initial segment did show a response until 7 days and in the caput and cauda regions until 28 days. Interestingly, epithelial cell height did not reach the control values in the initial segment and caput even after 28 days (Fig. 2A and B), but significantly increased compared to that of the control in the corpus and cauda regions (Fig. 2C and D).

Immunohistochemical Analysis for BrdU and PCNA in Control Rat Epididymides

In control animals, staining for both PCNA (Fig. 3a, c, e, and g) and BrdU (3b, d, f, and h) was observed in all regions. Most of the immunoreactivity was observed over principal cells for both PCNA and BrdU; however others cell types also showed

some staining. As shown clearly in the figure, we observed labeling in principal, basal, and narrow cells in the initial segment, halo cells in the caput, principal and clear cells in the corpus and cauda epididymidis. No staining was observed in the absence of the primary antibodies (data not shown).

Immunohistochemical Analysis for BrdU and PCNA in Orchidectomized, Regressed Rat Epididymides and Effect of Testosterone Administration

Because the changes in morphology were already observed 3 days after testosterone implantation, we determined the BrdU incorporation and PCNA expression at an earlier time point, 1 day, as well as at 3, 7 and 28 days after initiation of testosterone treatment.

Because of the similarity of the results observed, we only show BrdU and PCNA immunostaining in the initial segment and corpus regions at 3 and 28 days. After orchidectomy, immunoreactivity for BrdU was undetectable at all time points (Fig. 4a, c, d, and e), while after testosterone replacement the number of cells that immunostained positively for BrdU had clearly increased after 3 days. A similar pattern was observed for PCNA immunostaining with the exception that a few cells were stained in the regressed epididymis after 3 days (Fig. 5a and e); however, the number decreased to an undetectable level by 28 days (Fig. 5c and g). After testosterone replacement, PCNA-positive cells were detected after 1 day and increased over time (Fig. 5b, d, f, and h). The corpus epididymidis at 3 days appeared to show maximal staining (Fig. 5f). Tubules were deformed after orchidectomy, but they attained their normal shape after testosterone replacement.

Quantitative Study of Cell Proliferation

BrdU-incorporation and PCNA expression were seen in control rat epididymides in all regions (Fig. 6 and 7). BrdU incorporation was not seen at any time after orchidectomy (Fig. 6). A constant level of PCNA expression was seen in regressed epididymides; the relative number of PCNA-positive cells did not change by 7 days, but decreased sharply by day 28 in all segments (Fig. 7). After testosterone administration, BrdU incorporation and PCNA expression increased in a segment-specific and time-dependent manner. Neither marker was significantly increased in any region of the regressed epididymis 1 day after testosterone replacement. The incorporation of BrdU and expression of PCNA were relatively constant in the initial segment and were lower than those in controls (Fig. 6A and 7A). In the caput epididymidis, testosterone treatment returned the degree of incorporation of BrdU to control levels by 7 days and was not further exceeded (Fig. B), while the expression of PCNA reached control levels as early as 3 days and kept on rising to nearly three times that level by 28 days. The corpus epididymidis showed the highest levels for both markers and this was reached at the 3-day treatment time-point (Fig. 6C and 7C); after that time, the relative number of positive cells decreased suggesting that there is a limit to the number of new cells despite the constant level of testosterone. By 3 days after testosterone replacement, the number of BrdU- and PCNA-positive cells in the cauda epididymidis had returned to that of control; the number of BrdU-positive cells peaked at 7 days, while that of PCNA-positive cells was highest at 28 days.

The highest relative incorporation of BrdU in the regressed epididymides after testosterone replacement versus control was approximately 0.3 in the initial segment, 1 in the caput at 28 days, 5 in the corpus at 3 days, and 2.5 in the cauda at 7 days. Relative to BrdU, expression of PCNA was always higher in all regions at all time points; this may be because of an increase in the number of cells involved in repair.

Immunohistochemical Analysis for Different Cell Types

In order to determine whether cells undergoing division were of a specific cell type(s), we used selective markers for each cell type to identify the proportion of each cell type at 3 days after replacement with either empty implants or testosterone-filled implants in the regressed corpus epididymidis; the 3-day postimplantation time point was selected due to the large number of labeled nuclei observed at this time point in this region. Using GSTP1 as a basal cell marker for this region, we noted intense staining in the nuclei of these cells in all groups of animals; they made up nearly 20% of the total cells. The shape of basal cells was changed after orchidectomy; testosterone replacement could not restore the shape, as anticipated because of the lack internal luminal pressure (Fig. 8a, c, and e). Intense staining in apical regions of clear cells of the corpus epididymidis was seen in all groups of animals. The proportion of clear cells, as identified by ATP6V1B1 staining, seen in control and orchidectomized rats, with empty or testosterone implants, was nearly identical and represented approximately 7% of all cells (Fig. 8b, d, and f). The relative contribution of halo cells, identified as CD-4-positive cells, was

4% in control and slightly increased by 10% to 4.4% in orchidectomized rats with either empty or testosterone implants (data not shown). Sulfated glycoprotein-2 (clusterin) is localized in the cytoplasm of all principal cells along the epididymal duct (data not shown). No significant change was observed in the proportion of principal cells in treated animals compared to control. These results suggest that all the cell types can be divided in control groups. Although orchidectomy decreases the proliferating activity, testosterone replacement induces cell proliferation in all cell types. Thus, dividing cells have no impact on the relative cell populations in the epididymal epithelium.

Discussion

The results from the present study show that there are time-dependent and segment-specific responses to androgen in restoring the morphological changes along the epididymis of the adult rats after regression. Morphological changes are being reversed by re-expansion of existing cells, as assessed by increased epithelial cell height, and increasing the number of new cells in the epithelial compartment.

Although there is general agreement in the literature that orchidectomy results in a reduction in epididymal weight, tubule diameter, epithelial height, and enzyme activities (7;14), previous studies have not clarified the role of androgen action in maintaining the epithelial cell height and the number of new cells in the regressed epididymis and after testosterone replacement. In this study, we found that orchidectomy resulted in a major decrease in tubular diameter in all regions; even after treatment with testosterone for 28 days, this decrease in tubule diameter was

not reversed; presumably, this is due to the removal of the fluid that moves continuously from the testis to the epididymis in the intact animals. However, the tubule diameter is significantly increased in treated animals compared to those receiving empty implants (Fig. 1). From the changes in epithelial cell heights due to androgen withdrawal, it is apparent that there is an almost 40% decrease in the initial segment and a 40% increase in the cauda region. It appears that the caput and corpus are transition regions from the initial segment to the cauda; epithelial cell height was slightly decreased in the caput and slightly increased in the corpus regions, respectively (Fig. 2). In the cauda region after orchidectomy, the diminished luminal content and tubular diameter presumably result in decreased intra-luminal pressure, with a consequent increase in the epithelial height; one would expect the cell volume to remain unchanged. However, the large decrease in tubular diameter and cell height in the initial segment can be attributed to diminishing cell volume. A significant increase in epithelial cell height was found after testosterone replacement in regressed epididymides in all regions. We propose that, in the regressed epididymis, testosterone increases both the re-expansion of existing cells and the number of new cells.

In 2.5-month-old rats, 2.2% of principal cells and 1.4% of basal cells of the whole epididymis were labeled after a single injection of ^{3}H-thymidine; this number decreased with age (17). Immunohistochemical evaluation of BrdU-labeling of epithelial cells in different epididymal regions has established that the number of nuclei labeled was significantly higher in the caput than in the corpus and cauda (34). However, the number of cells that are proliferative in the epididymal epithelium

after regression and even after testosterone replacement has not been investigated previously. The present results are consistent with the previous reports for the percentage of cell proliferation in intact rats. The weights of the seminal vesicles, coagulating glands, and prostates increase 5-8 fold with 1 mg of testosterone over 2 weeks, after 1-month orchidectomy, while epididymal weights increase by only 2-fold (35). Orchidectomy decreases the mitotic activity of the accessory sex organs such as the seminal vesicles and prostate to non-detectable levels; testosterone treatment after 68 hours increases their labeling index to 34-fold, and 20-fold, respectively (36). Similarly, we found that in the regressed epididymis, the number of BrdU-labeled nuclei decreased to nondetectable levels at all time points. Using the PCNA antibody, we found that the number of nuclei labeled for PCNA is decreased to undetectable level at 28 days suggesting that there are cells involved in repair even after 7 days postregression, but not after 28 days. After testosterone replacement, epithelial cells respond to androgen; BrdU incorporation and PCNA expression were differentially increased in a segment-specific and time-dependent manner. Interestingly, the time course of labeling with PCNA appears to be similar to that with BrdU; this level is always higher in PCNA expression than that in BrdU incorporation. A probable explanation is that PCNA is also involved in DNA repair (37-39), suggesting that it may be expressed by cells that are not cycling; overall, its expression increases during the G_1-phase, peaks at the S-phase, and declines during G_2/M of the cell cycle (25). Moreover, the long half-life of PCNA is leading to its continuous expression in some cells that are not actively dividing (40;41). At 3 days post implantation, testosterone treatment caused an extensive mitotic activity,

followed by rapid decrease in corpus, slow increase in the caput and cauda and constant level in the initial segment (Fig. 5, 6), suggesting that rates and extent of cell proliferation in response to hormone are determine by regional considerations. The latter result agrees with those who reported that in addition to the regulation mediated by androgen, luminal factors also play a role in regulating epididymal function; these are crucial factor for initial segment (42-44). The highest mitotic activity shown in the corpus at 3 days indicates the specificity of this region in response to testosterone. That is presumably because of the differences in blood-epididymis barrier in this region.

Specific markers were used to assess the changes in the proportion of each cell type. As previously reported no changes were found in the proportion of clear cells after orchidectomy (8) or even after testosterone replacement. The non-significant change found in the proportion of halo cells at 3 days post-testosterone implantation in the corpus epididymidis could be explained by a transient increase in the number of halo cells in respond to testosterone. Halo cells consist of the main types of immune cells and their distribution in the epididymis increases during aging (32). In general, higher concentration of immune cells appeared to be correlated with major alterations of the epithelial structure, such as lysosome accumulation (45), suggesting that the recruitment of lymphocytes is an important factor for modification of the epithelial integrity.

Mammalian epididymides are often considered as a static tissue without significant cell renewal. However, our observations indicate that there is low cell proliferation in all cell types of the control rat epididymis. Although orchidectomy

reduces cell proliferation to undetectable level, testosterone replacement triggers cell proliferation in a segment-specific and time-dependent manner. The size of the tubules and epithelial cell heights were enlarged after testosterone administration in the regressed epididymides as judged by histological evaluation. These changes may indicate an increase in the number of new cells and re-expansion of existing cells; these findings illustrate the role of androgen in restoring cellular architecture in the regressed epididymis.

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Figure 1. Tubule diameter of cross sections of the regressed epididymis treated with testosterone or empty implants. Initial segment (**A**), caput (**B**), corpus (**C**), and cauda (**D**) regions of the epididymis. The letter a indicates that values between control and regressed epididymis implanted with testosterone (stripped bar) differ significantly; the letter b, that values between regressed epididymis implanted with testosterone or empty implant (white bar) at the same time point differ significantly, ($p < 0.05$).

Figure 1

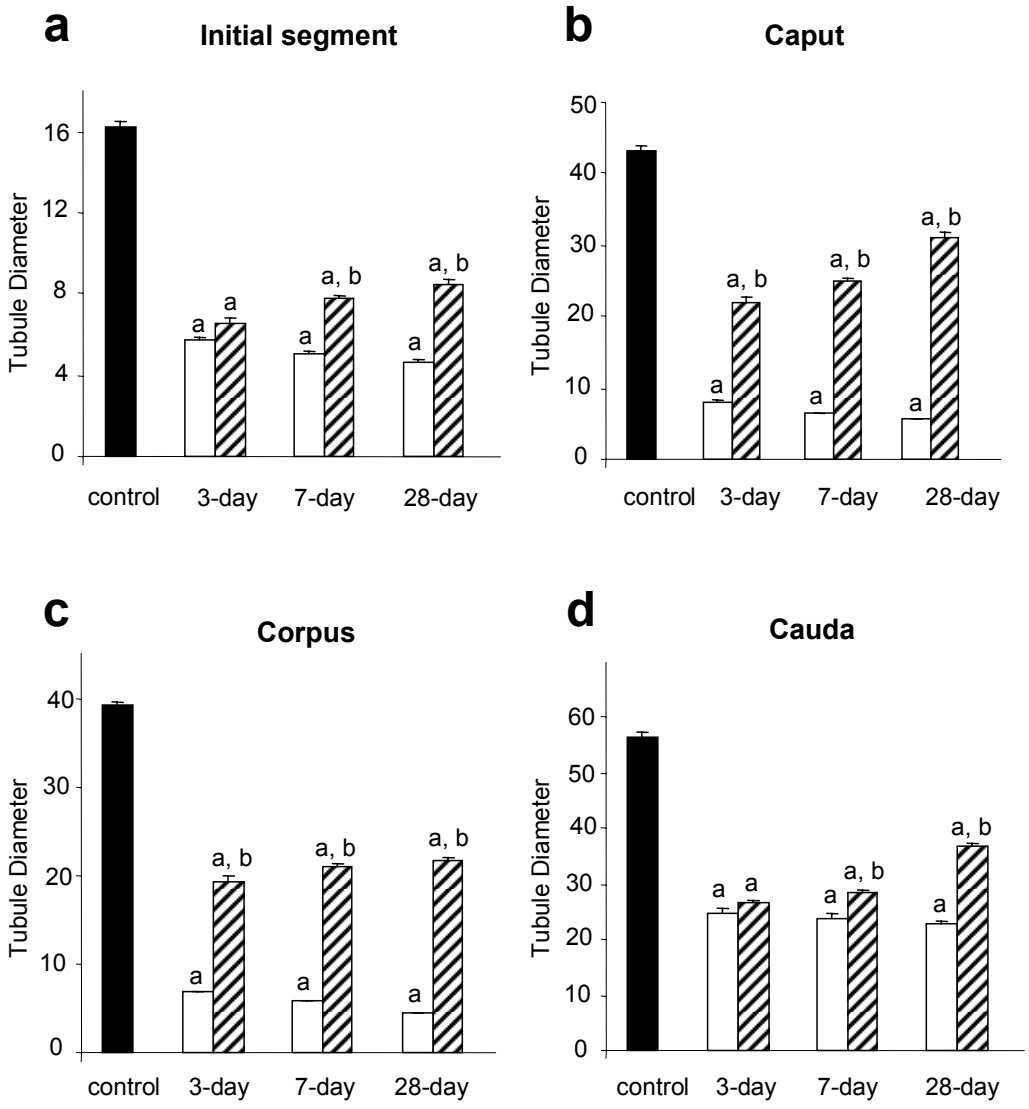

a
Initial segment
Tubule Diameter
16
12
8
4
0
a
a
a
a, b
a
a, b
control
3-day
7-day
28-day
b
Caput
Tubule Diameter
50
40
30
20
10
0
a
a, b
a
a, b
a
a, b
control
3-day
7-day
28-day
c
Corpus
Tubule Diameter
40
30
20
10
0
a
a, b
a
a, b
a
a, b
control
3-day
7-day
28-day
d
Cauda
Tubule Diameter
60
50
40
30
20
10
0
a
a
a
a, b
a
a, b
control
3-day
7-day
28-day

Figure 2. Epithelial cell height of tubular cross sections of the regressed epididymis treated with testosterone or empty implants. Initial segment (**A**), caput (**B**), corpus (**C**), and cauda (**D**) regions of the epididymis. The letter a indicates that values between control and regressed epididymis implanted with testosterone (stripped bar) differ significantly; the letter b, that values between regressed epididymis implanted with testosterone or empty implant (white bar) at the same time point differ significantly, ($p < 0.05$).

Figure 2

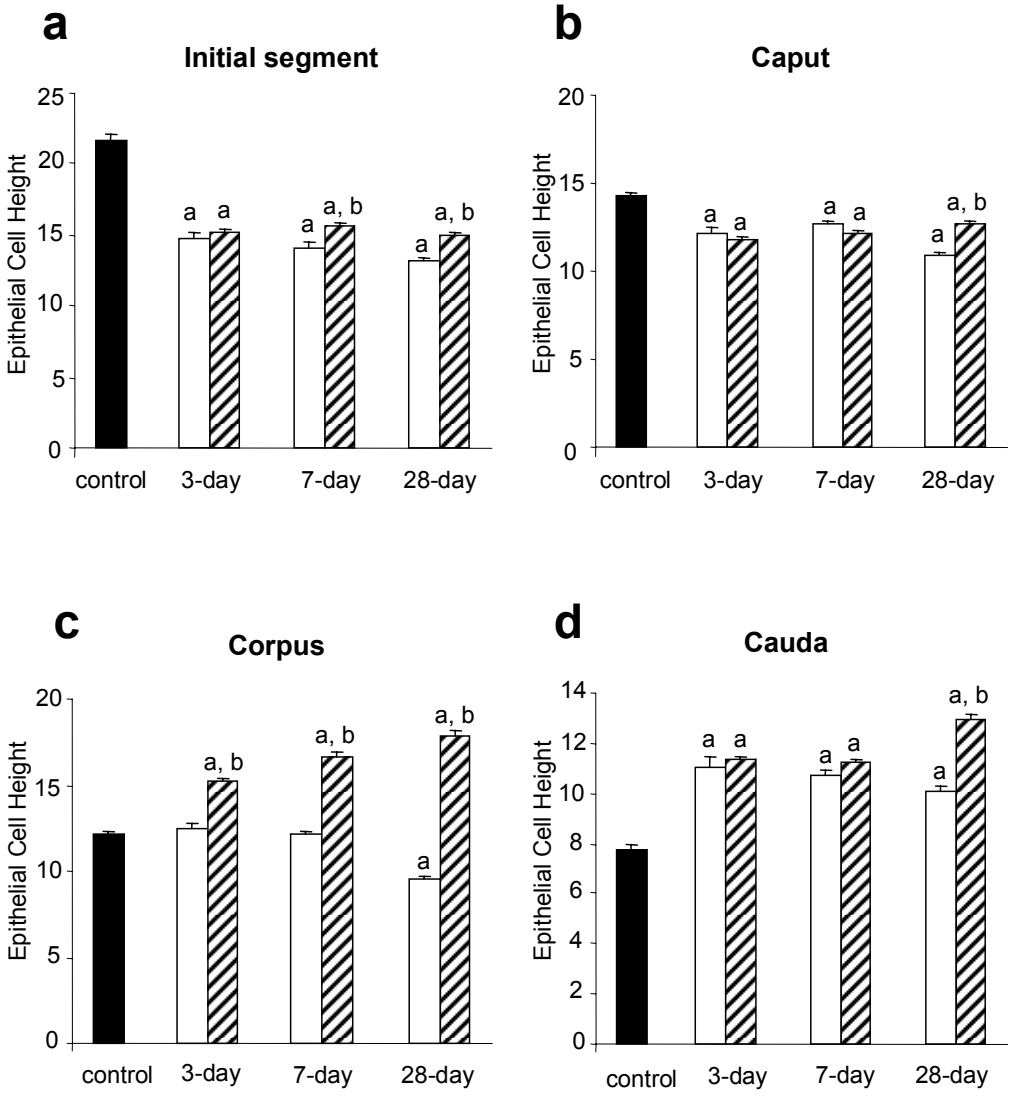

a
Initial segment
Epithelial Cell Height
25
20
15
10
5
0
a a
a a, b
a a, b
control
3-day
7-day
28-day
b
Caput
Epithelial Cell Height
20
15
10
5
0
a a
a a
a a, b
control
3-day
7-day
28-day
c
Corpus
Epithelial Cell Height
20
15
10
5
0
a, b
a, b
a
a, b
control
3-day
7-day
28-day
d
Cauda
Epithelial Cell Height
14
12
10
8
6
4
2
0
a a
a a
a a, b
control
3-day
7-day
28-day

Figure 3. Light micrographs of tubular cross sections of the initial segment (Is), caput (Ca), corpus (Co), and cauda (Cd) regions of the epididymis of control rats immunostained for PCNA (a, c, e, g) or BrdU (b, d, f, h). Arrows indicate nuclei of principal cell (P), basal cell (B), narrow cell (N), clear cell (C), and halo cell (H) that are intensely reactive. Scale bar = 20 μm.

Figure 3

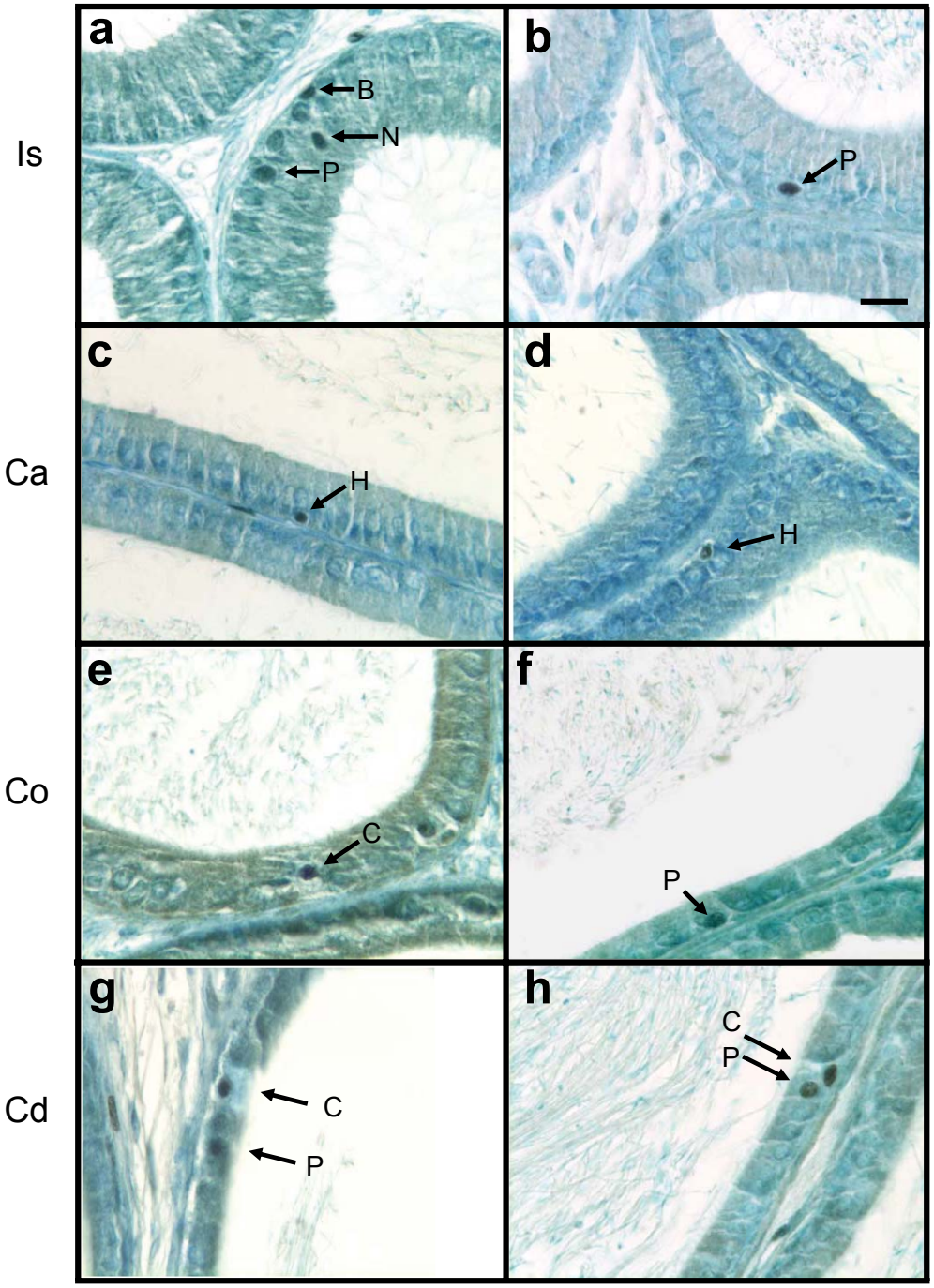


Figure 4. Light micrographs of tubular cross sections of the initial segment (Is) and corpus (Co) regions of regressed epididymides and after testosterone replacement immunostained for BrdU. Eight-day orchidectomized rat treated with empty implant (**a**, **c**, **e**, **g**) or testosterone implant (**b**, **d**, **f**, **h**) for 3 or 28 days. L indicates lumen; IT, intertubular space. Arrows indicate nuclei of cells that are intensely reactive. Scale bar = 20 μm.

Figure 4

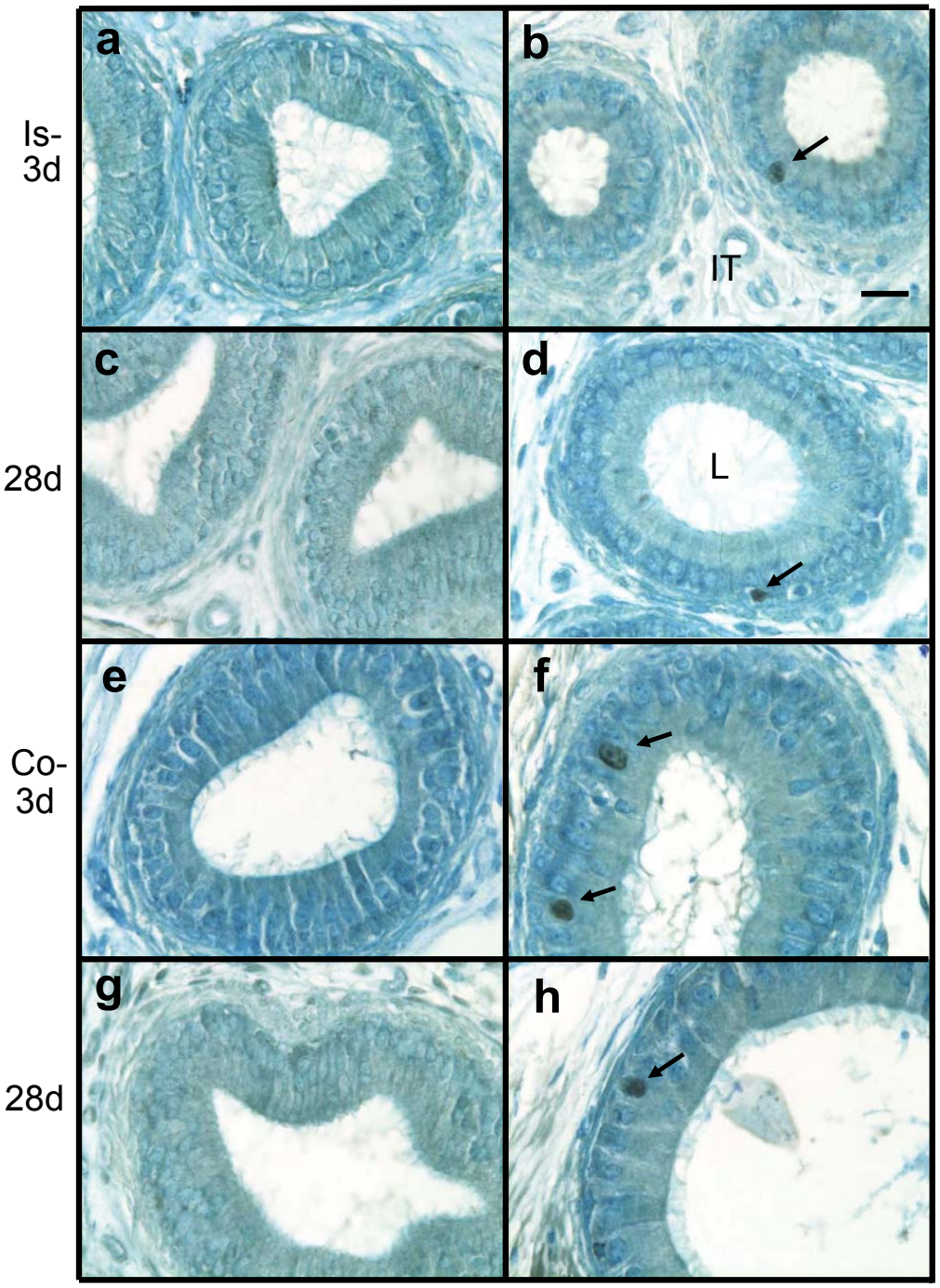


Figure 5. Light micrographs of tubular cross sections of the initial segment (Is) and corpus (Co) regions of regressed epididymides and after testosterone replacement immunostained for PCNA. Eight-day orchidectomized rat treated with empty implant (**a**, **c**, **e**, **g**) or testosterone implant (**b**, **d**, **f**, **h**) for 3 or 28 days. L indicates lumen; P, principal cell; IT, intertubular space. Arrows indicate nuclei of cells that are intensely reactive. Scale bar = 20 μm.

Figure 5

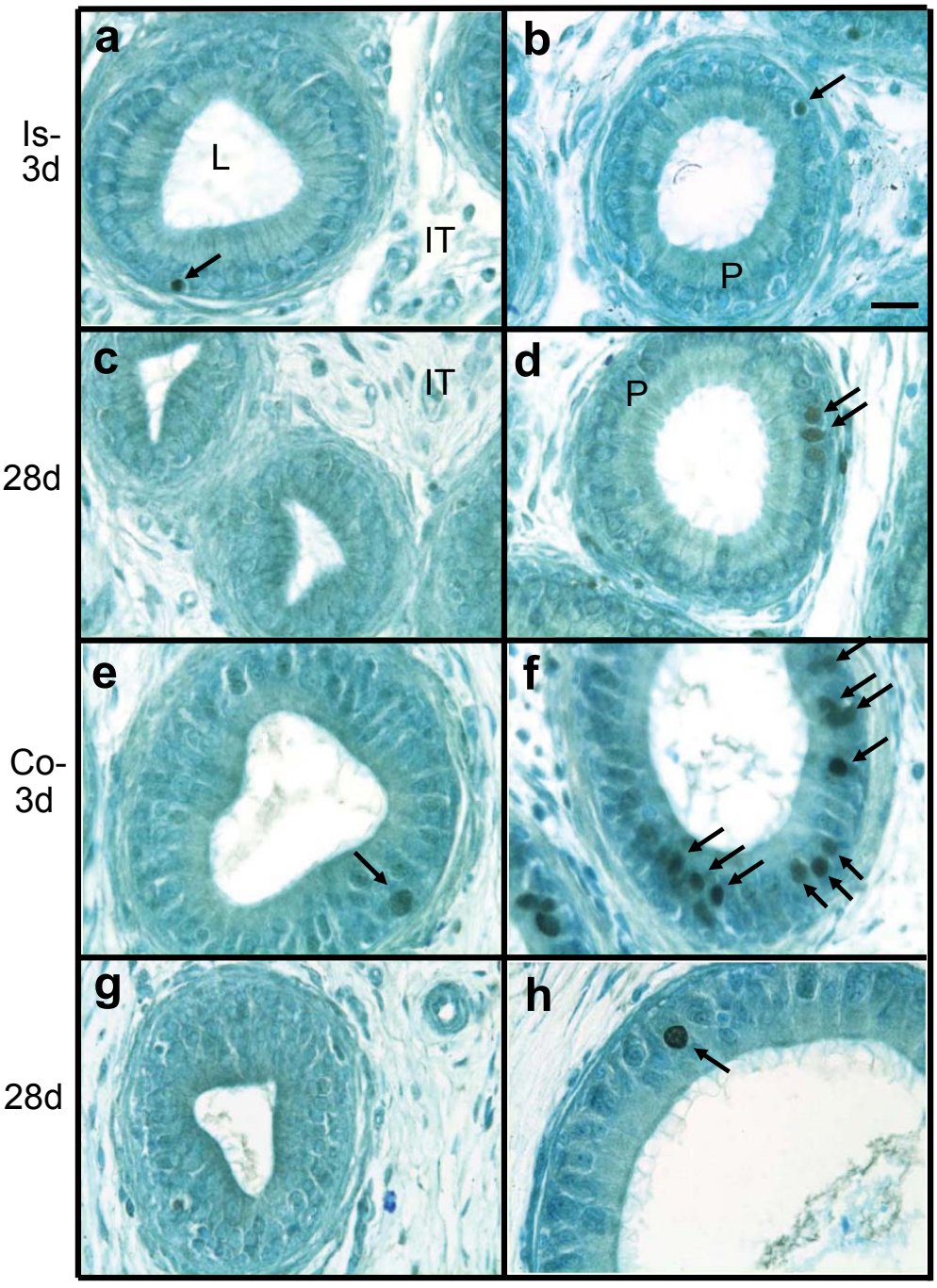


Figure 6. Total number of BrdU-positive cells per 100 tubules shown in the initial segment (A), caput (B), corpus (C), and cauda (D) regions of the epididymis. The letter a indicates that values between control and regressed epididymis implanted with testosterone (stripped bar) differ significantly; the letter b, that values between the regressed epididymis implanted with testosterone or empty implant (white bar) at the same time point differ significantly, ($p < 0.05$). Each bar (mean ± SEM) represents 4 or 5 replicates.

Figure 6

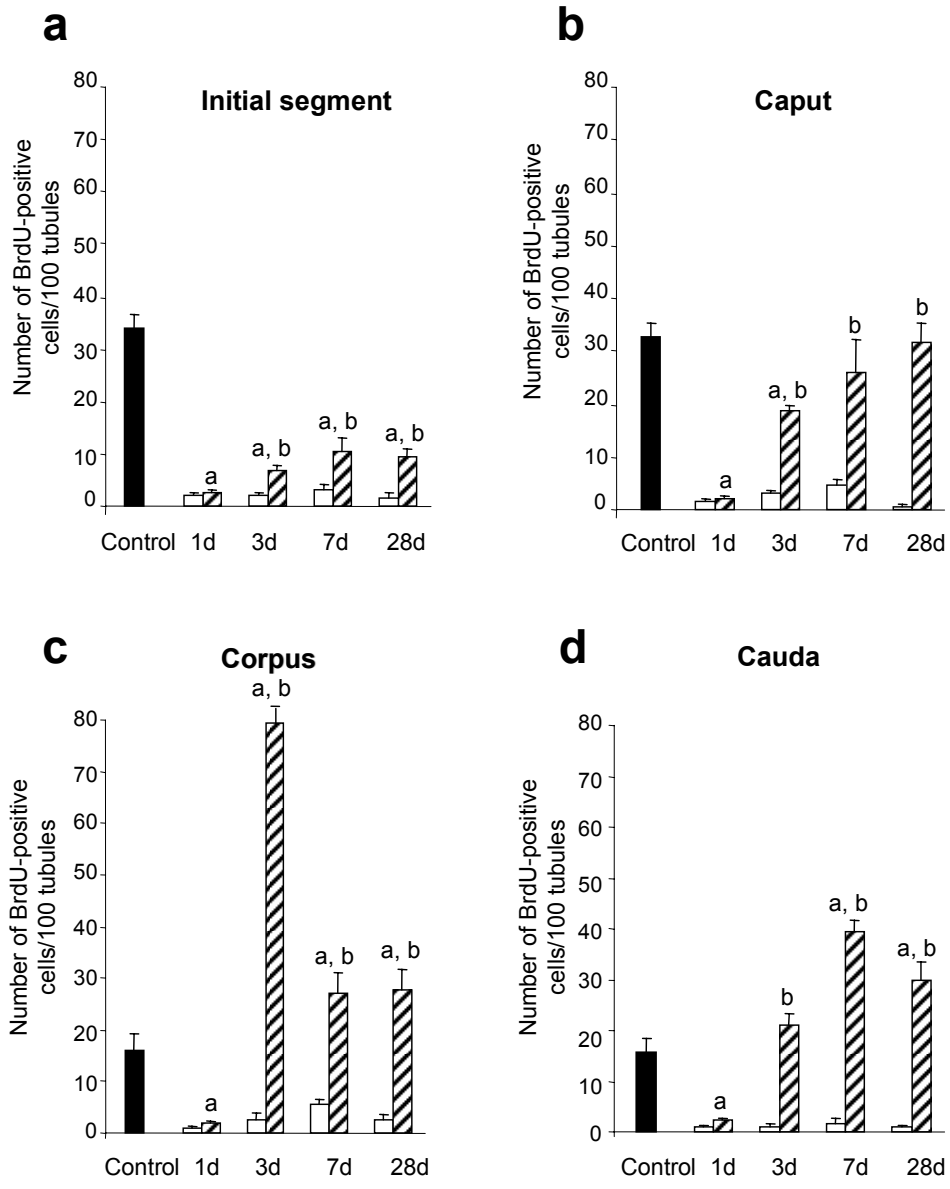

a
Initial segment
Number of BrdU-positive cells/100 tubules
0
10
20
30
40
50
60
70
80
a
a, b
a, b
a, b
Control
1d
3d
7d
28d
b
Caput
a
a, b
b
b
c
Corpus
a
a, b
a, b
a, b
d
Cauda
a
b
a, b
a, b

Figure 7. Total number of PCNA -positive cells per 100 tubules shown in the initial segment (A), caput (B), corpus (C), and cauda (D) regions of the epididymis. The letter a indicates that values between control and regressed epididymis implanted with testosterone (stripped bar) differ significantly; the letter b, that values between the regressed epididymis implanted with testosterone or empty implant (white bar) at the same time point differ significantly, ($p < 0.05$). Each bar (mean ± SEM) represents 4 or 5 replicates.

Figure 7

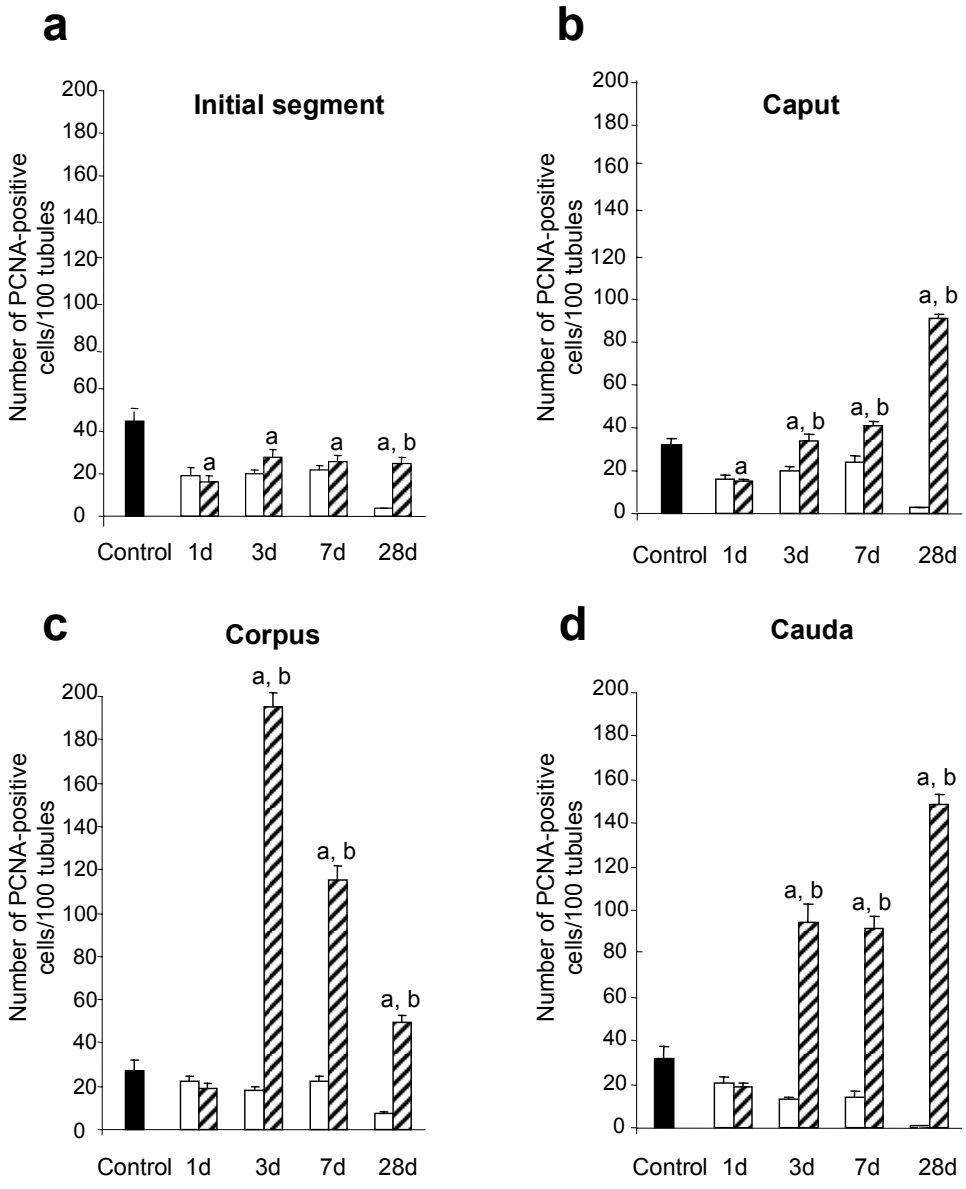
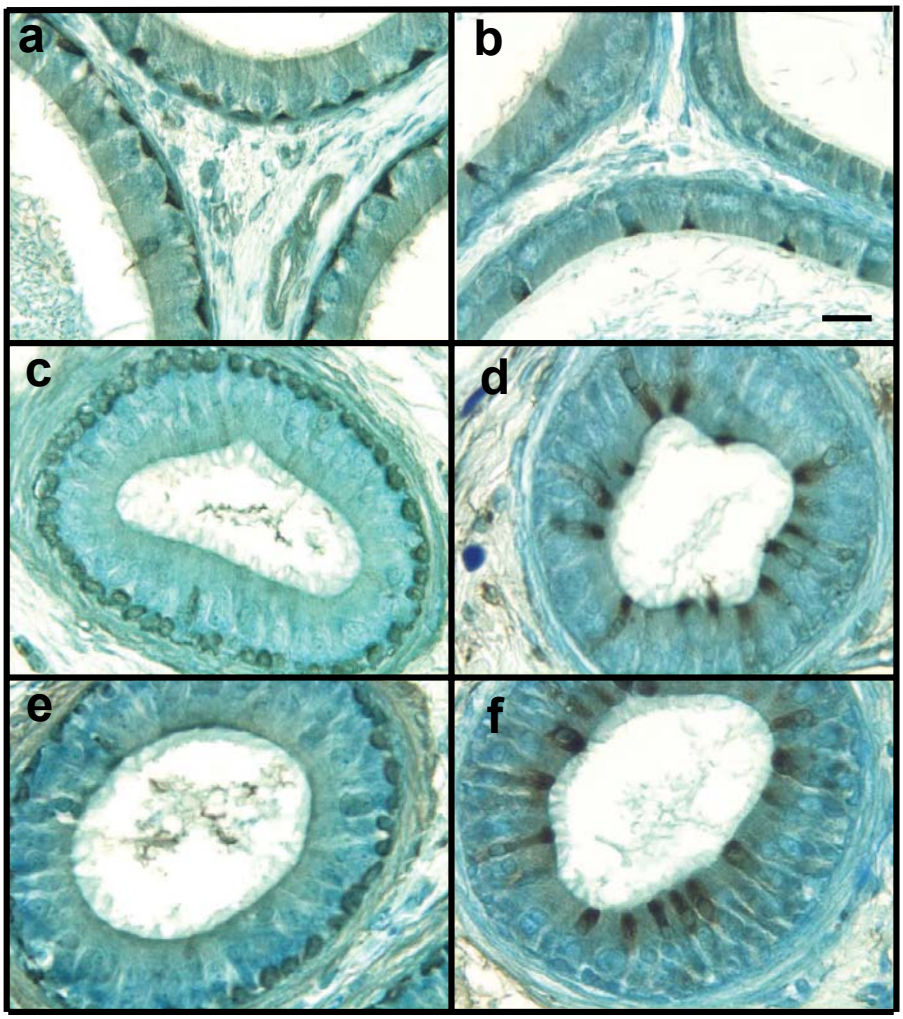

a
Initial segment
b
Caput
c
Corpus
d
Cauda
Number of PCNA-positive cells/100 tubules
0
20
40
60
80
100
120
140
160
180
200
Control
1d
3d
7d
28d
a
a, b

Figure 8. Immunolocalization of basal cells and clear cells of the corpus epididymidis at 3 days. Nuclei of the basal cells (**a**, **c**, **e**) and cytoplasm of the clear cells (**b**, **d**, **f**) immunoreact for GSTP1 and ATPVB1, respectively. (**a**, **b**) indicate corpus epididymidis in control rats, (**c**, **d**), orchidectomized rats with empty implants, and (**e**, **f**), orchidectomized rats with testosterone implants. Scale bar = 20 μm.

Figure 8



CONNECTING TEXT

In the previous chapter, histology studies revealed changes in the morphology and structure of the regressed androgen-deprived tissue in response to testosterone treatment. Size of the tubules with respect to the lumen diameter and epithelial cell height were increased in a region-specific and time-dependent manner, and tubules attained their normal shape. In addition, despite possessing a low mitotic index, epididymal epithelial cells were able to renew themselves and divide following hormone replacement. Therefore, testosterone triggers tubule enlargement and cell proliferation following epididymal regression. These morphological changes occur following a cascade of processes and differential gene expression. Several lines of evidence from the literature have confirmed that the main androgen acting is DHT rather than testosterone; moreover, the proximal region of the epididymis is very active in protein synthesis and secretion. For these reasons, in the next chapter, the sequence of genes/gene families that are first activated by DHT or E2, two active metabolites of testosterone, in the regressed initial segment and caput epididymidis are examined.

CHAPTER 3

Identification of Early Response Genes and Pathway Activated by Androgens in the Initial Segment and Caput Regions of the Regressed Rat Epididymis

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Abstract

To identify the initial response to androgens in the orchidectomized, regressed epididymis, we determined the gene expression changes triggered by the administration of either of two metabolites of testosterone, 5α-dihydrotestosterone (DHT) or 17β-estradiol (E2), in the regressed rat epididymis. Adult rats were orchidectomized and 8 days later implanted with either empty implants (control), DHT- or E2-filled-PDS implants. Rats were euthanized 12 h, 1 and 7 days later and RNA was extracted and probed on Rat 230-2.0 Affymetrix arrays. Probe sets that respond to DHT or E2 were identified at early time points; while the expression of some was repressed, the expression of many others was either transiently or chronically elevated. Nerve growth factor receptor (*Ngfr*) and S100 calcium binding protein G (*S100g*) were two E2 up-regulated genes detected at 12 h. Among the genes that showed a dramatic early response to DHT were endothelin 1(*Edn1*), bone morphogenetic protein 4 (*Bmp4*) and insulin-like growth factor binding protein 3 (*Igfbp3*), that were suppressed and insulin-like growth factor 1(*Igf1*), that was induced. Genes that were up- or down- regulated by DHT were classified based on biological function. Using PathwayStudio 4.0, we identified genes that were linked and directly influenced either the expression or regulation of one another. Epidermal growth factor (EGF) and IGF1 play an important role in the pathway due to their function in regulation and expression of many other genes. These results provide novel insights into the impact of androgen action on the expression of genes that are important for epididymal function.

Introduction

The mammalian epididymis is an androgen-dependent tissue comprised of a single coiled tubule that is divided into initial segment, caput, corpus, and cauda regions (1). The epididymal epithelium is composed of four major cell types: principal, basal, clear, and halo cells (2); it functions in both secretion and removal of specific ions, small organic molecules, and protein from the epididymal lumen (3;4). Maturation of spermatozoa, including the acquisition of motility and the ability to fertilize an oocyte, occurs during transit through the unique and dynamic microenvironment of the epididymal lumen (5-7). This specificity of the microenvironment is under the influence of several endocrine and paracrine factors, from the blood circulation and secreted directly by the testis, that ultimately regulate epididymal gene expression (8;9). The most active regulator of epididymal cell functions is dihydrotestosterone (DHT), the more potent 5α-reduced metabolite of testosterone (10-13). DHT binds to the androgen receptor (AR) and interacts with the androgen response element in the promoters of genes regulated by androgens. Immunostaining reveals that the androgen receptor is present in all regions of the epididymis and in most of the epididymal cell types (14-16). Moreover, recent evidence has accumulated suggesting that aromatase P450 (CYP19), an enzyme responsible for the conversion of androgen to estrogens, is present in spermatozoa (17-19) and that the epididymis has both alpha and beta estrogen receptors (14;16); estrogens have been postulated to play a role in regulation of luminal fluid and ion transport as well as maintaining a differentiated epithelial morphology (20-22).

Androgen deprivation has been used to study the role of androgens in the epididymis; this has been achieved through several methods, including bilateral orchidectomy (12;23), treatment with androgen antagonists (24;25), and treatment with GnRH antagonists (26;27). Orchidectomy, the most commonly used method, removes all circulating androgens as well as testicular factors, resulting in epididymal regression. Therefore, this regression is due not only to removal of circulating androgens but also to the removal of spermatozoa and the luminal fluid coming from the testis; removal of luminal components from the entire epididymis takes approximately 7 days (28).

A variety of epididymal functions are affected by orchidectomy. For instance, epididymal morphology, ion transport, synthesis and secretion of a number of proteins, and the activity of certain enzymes are under the control of androgens (29). At the mRNA level, several epididymal genes have been described as androgen dependent; the expression of these genes either declined after orchidectomy, such as Crisp-1 (30), glutathione peroxidase 3 (*Gpx3*) (31), carbonic anhydrase (32), cyclooxygenase 2 (33), and angiotensinogen (34) or was induced or not affected in the absence of androgens, such as clusterin (35), and transforming growth factor β (*Tgfβ)* (36) . In addition, testicular factors play a role in maintaining the epithelial structure and normal expression of proximal epididymal genes in the initial segment and caput epididymidis; these genes include basic fibroblast growth factor (*bfgf*), androgen binding protein (*Abp*), proenkephalin (*Penk1*) (37), cystatin-related epididymal specific (38), 5-a reductase 1 (*Srd5a1*) (39), and γ-glutamyl transpeptidase (*Ggt*) (40). Although the expression of many epididymal genes is

regulated by androgen, only a few of these have been shown to contain androgen response elements (ARE).

The blood flow to the initial segment is higher than to that of any other region of the epididymis (41). Due to the link between the volume of capillary flow and the local cellular metabolic rate (42), this segment is metabolically very active and is critically dependent both on factors secreted directly by the testis and on circulating androgens (43). The two proximal regions of the epididymis (initial segment and caput region) have high levels of estrogen and androgen receptors (16;44) and are most crucial for epididymal sperm maturation (45). Consequently, we targeted these regions for our studies.

Transcriptional profiling of the epididymis has been previously reported for the rat (23;46) and mouse (47), as well as for orchidectomized mice with treated with high doses of DHT (9) or 17ß-estradiol (E2) (48) supplementation. In the present study, we have done genome-wide profiling of mRNAs expressed in the initial segment and caput of the orchidectomized and regressed rat epididymides implanted with either empty implants, or capsules filled with DHT or E2. Using the Affymetrix Rat Genome 230-2 Microarray chips containing probe sets that span the entire rat genome, we determined which genes and gene families were first affected in the regressed initial segment and caput epididymidis after treatment with either DHT or E2. While the level of expression of some genes is repressed by androgens, many others are either transiently or chronically increased. It appears that physiologically relevant estradiol concentration plays a relatively minor role in early gene activation in the regressed epididymis. Furthermore, we identified early

response pathways activated by DHT. Besides highlighting the significance of early androgen responsive genes, these results are also important for understanding the normal events associated with the androgenic response.

Matherials and Methods

Animals and Treatments

Adult Brown Norway rats aged 10-12 weeks (n=5) were purchased from Harlan (Indianapolis, IN). Rats were housed at the McIntyre Animal Resources Center, McGill University, under controlled light (14-h light: 10-h dark) and temperature (22°C); animals had free access to food and water. 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 (Animal Use Protocol no. 206). They were divided in two groups: sham-operated or orchidectomized. After 8 days, orchidectomized rats were implanted subdermally with either empty polydimethylsiloxane (Silastic®) rods (control), DHT-, or estradiol-filled rods. Implants were prepared as previously described (49) from PDS (Dow Corning Medical Grade 0.078 mm ID, 0.125 mm OD). Filled implants containing DHT (Steraloids Inc, Newport, Rhode Island, 3×6.2 cm) and estradiol (Steraloids Inc, 1cm) released steroid at rates of 24 and 2.4 µg/cm/day, respectively. Rats were euthanized at 12 h, 1, 3 and 7 days post-implantation and the epididymides were removed and divided into three sections (initial segment-caput, corpus, cauda). Weights of the epididymidis and other sex accessory glands were measured and RNA of epididymal segments was extracted.

RNA of initial segment- caput of three rats at 12 h, 1d, and 7 d was purified for microarray analysis. In the present study, we used the Brown-Norway rat, an inbred rat model, to minimize inter-individual genetic variation in the microarray analysis.

Serum Hormone Analysis

At the time of sacrifice, blood was collected and serum was obtained by centrifuging the blood for 2 minutes at 12 000 × g. Supernatants were collected and frozen at -80 ^{o}C for further analysis. A commercially available dihydrotestosterone (DHT) enzyme-linked immunoassay kit (US Biological, Swampscott, MA) with sensitivity of 6 pg/ml, and estradiol (E2) immunoassay kit (BioSource International, Inc., Camarillo, CA) with sensitivity of 6 pg/ml were used to measure the total serum DHT and E2 concentrations in each group, according to the manufacturer's instructions. Serum DHT values were 297 ± 103 pg/ml in the sham control, 1518 ± 47 pg/ml in the orchidectomized group with DHT implants, and 59± 4 pg/ml in the orchidectomized group with empty implants. Serum E2 values were 14.4 ± 0.4 pg/ml in the sham control, 78.7 ± 15.4 pg/ml in the orchidectomized group with E2 implants, and 11.1 ± 2.7 pg/ml in the orchidectomized group with empty implants.

RNA Extraction and DNase Treatment

Epididymal regions were crushed under liquid nitrogen and RNA was extracted and DNaseI treated using the RNeasy Mini kit (Qiagen, Mississauga, ON, Canada) according to the manufacturer's instructions. RNA concentration was assessed by OD determination at 260 nm (DU7 spectrophotometer, Beckman,

Montreal, Quebec, Canada). RNA used for microarray analysis had an A260/A280 ratio greater than 1.8. To verify the quality of each sample, 2 μg RNA was run on a denaturing gel containing 1% agarose. The RNA quality was also assessed by Genome Quebec using a bioanalyzer (Agilent Technologies, Santa Clara, CA).

Microarray Process and Analysis

Microarrays were done at the DNA Microarray Centre at McGill University, Genome Quebec Innovation Centre, according to the one-cycle protocol provided by the manufacturer (Affymetrix Inc., Santa Clara, CA). In short, double stranded cDNA was synthesized from 5 μg total RNA, followed by a transcription reaction to produce biotin-labeled cRNA that was then hybridized to Rat Genome 230-2 Microarray chips (Affymetrix). The arrays were washed and stained with streptavidin phycoerythrin in Affymetrix Fluidics Station and scanned with an Affymetrix scanner. Expression analysis of all replicate microarray experiments was done by using GeneSpring 7.2 software (Agilent Technology). The Robust Multi-array Average (RMA) algorithm was applied and only the genes with an expression value ≥ 5 were considered as expressed. The raw data obtained were further normalized by data transformation from log base 2 to linear and by per gene to the median (GeneSpring). Comparisons were done between RNAs from tissues of sham-operated groups versus orchidectomized rats with empty implant or between orchidectomized rats with empty implant versus orchidectomized rats with either DHT or estradiol implant for each of the time points. Differential expression was defined as those transcripts that had a difference of 2-fold or greater and were statistically different (significance level

set at $p \leq 0.05$). Annotation and biological function of these genes were obtained from the NetAffx Analysis Center (http://www.affymetrix.com).

Quantitative Real-Time PCR Analysis

Quantitative real-time PCR was done using the Light-Cycler system (Roche Diagnostics, Laval, QC, Canada) with the Quantitect One-Step SYBR green RT-PCR kit (Qiagen) according to the manufacturer's protocol. The primers for *Ngfr, S100g, Igfbp3*, *Edn1*, *Gjb3*, *Ramp3*, and *Egln3* were provided by Qiagen; for *Igf1* and *cyclophilin A*, the specific primer sequences were designed using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi/). *Cyclophilin A* was chosen as the endogenous control because of its invariant expression throughout the epididymis and lack of response to androgen status (40). All standards and samples were assayed in duplicate. Average values for the target gene RNA concentrations of each sample were normalized to the average value for *cyclophilin* RNA concentration in the same sample. The results of RT- PCR experiments were confirmed by melting-curve analysis. The primer sequences designed are as follows: *cyclophilin A*: forward primer, 5'-GTGGTCTTTGGGAAGGTGAA-3'; reverse primer, 5'-GTTGTCCACAGTCGGAGATG-3' (NM-008907). *Igf1*: forward primer, 5'-GGCATTGTGGATGAGTGTTG-3; reverse primer, 5'-GTCTTGGGCATGTCAGTGTG-3' (NM-178866). The significant effects of E2 or DHT replacement were determined using t-test. The level of statistical significance was set as $p \leq 0.05$.

Western Blot Analysis

Protein extracts (n = 3) were collected for each group using the cellular fractionation kit (Cat. No: 40010) from Active Motif (Carlsbad, CA), according to the manufacturer's protocol. Using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA), we determined the protein concentration by the Bradford method. Protein samples (15 μg per group) were boiled with loading buffer for 5 minutes and fractionated by SDS-PAGE using 15% acrylamide gels. Prestained precision standards (Bio-Rad Laboratories) were used as molecular weight markers. The fractionated proteins were transferred to a Hybond-P membrane (Amersham Biosciences UK, Buckinghamshire, UK). They were blocked with 5% nonfat dried milk in 137mM NaCl, 29mM Tris (pH 7.4), and 0.1% Tween 20 at room temperature for 1 h and then incubated over night at 4 C with a primary mouse monoclonal antibody to IGF1 (1:1000, ab36532; Abcam, Cambridge, MA). Antibody binding was detected by incubating with antimouse IgG conjugated to horseradish peroxidase (1:10,000, NA931V; Amersham Pharmacia Biotech UK). Cylophilin A (1:5000, catalog no. 07-313; Upstate, Lake Placid, NY) was used as a loading control and was detected using a secondary, donkey antirabbit IgG-horseradish peroxidase antibody (1:10,000, NA934V; Amersham Biotech UK). Western blots were visualized with the Enhanced Chemiluminescence Plus kit and hyperfilm enhanced Chemiluminescence (Amersham Biosciences UK). Quantification of Western blot data was done by line densitometry using a chemiImager 4000 imaging system with AlphaEase (version 5.5 software; Alpha Innotech). IGF1 levels for each group were detected relative to the corresponding cyclophilin A value.

Pathway Analysis

Genes obtained from array analysis (orchidectomy with empty implant versus orchidectomy with DHT implant) were imported to PathwayStudio 4.0 software (Ariadne Genomics, Rockville, MD) according to the manufacturer's instructions. The software updated the list by excluding the redundant and non-annotated genes. PathwayStudio 4.0 and ResNet-3.0 database were used to visualize direct interactions between selected entities. Entities were limited to proteins and pathways and relationships limited to binding, expression, protein modification, and regulation.

Results

Sex Accessory Tissue Weights

The weights of the ventral prostate and seminal vesicles are indices of the circulating concentration of biologically active androgens (50). These tissues were weighed in control and treated rats in order to ascertain the androgenic status of the animals used in these experiments. It was found that the weight of all tissues was markedly diminished one week after orchidectomy. By 3 days after DHT replacement, the weights of the paired seminal vesicles (Fig. 1a) and the ventral prostate (Fig. 1b) were significantly increased compared to those in orchidectomized rats with empty implant. E2 treatment did not result in any weight change. The same pattern was found in paired epididymides (Fig. 1c). Orchidectomy (7 days) resulted in a 66% decrease (sham operated: 176 mg; orchidectomized: 60 mg) in initial segment-caput epididymal weight. Weights of the regressed epididymides did not change significantly at 12 h or 1 day after DHT treatment, but increased by 63% at 7

d post-implantation (99 mg). Estradiol treatment did not alter the epididymal weight at any time point examined (Fig. 1d).

Impact of DHT or E2 Treatment on Gene Expression in the Regressed Rat Epididymis

Of the probe sets on the array, 60% were expressed under all conditions. After orchidectomy (no hormonal treatment), 6-7% of the expressed probe sets (902 probe sets after 8.5 days, 950 after 9 days and 1059 after 15 days) were up- or down-regulated (approximately equal numbers were up- and down- regulated) (Fig. 2a). After DHT treatment, the expression levels of 90, 345, and 650 probe sets were altered at 12 h, 1 d, and 7 d, respectively (Fig. 2b). Interestingly, a large proportion of these probe sets were similar to the ones that changed after orchidectomy in the absence of hormonal treatment (67% at 12h, 70% at 1d, and 77% at 7d). The expression levels of only a very few probe sets were under the influence of estradiol; 6, 4, and 13 probe sets at 12 h, 1 day, and 7 days, respectively (Fig. 2c). After estradiol treatment, the expression levels of only 2 probe sets at 12 h, none at 1day, and 5 at 7 days were similar to those that changed after orchidectomy without hormonal treatment.

Effects of E2 or DHT on Induction or Repression of Gene Expression of the Regressed Rat Epididymis

The mRNA expression of some genes was significantly altered after E2 or DHT replacement in the regressed epididymis at early time points. These results were further confirmed by quantitative RT-PCR. For this purpose, we selected 2

genes for E2 (Fig. 3) and 6 genes for DHT (Fig. 4). The data obtained by qRT-PCR closely correlated with those from the array analysis. The expression of 2 genes (*Ngfr*, and *S100g*) was up-regulated by E2. The expression of 6 genes (*Igf1*, *Gjb3*, *Ramp3*) was up- or (*Igfbp3*, *Edn1*, *Egln3*) down-regulated significantly ($p \leq 0.05$) by DHT replacement in the regressed epididymis. Decrease in expression of Igfbp3 after orchidectomy in rat (23) and up-regulation of *Gjb3* by DHT in mouse caput were reported previously (9).

Classification of Genes Influenced by DHT or E2 in RegressedTtissue

Probe sets obtained by array analyses with known annotations (orchidectomy with empty implant versus orchidectomy with DHT or E2 replacement at 12 h, 1 and 7 days) were classified based on their biological function, such as signal transduction, metabolic process, transport, regulation of cell proliferation, cell-cell signaling, cell adhesion, regulation of transcription, proteolysis, immune response, and spermatogenesis (Supplemental Table 1.a, b, c, and 2). Significant changes in the expression of genes involved in the regulation of cell proliferation and growth were observed and the number of genes in these groups was increased over time. *Igf1* transcripts decreased after regression and increased after DHT replacement; a similar pattern was found for *Egf*. In contrast, orchidectomy induced the expression of *Igfbp3*, while DHT highly suppressed its expression, suggesting that there is a negative regulatory pathway involved between these two genes. The stability of IGF1 protein was examined by Western blot analysis; the level of IGF1 protein was undetectable after regression; however after 7 d, DHT increased the IGF1 protein level to 50% of that of the sham-operated group (Fig. 5). *Bmp4* and *Edn1* were two

DHT-regulated genes involved in regulation of cell proliferation; they showed an early response (12 h) and expressed significantly at all time points examined. Gap junction membrane family members were also regulated by DHT; *Gja1* was expressed significantly at 12 h and more members of this family, such as *Gjb3* and *Gja4,* were expressed at 1 and 7 days. The expression of many solute carrier family members was increased after DHT replacement; presumably, they play a significant role in restoring the epithelial cell morphology and integrity. The expression of interleukin 13 receptor (*Il13ra2*) and interleukin 1 receptor-like 1 (*Il1rl1*), mediators of the immune response pathway, was induced by DHT. While there were many genes regulated by DHT, we found few genes regulated by E2 in the epididymis at early time points. The expression level of calcium binding protein G (*S100g*) was increased by 12-fold after E2 replacement in the regressed epididymis and increased more over time, suggesting that it may have important role in the maintenance of epididymal cell integrity.

Pathway Activated by DHT after 12 h and 1 Day in the Regressed Rat Epididymis

To visualize known regulatory relationships among the genes that were significantly affected by DHT treatment, we used the Pathway Assist Software. Few genes were functionally linked together at 12 h; BMP4 influences the expression of GJA1 and SDC1, and EDN1 functions in GJA1 regulation (Fig. 6a). At 1 day, a large number of these genes were linked and influenced directly either the expression or regulation of one another. Epidermal growth factor (EGF) and insulin-like growth factor 1 (IGF1*)* had a central role in the pathway due to their linkage with many other

genes. Many of the genes shown at 12 h were consistently present at 1 day, such as EDN1, GJA1, and BMP4*,* others, including SDC1 and NOX4*,* were expressed transiently (Fig. 6b). In addition, similar to EDN1, GJA1, and BMP4 at 12 h and 1 day, EGF, IGF1, IGFBP1*,* IGFBP3, PTGS2, and AR at 1 d were involved in the common pathways, with potential roles in cell survival, cell proliferation, differentiation, secretion and motility.

Discussion

The goal of this study was to uncover initial androgen-mediated events that ultimately maintain epididymal function and integrity. The regulation of genes by androgen in the epididymis has been investigated extensively, but a sequential gene expression response to androgen replacement following epididymal regression has not been shown in previous studies. We employed both major metabolites of androgen, DHT and E2, to differentiate their roles in maintaining epididymal weight and function. Following regression, DHT, but not E2, significantly increased epididymal weights after 3 days, similar to the other sex accessory tissues.

A number of genes that are regionally expressed in the epididymis have been identified, including those encoding secretory proteins with putative roles as proteases, protease inhibitors, antioxidant enzymes, growth factors, neuropeptides, and transporters (44). Several of these genes are unique to the epididymis and may have specific roles in epididymal function. Within the epididymal regions, the highest number of androgen-regulated genes was observed in the proximal region (initial segment- caput); this suggests that more androgen-responsive genes are active in

this part, and that the level of protein synthesis is higher than in the rest of the epididymis (51). In the present study, we identified sequential activation or suppression of genes that are important for keeping the microenvironment of this segment unique and that may be important for sperm maturation (44). After orchidectomy, there is an overall change in gene expression (23); however DHT could reverse the changes in expression for a large proportion of these genes and this number is increased with increasing time post-DHT treatment. In contrast, few genes respond to E2 after epididymal regression.

Nerve growth factor receptor (*Ngfr*) is one of the genes up-regulated in response to E2 at 12 h. Nerve growth factor has an essential role in the survival, development and differentiation of neurons in the nervous system (52); however, its action is not restricted to the nervous system. Immunoreactivity to NGF and its receptors was detected in epithelial cells of efferent duct, epididymis, and vas deferens, as well as in seminal vesicles, prostate, and coagulating gland epithelium, Leydig cells and elongated spermatids, suggesting that it is an important growth factor in gonadal function and may act as a regulator of sperm maturation or transport in the male rat (53). S100 calcium binding protein G (*S100g*) is another early response gene that is highly up-regulated by E2 after orchidectomy and regression. Previously, it has been shown that calcium binding protein is mainly regulated by estrogen in the pituitary gland of rats (54), and that its expression is enhanced by E2 via ERα pathway in the uterus in immature rats (55).

Among the many genes that are regulated by DHT, we identified several that responded early to DHT, i.e., after 12 h. Endothelin 1 is one of the transcripts

identified as being androgen regulated and localized in the epididymis. In the human reproductive system, EDN1 or its receptor have been found in the uterus (56), testis (57) and seminal fluid (58). In the epididymis, Edn1 mRNA and its receptors were detected in all regions; it was localized in some principal cells and endothelin receptor A immunostaining was found mainly in basal cells in the proximal region of the epididymis (59;60), suggesting that these genes have a major role in controlling epididymal blood flow and regulating the contractile activity that causes the epididymal motility necessary for sperm transport. Consistent with our finding, it has been shown that *Edn1* is up-regulated in the orchidectomized rat prostate (61), indicating that androgen regulation in the epididymis has some repressive activity in addition to an ability to up-regulate gene expression.

Bone morphogenetic protein 4 (*Bmp4*) is another gene that responds early to DHT; it is up-regulated more that 2 fold after orchidectomy, and its expression significantly declines with DHT replacement. BMPs play essential roles in many aspects of developmental biology. *Bmp4* is the most widely expressed *Bmp* gene during mouse embryogenesis and in adults; most *Bmp4* homozygous mutants die during early gastrulation with defects in mesoderm and germ cell formation (62;63). Its expression is detected in the epididymis and testis throughout postnatal development, and it has a function in the maintenance of spermatogenesis and of epididymal integrity in adult mice (64). Gap junction membrane channel protein beta 3 (*Gjb3*) is enriched in the caput epididymidis, as previously reported (9), and is highly up-regulated by DHT at an early time point. It is also localized in the seminiferous epithelium of the rat testes, indicating that local networks of cell-cell

communication in the tubules that are regulated by T are important for the initiation and maintenance of spermatogenesis.

Our study has established that the expression of several genes is activated by DHT replacement in the regressed epididymis over time. Androgen receptor mediated androgen action is itself regulated by androgens. In the rat epididymis, androgen receptor mRNA levels were increased almost 1.5 fold with androgen withdrawal and decreased below control levels after androgen stimulation (65). Similarly, we observed an increase in mRNA levels following orchidectomy, and DHT replacement decreased the levels down to control (sham-operated) levels after 12 h and 1day, and even less than the control levels at 7 days.

We found that insulin-like growth factor 1(*Igf1)* and insulin-like growth binding protein 3 (*Igfbp3)* were regulated by DHT; this result was confirmed by real-time PCR. IGF1 is immunolocalized to the epithelial cells of the caput and cauda epididymidis during postnatal development (66). IGF1, an anabolic growth factor responsible for normal growth and development, stimulates protein synthesis (67) , has a major role in proliferation and differentiation of satellite cells (68), suppresses proteolysis in muscle (69), and exerts anti-apoptotic and mitogenic effects in the prostate (70). Similarly, IGF1 plays a crucial role in Leydig cell maturation in the testis and absence of this growth factor results in decreased levels of androgens and fertility (71;72). Insulin-like growth factor binding protein 3 (IGFBP3) is one of the 7 proteins of IGFBPs family that bind to IGFs with high affinity and control the distribution of IGFs (73). It has been hypothesized that IGFBPs attenuate the cellular response to IGF1 through the high affinity binding of IGF1 to IGFBPs. The IGF and

IGFBP3 system plays an important role in prostate epithelial cell proliferation, apoptosis and tumor progression (74). In silico analysis identified a putative androgen response element (ARE) in the *Igf1* (75) and *Igfbp3* (76) promoters. Following orchidectomy, the expression of *Igfbp3* gene in the initial segments and caput of the epididymis (23) and in the ventral prostate is rapidly increased (77). Similarly, we observed that the mRNA expression level of this gene is highly increased after orchidectomy; DHT replacement causes a highly significant decline, when compared to the control after 1 day, suggesting that *Igfbp3* expression is androgen dependent and negatively regulates growth and promotes apoptosis in the epididymis.

We found that EGF may have a central role in the early response pathways activated by DHT manipulation in the regressed epididymis as it can directly influence the regulation and expression of many other genes affected by DHT at 1 day. Orchidectomy caused a significant decrease in its expression level and DHT replacement reversed this effect. Epidermal growth factor (EGF) was immunoreactivity is found in mouse and human testis and has been implicated in playing an important role in the regulation of spermatogenesis (78;79). EGF precursor is also known to be produced in the testis (80). Testosterone interacts with EGF to influence the Wolffian duct formation during development (81). Androgen increases the *Egf* binding sites and modulates the cellular proliferation in the rat prostate (82), thus suppression of EGF receptor signaling results in a reduction of the incidence of prostate cancer metastasis (83). Moreover, it has been found that EGF and IGF1 have the ability to suppress apoptosis induced by chemotherapeutic

treatments in salivary acinar cells (84). Interestingly, the presence and activity of growth factors in the epididymis and some of their potential target genes have been reported recently; *Plau* and *Ptgs2* are two of the targeted genes (85), similar to what we identified in our depicted pathway.

In conclusion, we have identified a set of novel early androgen-regulated genes in the proximal epididymis. Androgen regulates genes that are involved with cell survival, proliferation and growth, secretion, and motility pathways, with significant cross talk. EGF and IGF1 are at the core of these pathways. We postulated that these genes might play an important role with regard to the development, regulation, and integrity of the epididymis. Therefore, the present study provides a novel insight into the specific cascade of events that are mediated by androgens to restore the epididymal structure and function.

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Figure 1. Seminal vesicles, ventral prostate, paired epididymides, and paired initial segment and caput epididymal weights. Sham-operated groups are shown with diagonally striped bars. Black bars represent the orchidectomy and empty implant group, grey bars indicate orchidectomy and DHT implants, and white bars indicate orchidectomy and E2 implants at all four time points. Each bar represents the mean of 5 observations ± SEM. For all four parameters, the values for the orchidectomy and DHT implant groups are significantly greater than those of orchidectomy and empty implant groups, at 3 and 7 days ($p \leq 0.05$).

Figure 1

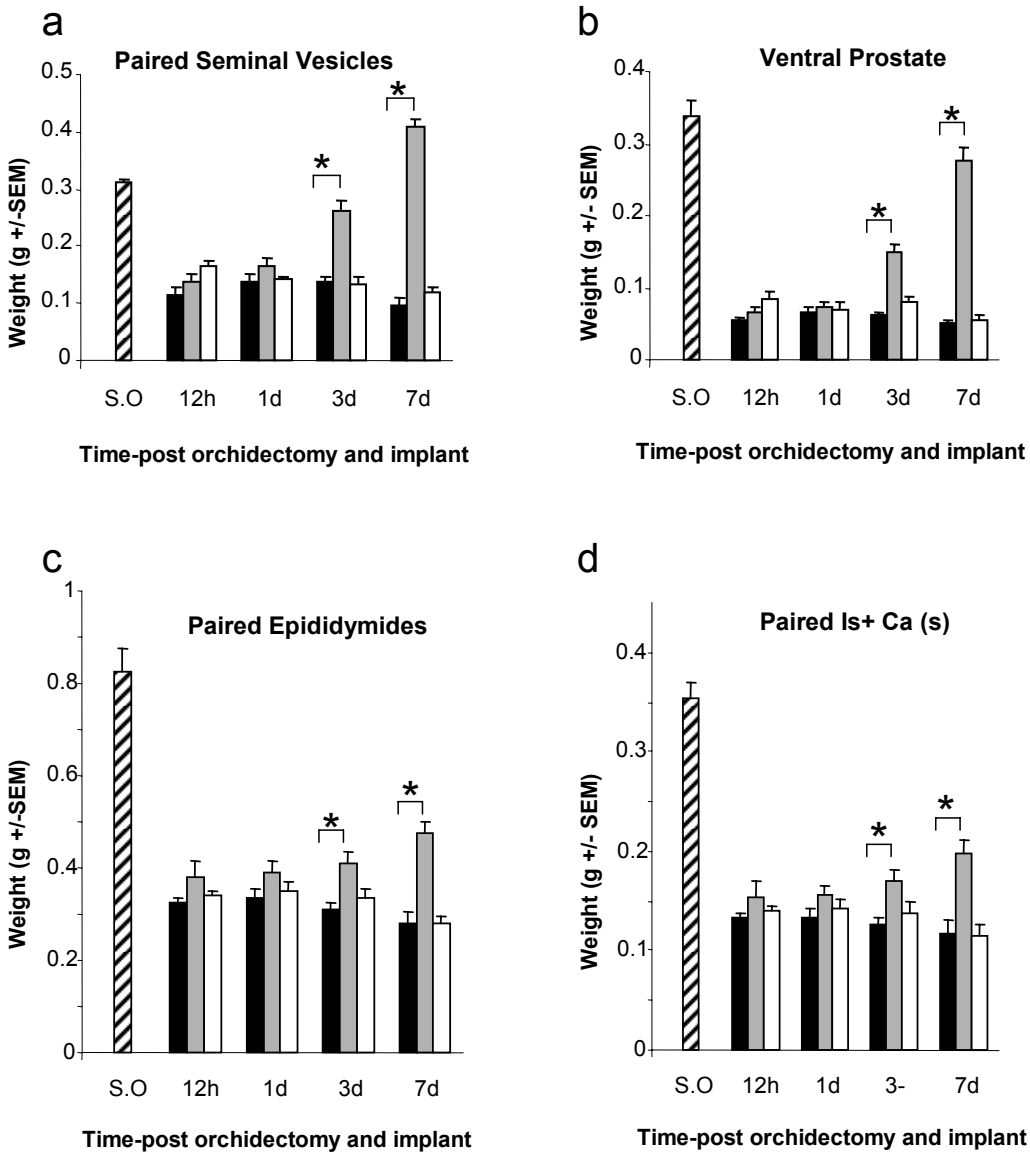

a
Paired Seminal Vesicles
Weight (g +/-SEM)
0.5
0.4
0.3
0.2
0.1
0
S.O
12h
1d
3d
7d
*
*
Time-post orchidectomy and implant
b
Ventral Prostate
Weight (g +/- SEM)
0.4
0.3
0.2
0.1
0
S.O
12h
1d
3d
7d
*
*
Time-post orchidectomy and implant
c
Paired Epididymides
Weight (g +/-SEM)
1
0.8
0.6
0.4
0.2
0
S.O
12h
1d
3d
7d
*
*
Time-post orchidectomy and implant
d
Paired Is+ Ca (s)
Weight (g +/- SEM)
0.4
0.3
0.2
0.1
0
S.O
12h
1d
3-
7d
*
*
Time-post orchidectomy and implant

Figure 2. Changes in the numbers of probe sets expressed under different conditions at different time points in the initial segment and caput epididymidis. a) Probe set expression changes after orchidectomy. The numbers of probe sets with up- or down-regulated expression is increased over time. **b)** Probe set expression is up- or down-regulated by DHT in the orchidectomized and regressed epididymis. **c)** Probe set expression is up- or down-regulated by E2 in the orchidectomized and regressed epididymis. Differential expression is defined as those transcripts that have a difference of 2-fold or greater and statistically different in both groups (n = 3 replicates).

Figure 2

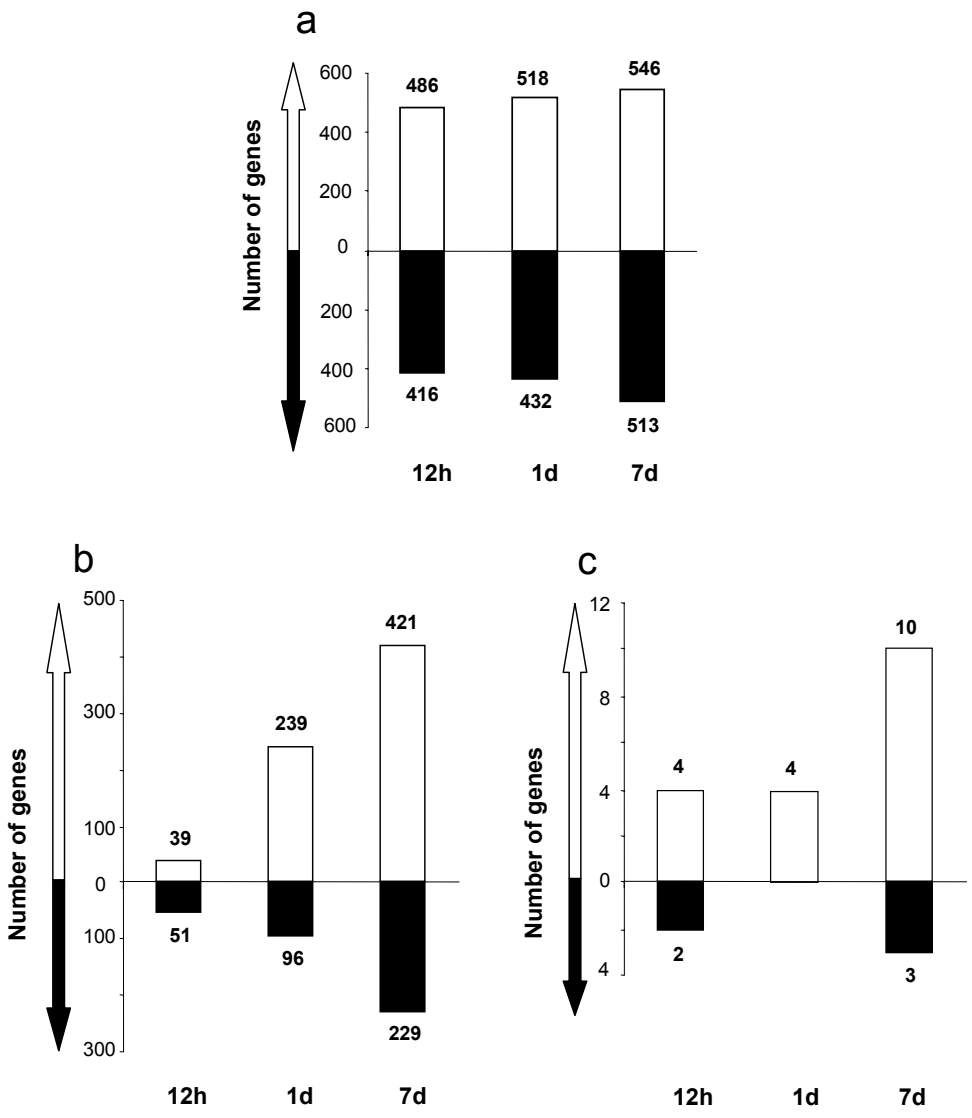

a
Number of genes
600
400
200
0
200
400
600
486
518
546
416
432
513
12h
1d
7d
b
Number of genes
500
300
100
0
100
300
39
239
421
51
96
229
12h
1d
7d
c
Number of genes
12
8
4
0
4
4
4
10
2
3
12h
1d
7d

Figure 3. Real-time quantitative PCR analysis of up-regulated genes in the regressed initial segment and caput epididymidis at 12 h, 1, and 7 days. Genes were regulated by E2. Primers used are for *Ngfr* and *S100g*. The first two bars indicate the array results and the two latter indicate the real-time PCR results. The control (orchidectomy with empty implant) data are depicted by black or stripe bars and white or gray bars indicate the orchidectomy and E2 implant group. Each bar represents the mean of 4 replicates ± SEM.

Figure 3

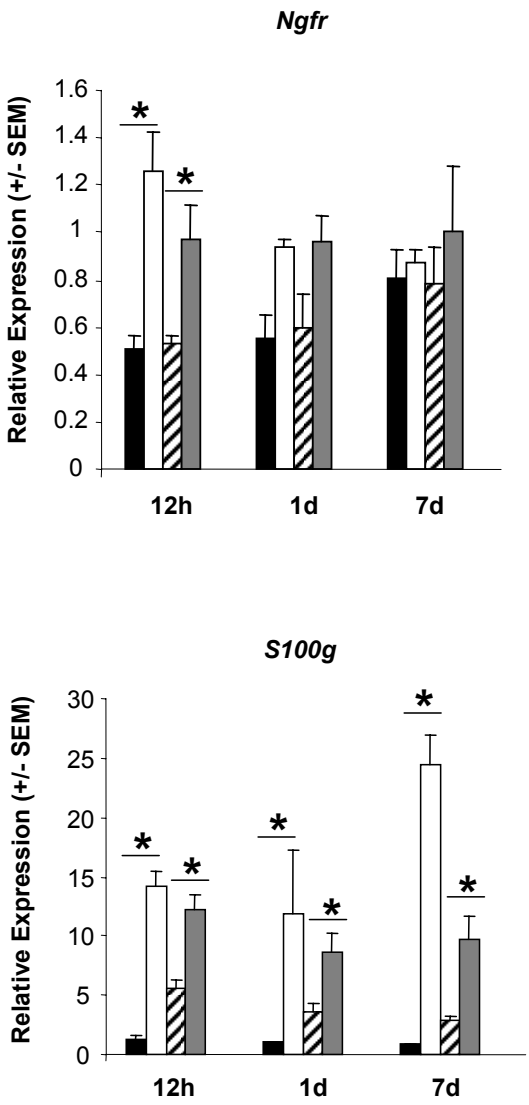

Ngfr
Relative Expression (+/- SEM)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
12h
1d
7d
S100g
Relative Expression (+/- SEM)
30
25
20
15
10
5
0
*
*
*
*
*
*
12h
1d
7d

Figure 4. Real-time quantitative PCR analysis of up- and down-regulated genes in the regressed initial segment and caput epididymidis at 12 h, 1, and 7 days. Genes were regulated by DHT. Primers used are for *Igf1*, *Gjb3*, *Ramp3*, *Igfbp3*, *Edn1*, and *Egln3*. The relative expression of *Igf1* and *Igfbp3* genes was significantly greater or smaller compared to that of orchidectomized with empty-implanted group, respectively at 1 and 7 days ($p \leq 0.05$). The other genes responded at 12 h; this response was sustained at 1 and 7 days. The first two bars indicate the array results and the two latter indicate the real-time PCR results. The control (orchidectomy with empty implant) data are depicted by black or stripe bars and white or gray bars indicate the orchidectomy and DHT implant group. Each bar represents the mean of 4 replicates ± SEM.

Figure 4

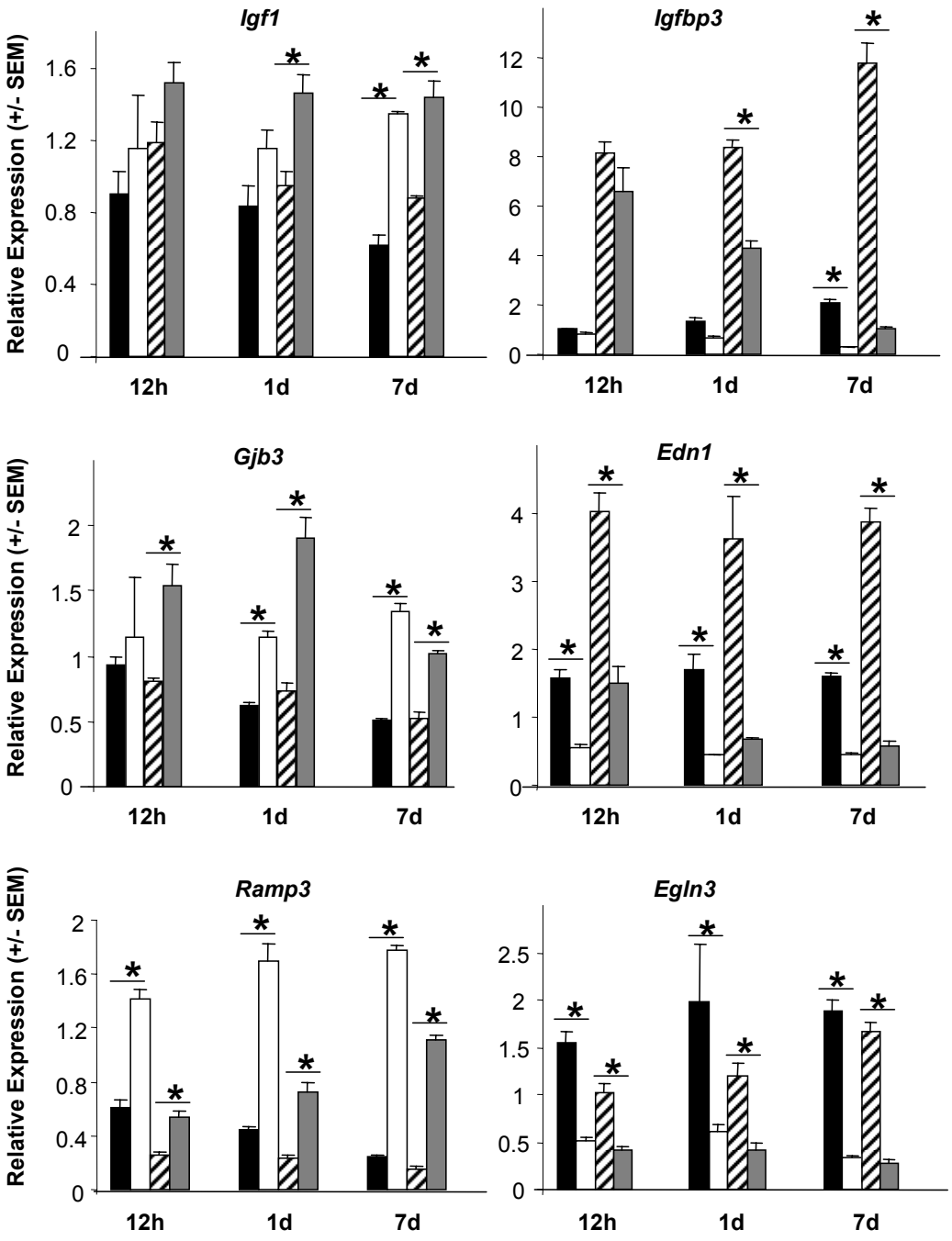

Igf1
Igfbp3
Gjb3
Edn1
Ramp3
Egln3
Relative Expression (+/- SEM)
12h
1d
7d
*

Figure 5. Analysis of IGF1 protein expression in the orchidectomized and regressed epididymis before and after DHT replacement at 7 days. Fifteen micrograms of protein extract/group (n = 3) were fractionated by SDS-PAGE, transferred to PVDF membrane and probed with an anti-IGF1 antibody. A band was detected at approximately 17 kDa. Cyclophilin A was used as an internal control for protein loading. The vertical axis of the graph represents relative intensity that was measured by densitometry and is expressed as a ratio of IGF1 to cyclophilin A relative to control. The horizontal axis indicates the different treatment groups. Each bar represents the mean of 3 replicates ± SEM.

Figure 5

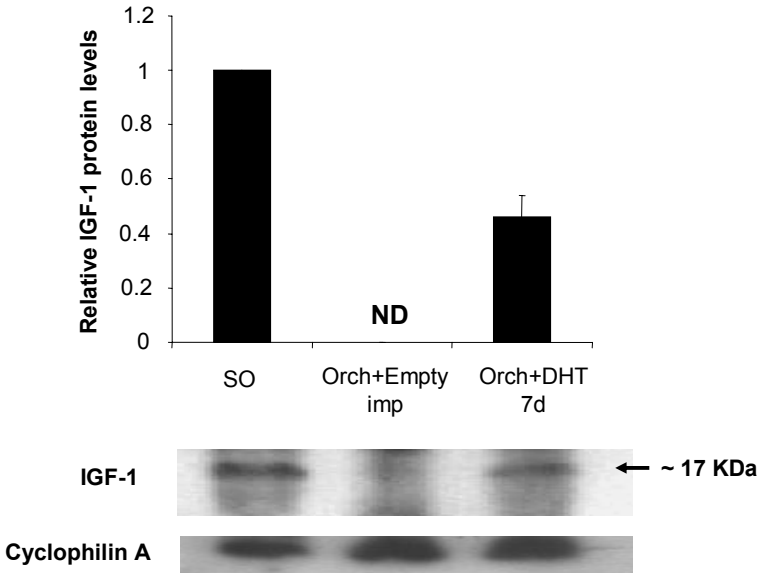
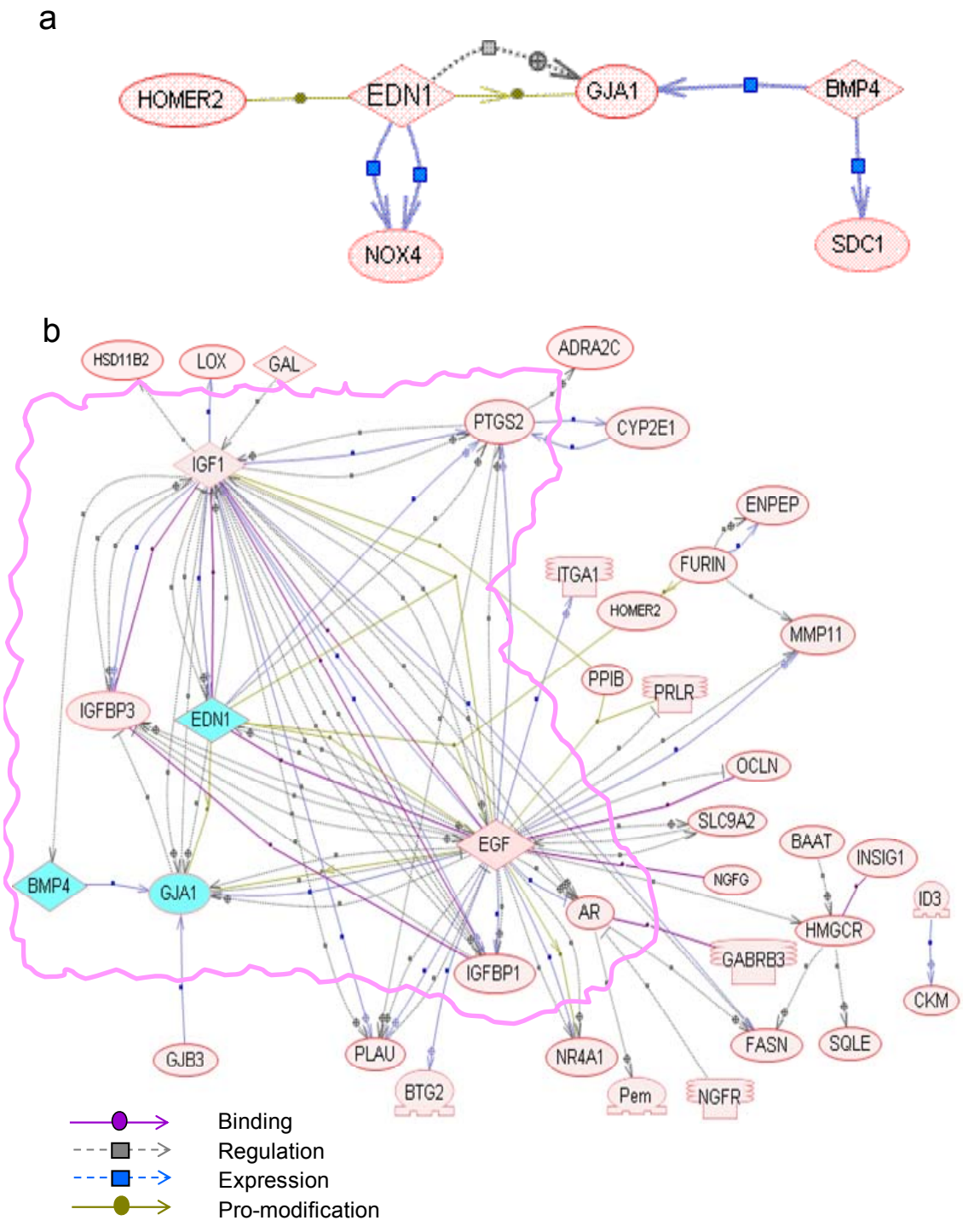

Relative IGF-1 protein levels
1.2
1
0.8
0.6
0.4
0.2
0
ND
SO
Orch+Empty imp
Orch+DHT 7d
IGF-1
~ 17 KDa
Cyclophilin A

Figure 6. Potential direct functional linkages between genes responding to DHT in the orchidectomized and regressed epididymis at early time points, a) 12 h and b) 1 day. Only genes that have been directly linked to one another via a primary interaction in the literature to date are shown. Arrows indicate the direction of the interactions, + indicates positive influence, - indicates inhibition, and the color of the linkages indicates the type of interaction between the two genes (binding, regulation, expression, and protein modification). The blue color represents common genes at 12 h and 1 day in the same pathway.

Figure 6


a
HOMER2
EDN1
GJA1
BMP4
NOX4
SDC1
b
HSD11B2
LOX
GAL
ADRA2C
PTGS2
CYP2E1
IGF1
ENPEP
FURIN
ITGA1
HOMER2
MMP11
PPIB
PRLR
IGFBP3
EDN1
OCLN
SLC9A2
BAAT
INSIG1
EGF
NGFG
BMP4
GJA1
AR
ID3
HMGCR
GABRB3
IGFBP1
CKM
GJB3
PLAU
NR4A1
FASN
SQLE
BTG2
Pem
NGFR
Binding
Regulation
Expression
Pro-modification

Supplemental Table 1. List of DHT- regulated genes with known annotations in the initial segment and caput epididymis after regression and their classification based on biological function at: **a)** 12 h **b)** 1 day **c)** 7 days. Genes symbol are shown in bold if they have a similar expression pattern between two groups: orchidectomy versus sham operated and orchidectomy versus orchidectomy plus DHT.

Suppl Table 1a

Common gene name	Gene symbol	GenBank accession no.	Fold change	
			Orch. vs Orch. + DHT	Orch. vs Sham operated
Signaling transduction/GTPase				
Arg/Abl-interacting protein	***Argbp2***	NM_053770	2.05	2.12
Chimerin (chimaerin) 1	*Chn1*	NM_032083	2.54	
FGF receptor activating protein 1	***Frag1***	NM_053895	0.36	0.25
G-protein coupled receptor 12	***Gpcr12***	NM_030831	2.23	2.4
Homer, neuronal immediate early gene2	***Homer2***	NM_053309	0.38	0.21
Receptor (calcitonin) activity modifying protein 3	***Ramp3***	NM_020100	0.43	0.16
Tropomyosin 1, alpha	*Tpm1*	NM_019131	2.1	
Metabolism/biosynthesis/enzyme				
Bile acid-Coenzyme A: amino acid N-acyltransferase	*Baat*	NM_017300	2.1	
Cytosolic cysteine dioxygenase 1	***Cdo1***	NM_052809	0.5	0.42
Carboxylesterase 3	*Ces3*	NM_133295	0.34	
Glutamate decarboxylase 1	*Gad1*	NM_017007	5.48	
Polypeptide GalNAc transferase T1	*Galnt1*	NM_024373	0.5	
Glycerol 3-phosphate dehydrogenase 1	*Gpd1*	NM_022215	0.48	
Hydroxysteroid 11-beta dehydrogenase 2	***Hsd11b2***	NM_017081	2.64	3
Aminoadipate aminotransferase	*Aadat*	NM_017193	2.44	
cytochrome P450, family 2, subfamily b, polypeptide 21	***Cyp2b21***	NM_022850	0.36	0.05

NADPH oxidase 4	*Nox4*	NM_053524	2	
Pyruvate carboxylase	***Pc***	NM_012744	0.48	0.28
6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4	***Pfkfb4***	NM_019333	0.4	0.3
Transport				
Gamma-aminobutyric acid receptor, subunit beta 3	***Gabrb3***	NM_017065	2.6	3.11
Mink-related peptide 2	*Kcne3*	NM_022235	4.32	
Potassium inwardly-rectifying channel, subfamily J, member 15	*Kcnj15*	NM_133321	0.39	
Potassium intermediate/small conductance calcium-activated channel, subfamily N, member 2	***Kcnn2***	NM_019314	2.26	2.81
Solute carrier family 13, member 2	*Slc13a2*	NM_031746	3.07	
Synaptorin	***Synpr***	NM_023974	4.38	15.73
Solute carrier family 7, member 5	***Slc7a5***	NM_017353	0.27	0.31
Regulation of cell proliferation				
Bone morphogenetic protein 4	***Bmp4***	NM_012827	2.64	3.7
Endothelin 1	***Edn1***	NM_012548	2.81	4.26
EGL nine homolog 3 (C. elegans)	*Egln3*	NM_019371	3.05	
Cell-cell signaling				
Guanylate kinase associated protein	*Dlgap1*	NM_022946	2.1	
Gap junction membrane channel protein alpha 1	***Gja1***	NM_012567	2.1	3.25
Syndecan 1	***Sdc1***	NM_013026	2.06	2.27
Regulation of transcription				
Forkhead box Q1	*Foxq1*	NM_022858	2.49	
SEC14 (S. cerevisiae)-like 2	***Sec14l2***	NM_053801	0.5	0.21

Cell adhesion				
Calcium and integrin binding 1 (calmyrin)	***Cib1***	NM_031145	0.41	0.39
Neuron-glia-CAM-related cell adhesion molecule	*Nrcam*	NM_013150	2.55	
Protein modification				
Kua homolog	***Kua***	XM_342588	0.5	0.36
Protein folding				
Cyclophilin B	*Ppib*	NM_022536	0.26	
Other				
Resistin	*Retn*	NM_144741	0.49	

Suppl Table 1b

Common gene name	Gene symbol	GenBank accession no.	Fold change	
			Orch. vs Orch. + DHT	Orch. vs Sham operated
Signaling transduction/GTPase				
Arg/Abl-interacting protein	***Argbp2***	NM_053770	2.03	2.3
FGF receptor activating protein 1	***Frag1***	NM_053895	0.29	0.19
Homer, neuronal immediate early gene2	***Homer2***	NM_053309	0.26	0.2
Receptor (calcitonin) activity modifying protein 3	***Ramp3***	NM_020100	0.26	0.15
Adrenergic receptor, alpha 2c	***Adra2c***	NM_138506	0.25	0.31
Breast carcinoma amplified sequence 1	***Bcas1***	NM_145670	0.45	0.31
EH-domain containing 3	***Ehd3***	NM_138890	0.28	0.33
MAS1 oncogene	***Mas1***	NM_012757	2.6	2.26
EF hand calcium binding protein 2	*Efcbp2*	NM_133415	0.38	
Sectm1b secreted and transmembrane 1B	***Sectm1b***	NM_199082	2.21	3.76
Phosphodiesterase 1A	***Pde1a***	NM_030871	2.24	4.02
Prolactin receptor	*Prlr*	NM_012630	2.22	
RASD family, member 2	***Rasd2***	NM_133568	0.35	0.42
Regucalcin	***Rgn***	NM_031546	0.47	0.18
Metabolism/biosynthesis/enzyme				
Bile acid-Coenzyme A: amino acid N-acyltransferase	*Baat*	NM_017300	2.23	
Cytosolic cysteine dioxygenase 1	***Cdo1***	NM_052809	0.48	0.37

Carboxylesterase 3	*Ces3*	NM_133295	0.32	
Glutamate decarboxylase 1	*Gad1*	NM_017007	3.42	
Hydroxysteroid 11-beta dehydrogenase 2	***Hsd11b2***	NM_017081	3.4	2.64
Aminoadipate aminotransferase	*Aadat*	NM_017193	2.57	
6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4	***Pfkfb4***	NM_019333	0.44	0.31
4-aminobutyrate aminotransferase	*Abat*	NM_031003	0.39	
ATP citrate lyase	***Acly***	NM_016987	0.44	0.47
Adenylate kinase 3-like 1	***Ak3l1***	NM_017135	0.36	0.31
Aldehyde dehydrogenase family 1, member A1	***Aldh1a1***	NM_022407	0.47	0.38
Arginosuccinate synthetase	*Ass*	NM_013157	2.24	
Creatine kinase, muscle	***Ckm***	NM_012530	0.48	0.14
CTL target antigen	***Cth***	NM_017074	0.15	0.14
Putative fatty acid desaturase	***Fads3***	NM_173137	0.49	0.42
Fatty acid synthase	*Fasn*	NM_017332	0.37	
Farensyl diphosphate synthase	***Fdps***	NM_031840	0.32	0.33
Glycine amidinotransferase (L-arginine:glycine amidinotransferase)	*Gatm*	NM_031031	0.49	
Glycine decarboxylase	***Gldc***	XM_219785	0.28	0.12
3-hydroxy-3-methylglutaryl-Coenzyme A reductase	***Hmgcr***	NM_013134	0.46	0.46
3-hydroxy-3-methylglutaryl-Coenzyme A synthase1	*Hmgcs1*	NM_017268	0.38	
Isopentenyl-diphosphate delta isomerase	***Idi1***	NM_053539	0.36	0.31
Growth response protein (CL-6)	***Insig1***	NM_022392	0.37	0.38
Keratin complex 2, basic, gene 8	***Krt2-8***	NM_199370	0.45	0.44

Protein phosphatase 1, regulatory (inhibitor) subunit 3B	*Ppp1r3b*	NM_138912	0.49	
Lecithin-retinol acyltransferase	*Lrat*	NM_022280	0.49	
Methionine adenosyltransferase II, alpha	***Mat2a***	NM_134351	0.43	0.38
Membrane protein, palmitoylated 3 (MAGUK p55 subfamily member 3)	***Mpp3***	XM_001081481	0.22	0.21
Mevalonate pyrophosphate decarboxylase	*Mvd*	NM_031062	0.38	
Phenylalanine hydroxylase	***Pah***	NM_012619	6.78	2.04
peroxisomal membrane protein Pmp26p (Peroxin-11)	*Pex11a*	NM_053487	0.41	
Phosphofructokinase, platelet	***Pfkp***	NM_206847	0.34	0.26
3-phosphoglycerate dehydrogenase	***Phgdh***	NM_031620	0.33	0.3
Retinol dehydrogenase 2	*Rdh2*	NM_199208	0.22	
Sterol-C4-methyl oxidase-like	***Sc4mol***	NM_012664	0.35	0.4
Surfactant, pulmonary-associated protein B	***Sftpb***	NM_138842	0.07	0.07
Squalene epoxidase	***Sqle***	NM_017136	0.38	0.28
steroid-5-alpha-reductase, alpha polypeptide 2	***Srd5a2***	NM_022711	0.37	0.4
Spermidine synthase	*Srm*	NM_053464	0.48	
Sulfotransferase family 1D, member 1	***Sult1d1***	NM_021769	0.24	0.27
UDP glycosyltransferase 1 family, polypeptide A6	*Ugt1a6*	NM_001039691	2.15	
Ubiquitin specific protease 2	*Usp2*	NM_053774	0.49	
Transport				
Gamma-aminobutyric acid receptor, subunit beta 3	*Gabrb3*	NM_017065	3.15	
Mink-related peptide 2	*Kcne3*	NM_032065	2.58	
Potassium inwardly-rectifying channel, subfamily	***Kcnj15***	NM_133321	0.32	0.27

J, member 15				
Synaptorin	***Synpr***	NM_023974	20.56	20.35
Solute carrier family 7, member 5	***Slc7a5***	NM_017353	0.26	0.31
Solute carrier family 1 (neutral amino acid transporter), member 5	***Slc1a5***	NM_175758	0.46	0.37
Blocked early in transport 1 homolog (S.cerevisiae)	*Bet1*	NM_019251	0.49	
Cytochrome P450, subfamily 2E, polypeptide 1	***Cyp2e1***	NM_031543	4	17.4
Cytochrome P450, subfamily 4B, polypeptide 1	***Cyp4b1***	NM_016999	2.12	3.45
Cytochrome P450 4F4	***Cyp4f4***	NM_173123	0.2	0.14
Putative potassium channel TWIK	***Kcnk1***	NM_021688	0.39	0.42
Solute carrier family 44, member 4	***Slc44a4***	NM_212541	0.35	0.22
Purinergic receptor P2X, ligand-gated ion channel, 2	*P2rx2*	NM_053656	2.06	
PDZ domain containing 1	***Pdzk1***	NM_031712	0.41	0.3
Prominin 2	*Prom2*	NM_138857	0.44	
Rh type C glycoprotein	***Rhcg***	NM_183053	0.38	0.25
Solute carrier family 6 (neurotransmitter transporter, betaine/GABA), member 12	***Slc6a12***	NM_017335	0.3	0.18
Potassium channel, subfamily T, member 1	*Kcnt1*	NM_021853	0.36	
Solute carrier family 22, member 5	***Slc22a5***	NM_019269	0.45	0.1
Solute carrier family 30, member 2	*Slc30a2*	NM_012890	0.37	
Solute carrier family 9, member 2	***Slc9a2***	NM_012653	0.37	0.24
Sulfite oxidase	***Suox***	NM_031127	0.28	0.28
Synaptic vesicle glycoprotein 2 b	*Sv2b*	NM_057207	0.47	
Synaptotagmin XII	***Syt12***	NM_138835	0.27	0.14
Solute carrier family 30 (zinc transporter),	*Slc30a3*	NM_0010132	0.48	

member3		43		
Regulation of cell proliferation/growth				
Bone morphogenetic protein 4	***Bmp4***	NM_012827	2.17	3.24
Endothelin 1	***Edn1***	NM_012548	3.94	4.28
Epidermal growth factor	***Egf***	NM_012842	0.5	0.32
C-fos induced growth factor (vascular endothelial growth factor D)	***Figf***	NM_031761	2.68	3.95
Insulin-like growth factor binding protein 1	***Igfbp1***	NM_013144	2.55	3.83
Prostaglandin-endoperoxide synthase 2	*Ptgs2*	NM_017232	0.47	
Cell-cell signaling				
Guanylate kinase associated protein	*Dlgap1*	NM_022946	2.12	
Gap junction membrane channel protein alpha 1	***Gja1***	NM_012567	2.31	3.3
Ephrin B1	***Efnb1***	NM_017089	0.25	0.38
Gap junction membrane channel protein alpha 4	***Gja4***	NM_021654	0.42	0.48
Gap junction membrane channel protein beta 3	***Gjb3***	NM_019240	0.44	0.43
Occludin	***Ocln***	NM_031329	0.12	0.09
Regulation of transcription				
Forkhead box Q1	*Foxq1*	NM_022858	2.6	
SEC14 (S. cerevisiae)-like 2	***Sec14l2***	NM_053801	0.33	0.19
Androgen receptor	***Ar***	NM_012502	2.28	2.21
B-cell translocation gene 2, anti-proliferative	***Btg2***	NM_017259	2.09	2.02
Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 4	***Cited4***	NM_053699	0.39	0.28
Hairy/enhancer-of-split related with YRPW motif 1	*Hey1*	XM_342216	2.09	
Inhibitor of DNA binding 3, dominant negative helix-loop-helix protein	***Id3***	NM_013058	2.56	5.45

Basic helix-loop-helix domain containing, class B, 8	*Bhlhb8*	NM_012863	0.25	
Immediate early gene transcription factor NGFI-B	***Nr4a1***	NM_024388	0.18	0.27
Placentae and embryos oncofetal gene	***Pem***	NM_022175	0.42	0.42
WNK lysine deficient protein kinase 4	*Wnk4*	NM_175579	2.45	
Cell adhesion				
Calcium and integrin binding 1 (calmyrin)	***Cib1***	NM_031145	0.46	0.42
Amphoterin induced gene and ORF 3	***Amigo3***	NM_178144	0.25	0.32
Similar to M-cadherin (LOC361432), mRNA	*Cdh15*	NM_207613	0.31	
Carbohydrate sulfotransferase 10	***Chst10***	NM_080397	0.36	0.19
Integrin alpha 1	***Itga1***	NM_030994	2.09	2.03
Spondin 2, extracellular matrix protein	***Spon2***	NM_138533	2.3	2.26
Neural cell adhesion molecule 1	***Ncam1***	NM_031521	2.43	3.81
Trophoblast glycoprotein	***Tpbg***	NM_031807	2.29	5.43
Immune response				
Interleukin 13 receptor, alpha 2	***Il13ra2***	NM_133538	0.19	0.11
Interleukin 1 receptor-like 1	***Il1rl1***	NM_013037	0.3	0.26
Protein modification				
Kua homolog	***Kua***	XM_342588	0.45	0.33
Lysyl oxidase	*Lox*	NM_017061	2.46	
Protein folding				
Cyclophilin B	*Ppib*	NM_022536	0.2	
Response to stress				
Crystallin, alpha B	*Cryab*	NM_012935	2.16	
Galanin	***Gal***	NM_033237	0.38	0.23

Hypoxia up-regulated 1	***Hyou1***	NM_001034028	0.37	0.43
Peroxiredoxin 6	***Prdx6***	NM_053576	0.42	0.32
Apoptosis				
Nerve growth factor receptor (TNFR superfamily, member 16)	***Ngfr***	NM_012610	0.25	0.33
Programmed cell death 8 (apoptosis-inducing factor)	***Pdcd8***	NM_031356	0.46	0.45
Proteolysis				
Calpain 10	***Capn10***	NM_031673	0.45	0.35
Cathepsin C	***Ctsc***	NM_017097	2.38	4.65
Aminopeptidase A	***Enpep***	NM_022251	2.02	2.01
Epoxide hydrolase 1	***Ephx1***	NM_012844	0.46	0.41
Matrix metalloproteinase 11	***Mmp11***	NM_012980	2.01	3.11
Nerve growth factor, gamma	***Ngfg***	NM_031523	2.11	2.42
Transmembrane protein 27	*Tmem27*	NM_020976	3.48	
Proprotein convertase subtilisin/kexin type3	***Pcsk3***	NM_019331	0.31	0.23
Plasminogen activator, urokinase	***Plau***	NM_013085	2.17	3.28
Sperm associated antigen 5	*Spag5*	NM_001044224	0.37	
Transmembrane protease, serine 2	*Tmprss2*	NM_130424	2.05	
Spermatogenesis				
Citron	***Cit***	NM_031790	0.47	0.33
Gamma-glutamyltransferase 1	***Ggt1***	NM_053840	0.33	0.27
Others				
CD52 antigen	***Cd52***	NM_053983	0.02	0.01

Espin	***Espn***	NM_019622	0.25	0.21
Surface protein MCA-32	***Mca32***	NM_021585	0.48	0.4
Olfactomedin related ER localized protein	*Olfm1*	NM_053573	0.36	
Opioid receptor, sigma 1	***Oprs1***	NM_030996	0.5	0.42
Orosomucoid 1	*Orm1*	NM_053288	0.48	
Plastin 3 (T-isoform)	*Pls3*	XM_343776	0.48	
ProSAPiP1 protein	***Prosapip1***	NM_172022	2.05	2.66
Testis expressed gene 101	*Tex101*	NM_139037	0.19	
Troponin I, slow isoform	*Tnni1*	NM_017184	0.33	

Suppl Table 1c

Common gene name	Gene symbol	GenBank accession no.	Fold change	
			Orch. vs Orch. + DHT	Orch. vs Sham operated
Signaling transduction/GTPase				
Arg/Abl-interacting protein	***Argbp2***	NM_053770	2.14	2.38
FGF receptor activating protein 1	***Frag1***	NM_053895	0.2	0.18
G-protein coupled receptor 12	***Gpcr12***	NM_001037295	2.54	2.51
Homer, neuronal immediate early gene2	***Homer2***	NM_053309	0.23	0.2
Receptor (calcitonin) activity modifying protein 3	***Ramp3***	NM_020100	0.14	0.11
Adrenergic receptor, alpha 2b	***Adra2b***	NM_138505	0.47	0.4
Adrenergic receptor, alpha 2c	***Adra2c***	NM_138506	0.3	0.31
Cysteine-rich secretory protein 1	***Crisp1***	NM_022859	0.18	0.15
Angiotensin II receptor, type 1 (AT1A)	***Agtr1a***	NM_030985	2.18	2.16
Angiopoietin-like 2	***Angptl2***	NM_133569	2.32	2.38
Breast carcinoma amplified sequence 1	***Bcas1***	NM_145670	0.45	0.32
Calcitonin receptor-like	***Calcrl***	NM_012717	2.03	2.45
Calcium/calmodulin-dependent protein kinase II beta subunit	*Camk2b*	NM_001042354	0.45	
Caveolin 2 (Cav2), mRNA	***Cav2***	NM_131914	2.38	2.94
Chimerin (chimaerin) 2	*Chn2*	NM_032084	2.03	
Citron	***Cit***	NM_001029911	0.39	0.29
Chemokine receptor (LCR1)	*Cxcr4*	NM_022205	2.25	

Dickkopf homolog 3 (Xenopus laevis)	***Dkk3***	NM_138519	2.36	2.98
EH-domain containing 3	***Ehd3***	NM_138890	0.29	0.28
Coagulation factor II receptor	***F2r***	NM_012950	2.1	2.75
Prepro bone inducing protein	*Gdf10*	NM_024375	0.43	
Purinergic receptor P2Y, G-protein coupled, 14	***P2ry14***	NM_133577	2.14	2.08
Leucine-rich repeat-containing G protein-coupled receptor 4	***Lgr4***	NM_173328	0.44	0.43
Growth factor receptor bound protein 14	***Grb14***	NM_031623	2.2	2.15
Gastrin-releasing peptide	*Grp*	NM_133570	0.27	
Guanylate cyclase 1, soluble, alpha 3	***Gucy1a3***	NM_017090	2.02	2.78
Homer, neuronal immediate early gene, 3	***Homer3***	NM_053310	0.49	0.48
CUB and zona pellucida-like domains 1	*Cuzd1*	NM_054005	0.07	
Inositol 1, 4, 5-triphosphate receptor 3	***Itpr3***	NM_013138	0.45	0.34
Phosphodiesterase 4D interacting protein (myomegalin)	***Pde4dip***	NM_022382	0.28	0.23
MAS1 oncogene	*Mas1*	NM_012757	2.14	
Sectm1b secreted and transmembrane 1B	***Sectm1b***	NM_199082	2.58	3.32
Tubulin, beta 2c	*Tubb2c*	NM_199094	0.47	
EF hand calcium binding protein 2	*Efcbp2*	NM_133415	0.26	
Phosphodiesterase 1A	***Pde1a***	NM_030871	3.45	4.73
Phosphodiesterase 4B	***Pde4b***	NM_017031	2.04	2.73
Rab38, member of RAS oncogene family	***Rab38***	NM_145774	3	2.79
RASD family, member 2	***Rasd2***	NM_133568	0.42	0.45
Regucalcin	***Rgn***	NM_031546	0.17	0.12
Regulator of G-protein signaling 1	***Rgs1***	NM_019336	2.2	2.76

Reticulon 1	*Rtn1*	NM_053865	2.21	
Membrane associated guanylate kinase, WW and PDZ domain containing 3	***Magi3***	NM_139084	2.08	2.14
Stathmin-like 2	*Stmn2*	NM_053440	2.41	
Tenascin XA	***Tnxa***	ID: 25602	2.69	3.37
Metabolism/biosynthesis/enzyme				
Cytosolic cysteine dioxygenase 1	***Cdo1***	NM_052809	0.44	0.37
Carboxylesterase 3	*Ces3*	NM_133295	0.37	
Glutamate decarboxylase 1	*Gad1*	NM_017007	3.8	
Polypeptide GalNAc transferase T1	*Galnt1*	NM_024373	0.49	
Hydroxysteroid 11-beta dehydrogenase 2	***Hsd11b2***	NM_017081	3.56	3.43
Pyruvate carboxylase	***Pc***	NM_012744	0.36	0.28
6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4	***Pfkfb4***	NM_019333	0.44	0.3
ATP citrate lyase	***Acly***	NM_016987	0.46	0.44
Adenosine kinase	*Adk*	NM_012895	0.47	
Adenylate kinase 3-like 1	***Ak3l1***	NM_017135	0.25	0.25
Aldehyde dehydrogenase family 1, member A1	***Aldh1a1***	NM_022407	0.34	0.34
Aldolase C, fructose-biphosphate	***Aldoc***	NM_012497	0.25	0.26
Apolipoprotein C-I	***Apoc1***	NM_012824	2.55	3.15
Cd36 antigen	***Cd36***	NM_031561	2.96	4.25
Carboxylesterase 1	***Ces1***	NM_031565	0.34	0.42
Carboxylesterase 2 (intestine, liver)	***Ces2***	NM_133586	0.21	0.11
Cellular retinoic acid binding protein 2	***Crabp2***	NM_017244	0.46	0.46
CTL target antigen	***Cth***	NM_017074	0.13	0.13

Cubilin (intrinsic factor-cobalamin receptor)	***Cubn***	NM_053332	0.25	0.15
Diazepam binding inhibitor	*Dbi*	NM_031853	0.34	
Phenylalkylamine Ca2+ antagonist (emopamil) binding protein	***Ebp***	NM_057137	0.31	0.31
Ectonucleotide pyrophosphatase/phosphodiesterase 1	***Enpp1***	NM_053535	0.42	0.41
Putative fatty acid desaturase	***Fads3***	NM_173137	0.46	0.41
Fatty acid synthase	*Fasn*	NM_017332	0.42	
Farnesyl diphosphate farnesyl transferase 1	*Fdft1*		0.42	
Farensyl diphosphate synthase	***Fdps***	NM_031840	0.25	0.39
Fetuin beta	***Fetub***	NM_053348	27.62	28.03
Alpha 1,3-fucosyltransferase Fuc-T (similar to mouse Fut4)	***Fut4***	NM_022219	0.41	0.49
UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferase T5	*Galnt5*	NM_031796	0.29	
Glycine amidinotransferase (L-arginine:glycine amidinotransferase)	*Gatm*	NM_031031	0.47	
Glucokinase	***Gck***	NM_012565	0.41	0.3
Enzymatic glycosylation-regulating gene	***Gcnt1***	NM_022276	0.4	0.45
Glycine cleavage system protein H (aminomethyl carrier)	***Gcsh***	NM_133598	0.45	0.4
Galactosidase, beta 1	***Glb1***	XM_236675	0.29	0.21
Glycine decarboxylase	***Gldc***	XM_219785	0.09	0.09
Uroguanylin	***Guca2b***	NM_022284	12.76	15.58
Histone deacetylase 6	*Hdac6*	XM_228753	0.31	
3-hydroxy-3-methylglutaryl-Coenzyme A reductase	***Hmgcr***	NM_013134	0.44	0.49

3-hydroxy-3-methylglutaryl-Coenzyme A synthase1	*Hmgcs1*	NM_017268	0.42	
Heat-responsive protein 12	***Hrsp12***	NM_031714	0.47	0.5
Hydroxysteroid dehydrogenase 17 beta, type 7	*Hsd17b7*	NM_017235	0.48	
Isopentenyl-diphosphate delta isomerase	***Idi1***	NM_053539	0.25	0.37
Growth response protein (CL-6)	***Insig1***	NM_022392	0.34	0.46
Keratin complex 2, basic, gene 8	***Krt2-8***	NM_199370	0.47	0.49
Acetoacetyl-CoA synthetase	***Aacs***	NM_023104	0.36	0.34
Lipoprotein lipase	***Lpl***	NM_012598	2.71	4.35
Low density lipoprotein receptor-related protein 2	***Lrp2***	NM_030827	0.3	0.24
2,3-oxidosqualene: lanosterol cyclase	***Lss***	NM_031049	0.48	0.47
Methionine adenosyltransferase II, alpha	***Mat2a***	NM_134351	0.47	0.41
Microsomal glutathione S-transferase 1	***Mgst1***	NM_134349	2.08	2.47
Membrane protein, palmitoylated 3 (MAGUK p55 subfamily member 3)	***Mpp3***	XM_001081481	0.27	0.13
Mevalonate pyrophosphate decarboxylase	***Mvd***	NM_031062	0.38	0.49
Phenylalanine hydroxylase	***Pah***	NM_012619	4.29	2.05
Phosphoenolpyruvate carboxykinase 1	***Pck1***	NM_198780	6.36	6.34
Perosisomal 2-enoyl-CoA reductase	*Pecr*	NM_133299	0.32	
Phosphatidylethanolamine N-methyltransferase	***Pemt***	NM_013003	0.36	0.3
Peroxisomal membrane protein Pmp26p (Peroxin-11)	*Pex11a*	NM_053487	0.313	
Phosphofructokinase, platelet	***Pfkp***	NM_206847	0.25	0.22
3-phosphoglycerate dehydrogenase	***Phgdh***	NM_031620	0.26	0.25
Phospholipase A2, group IIA	***Pla2g2a***	NM_031598	2.22	3.64

Phospholipase A2, group 5	***Pla2g5***	NM_017174	2.1	2.13
Prostaglandin D2 synthase	***Ptgds***	NM_013015	0.45	0.38
Protein tyrosine phosphatase, non-receptor type 5	*Ptpn5*	NM_019253	2.3	
Protein tyrosine phosphatase, receptor type, R	***Ptprr***	NM_053594	2.23	3.03
Epididymal secretory protein 4	*re4*	NM_173109	2.21	
Retinol dehydrogenase 2	***Rdh2***	NM_199208	0.23	0.15
Sterol-C4-methyl oxidase-like	***Sc4mol***	NM_012664	0.37	0.48
Sterol-C5-desaturase (fungal ERG3, delta-5-desaturase)-like	***Sc5d***	NM_053642	0.46	0.42
Surfactant, pulmonary-associated protein B	***Sftpb***	NM_138842	0.06	0.06
ST3 beta-galactoside alpha-2,3-sialyltransferase 2	***St3gal2***	NM_031695	0.38	0.32
Squalene epoxidase	***Sqle***	NM_017136	0.26	0.26
steroid-5-alpha-reductase, alpha polypeptide 2	***Srd5a2***	NM_022711	0.45	0.4
Sulfotransferase family, cytosolic, 1C, member 2	*Sult1c2*	NM_133547	0.35	
Sulfotransferase family 1D, member 1	***Sult1d1***	NM_021769	0.36	0.24
Thyroid hormone responsive protein	***Thrsp***	NM_012703	0.44	0.42
Ubiquitin specific protease 2	***Usp2***	NM_053774	0.36	0.36
Vanin 1	***Vnn1***	NM_001025623	2.48	2.28
Transport				
Gamma-aminobutyric acid receptor, subunit beta 3	***Gabrb3***	NM_017065	3.29	4.08
Mink-related peptide 2	*Kcne3*	NM_032065	2.35	
Potassium inwardly-rectifying channel, subfamily J, member 15	***Kcnj15***	NM_133321	0.3	0.28

Potassium intermediate/small conductance calcium-activated channel, subfamily N, member 2	***Kcnn2***	NM_019314	4.32	3.64
Solute carrier family 13, member 2	*Slc13a2*	NM_031746	2.21	
synaptorin	***Synpr***	NM_023974	22.35	17.58
Solute carrier family 7, member 5	***Slc7a5***	NM_017353	0.24	0.33
ATP-binding cassette, sub-family G (WHITE), member 1	***Abcg1***	NM_053502	2.53	2.68
Albumin	***Alb***	NM_134326	4.35	6.4
Solute carrier family 1 (neutral amino acid transporter), member 5	*Slc1a5*	NM_175758	0.48	
ATPase, Ca++-sequestering	***Atp2c1***	NM_131907	0.44	0.37
ATPase, class II, type 9A	***Atp9a***	ID: 84011	0.31	0.32
Chloride channel Kb	*Clcnkb*	NM_173103	2.12	
Cytochrome P450, subfamily 11A	***Cyp11a***	NM_017286	0.4	0.41
Cytochrome P450, subfamily 2E, polypeptide 1	***Cyp2e1***	NM_031543	9.77	21.59
Cytochrome P450, subfamily 4B, polypeptide 1	***Cyp4b1***	NM_016999	2.77	3.52
Cytochrome P450 4F4	***Cyp4f4***	NM_173123	0.07	0.07
Cytochrome P450, subfamily 51	***Cyp51***	NM_012941	0.43	0.5
Fatty acid binding protein 4	***Fabp4***	NM_053365	2.91	4.65
FXYD domain-containing ion transport regulator 6	***Fxyd6***	NM_022005	2.93	2.55
Solute carrier family 37 (glycerol-6-phosphate transporter), member 4	***Slc37a4***	NM_031589	0.47	0.46
Glutamate receptor, ionotropic, kainate 2	***Grik2***	NM_019309	2.11	2.7
Putative potassium channel TWIK	***Kcnk1***	NM_021688	0.43	0.41
Cytochrome P450, family 2, subfamily b, polypeptide 21	***Cyp2b21***	NM_198733	0.06	0.03

PDZK1 interacting protein 1	*Pdzk1ip1*	NM_130401	0.33	
Solute carrier family 44, member 4	***Slc44a4***	NM_212541	0.22	0.2
Alpha-2u globulin PGCL4	***Obp3***	NM_001033958	0.34	0.28
Purinergic receptor P2X, ligand-gated ion channel, 2	*P2rx2*	NM_053656	2.88	
Phosphatidylcholine transfer protein	***Pctp***	NM_017225	0.22	0.16
PDZ domain containing 1	***Pdzk1***	NM_031712	0.36	0.31
G protein-coupled receptor associated sorting protein 1	***Gprasp1***	NM_134386	2.53	3.24
Retinol binding protein 4	***Rbp4***	XM_215285	3.49	4.3
Rh type C glycoprotein	***Rhcg***	NM_183053	0.17	0.12
Solute carrier family 6 (neurotransmitter transporter, betaine/GABA), member 12	***Slc6a12***	NM_017335	0.26	0.19
Sideroflexin 5	***Sfxn5***	NM_153298	2.33	2.89
Potassium channel, subfamily T, member 1	*Kcnt1*	NM_021853	0.29	
Solute carrier family 12, member 3	***Slc12a3***	NM_019345	0.42	0.28
Solute carrier family 15, member 2	***Slc15a2***	NM_031672	0.44	0.3
Solute carrier family 16 (monocarboxylic acid transporters), member 6	***Slc16a6***	NM_198760	0.43	0.41
Solute carrier family 1, member 1	*Slc1a1*	NM_013032	2.47	
Solute carrier family 22, member 5	***Slc22a5***	NM_019269	0.13	0.1
Fatty acid transport protein	***Slc27a1***	NM_053580	2.07	2.3
Solute carrier family 30, member 2	*Slc30a2*	NM_012890	0.42	
Solute carrier family 34 (sodium phosphate), member 2	*Slc34a2*	NM_053380	0.11	
Solute carrier family 5, member 6	***Slc5a6***	NM_130746	0.48	0.43
Solute carrier family 7, member 1	***Slc7a1***	NM_013111	0.49	0.43

Solute carrier family 9, member 2	***Slc9a2***	NM_012653	0.33	0.24
Sulfite oxidase	***Suox***	NM_031127	0.29	0.29
Synaptic vesicle glycoprotein 2 b	*Sv2b*	NM_057207	0.4	
Synaptotagmin XII	***Syt12***	NM_138835	0.19	0.1
T-cell receptor gamma chain	***Tcrg***	ID: 24821	2.02	2.36
Transferrin receptor	***Tfrc***	XM_340999	0.28	0.26
Transient receptor potential cation channel, subfamily V, member 6	***Trpv6***	NM_053686	0.45	0.48
Xanthine dehydrogenase	***Xdh***	NM_017154	2.93	3.57
Plasma membrane proteolipid	***Pllp***	NM_022533	2.1	2.07
Solute carrier family 30 (zinc transporter), member3	***Slc30a3***	NM_001013243	0.35	0.49
Regulation of cell proliferation/growth				
Bone morphogenetic protein 4	***Bmp4***	NM_012827	3.2	3.47
Endothelin 1	***Edn1***	NM_012548	3.59	4.88
EGL nine homolog 3 (C. elegans)	*Egln3*	NM_012842	5.48	
Actin alpha 1	***Acta1***	NM_019212	0.21	0.26
Cyclin F	***Ccnf***	XM_340763	0.44	0.32
Cyclin-dependent kinase inhibitor 1C, p57	***Cdkn1c***	NM_001033757	2.1	3.02
Epidermal growth factor	***Egf***	NM_012842	0.44	0.3
Similar to replication protein A3 (LOC296883), mRNA	*Epb4.1l3*	NM_053927	0.45	
Endothelial cell-specific molecule 1	*Esm1*	NM_022604	0.31	
C-fos induced growth factor (vascular endothelial growth factor D)	***Figf***	NM_031761	3.75	4.07
Growth arrest specific 7	***Gas7***	NM_053484	2.32	2.85

Insulin-like growth factor 1	*Igf1*	NM_001082477	0.47	
Insulin-like growth factor binding protein 3	***Igfbp3***	NM_012588	7.8	10.18
Kruppel-like factor 5 (intestinal)	***Klf5***	NM_053394	2.77	3.26
Kinesin family member 11	*Kif11*	XM_001060913	0.26	
Kinesin family member 2C	***Kif2c***	NM_001085369	0.46	0.37
Immunoglobulin superfamily, member 10	***Igsf10***	NM_198768	2.23	4.04
Microphthalmia-associated transcription factor	***Mitf***	XM_001065759	2.22	2.09
Metallothionein 3	***Mt3***	NM_053968	0.22	0.23
Platelet-derived growth factor, C polypeptide	***Pdgfc***	NM_031317	2.06	2.54
Response gene to complement 32	***Rgc32***	NM_054008	3.16	2.76
Transforming growth factor, beta induced, 68 kDa	***Tgfbi***	XM_573983	2.6	3.7
LR8 protein	***Lr8***	NM_134390	2.12	2.77
Cell-cell signaling				
Gap junction membrane channel protein alpha 1	***Gja1***	NM_012567	3.19	4.2
Calbindin 2	***Calb2***	NM_053988	0.2	0.15
Ephrin B1	***Efnb1***	NM_017089	0.24	0.35
Frizzled homolog 1 (Drosophila)	***Fzd1***	NM_021266	2.41	2.93
Gap junction membrane channel protein alpha 4	***Gja4***	NM_021654	0.36	0.4
Gap junction membrane channel protein beta 3	***Gjb3***	NM_019240	0.33	0.36
Occludin	***Ocln***	NM_031329	0.08	0.06
Syndecan 2	***Sdc2***	NM_013082	2.04	2.45
SPARC-like 1	***Sparcl1***	NM_012946	2.06	2.48

Regulation of transcription

SEC14 (S. cerevisiae)-like 2	***Sec14l2***	NM_053801	0.16	0.15
Androgen receptor	***Ar***	NM_012502	3.27	2.53
Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 4	***Cited4***	NM_053699	0.23	0.23
Four and a half LIM domains 1	***Fhl1***	NM_001033926	2.35	2.48
Four and a half LIM domains 2	***Fhl2***	NM_031677	2.03	2.7
GA repeat binding protein, beta 1	*Gabpb1*	XM_344606	0.48	
General transcription factor III A	***Gtf3a***	ID: 246299	0.47	0.49
Hepatoma-derived growth factor, related protein 2	***Hdgfrp2***	NM_133548	2.22	2.37
Hairy/enhancer-of-split related with YRPW motif 1	***Hey1***	XM_342216	2.7	2.35
Inhibitor of DNA binding 3, dominant negative helix-loop-helix protein	***Id3***	NM_013058	2.96	5.18
V-jun sarcoma virus 17 oncogene homolog (avian)	***Jun***	NM_021835	2.01	
V-maf musculoaponeurotic fibrosarcoma oncogene family, protein B (avian)	***Mafb***	NM_019316	2.02	2.83
Nuclear receptor subfamily 1, group H, member 3	***Nr1h3***	NM_031627	2.14	2.01
Nuclear receptor subfamily 3, group C, member 2	*Nr3c2*	NM_013131	2.21	
Immediate early gene transcription factor NGFI-B	***Nr4a1***	NM_024388	0.28	0.33
Placentae and embryos oncofetal gene	***Pem***	NM_022175	0.31	0.43
POU domain, class 3, transcription factor 1	*Pou3f1*	NM_138838	0.1	
Peroxisome proliferator activated receptor, gamma	***Pparg***	NM_013124	2.1	2.71
MLX interacting protein-like	***Mlxipl***	NM_133552	0.4	0.29

Cell adhesion				
Calcium and integrin binding 1 (calmyrin)	***Cib1***	NM_031145	0.36	0.37
Amphoterin induced gene and ORF 3	***Amigo3***	NM_178144	0.35	0.29
Amine oxidase, copper containing 3	***Aoc3***	NM_031582	2.07	2.12
Ras-related C3 botulinum toxin substrate 1	***Rac1***	NM_134366	2.92	2.67
CD9 antigen	***Cd9***	NM_053018	2	2.64
Cadherin 13	*Cdh13*	NM_138889	2.48	
Similar to M-cadherin (LOC361432), mRNA	*Cdh15*	NM_207613	0.29	
Carbohydrate sulfotransferase 10	***Chst10***	NM_080397	0.21	0.17
Coagulation factor 5	*F5*	NM_001047878	0.43	
Immunoglobulin superfamily, member 1	***Igsf1***	NM_175763	0.21	0.37
Spondin 2, extracellular matrix protein	***Spon2***	NM_138533	2.79	2.88
Neural cell adhesion molecule 1	***Ncam1***	NM_031521	3.15	3.78
Immune response				
Adenosine deaminase	***Ada***	NM_130399	0.25	0.21
Sperm associated antigen 11	***Spag11***	NM_145087	0.11	0.07
Complement component 4a	***C4a***	NM_031504	2.15	3.14
Complement component 4 binding protein, alpha	***C4bpa***	NM_012516	8.72	8.72
Defensin beta 1	***Defb1***	NM_031810	0.16	0.13
GTPase, IMAP family member 4	***Gimap4***	NM_173153	2.24	2.54
GTPase, IMAP family member 5	***Gimap5***	NM_001033913	2.11	2.26
Interleukin 13 receptor, alpha 2	***Il13ra2***	NM_133538	0.05	0.04
Interleukin 1 receptor-like 1	***Il1rl1***	NM_013037	0.24	0.21
Interleukin 2 receptor, alpha chain	*Il2ra*	NM_013163	0.48	

RT1 class Ib, locus S3	***RT1-S3***	NM_001008886	2.32	2.82
Cell surface alloantigen 6 (mapped)	***Rt6_mapped***	ID: 24757	2.33	2.59
ADP-ribosyltransferase 2b	*Art2b*	NM_198735	2.33	
Serine (or cysteine) proteinase inhibitor, clade G (C1 inhibitor), member 1, (angioedema, hereditary)	***Serping1***	NM_199093	2.06	2.56
Src family associated phosphoprotein 1	*Scap1*	NM_173311	0.45	
Toll-like receptor 4	***Tlr4***	NM_019178	2.48	3.09
Protein modification				
Kua homolog	***Kua***	XM_342588	0.31	0.31
Palmitoyl-protein thioesterase 1	*Ppt1*	NM_022502	2.14	
Protein folding				
Cyclophilin B	*Ppib*	NM_022536	0.08	
FK506 binding protein 1b	*Fkbp1b*	NM_022675	0.47	
Response to stress				
Aryl hydrocarbon receptor	***Ahr***	NM_013149	2.16	3.3
Carbonic anhydrase 3	***Ca3***	NM_019292	3.66	6.06
Glutathione peroxidase 3	***Gpx3***	NM_022525	2.12	2.14
Peroxiredoxin 6	***Prdx6***	NM_053576	0.37	0.32
Cytoglobin	***Cygb***	NM_130744	2.34	2.86
Apoptosis				
Activin A receptor, type IC	*Acvr1c*	NM_139090	2.3	
Nerve growth factor receptor (TNFR superfamily, member 16)	*Ngfr*	NM_012610	0.19	
Programmed cell death 8 (apoptosis-inducing factor)	***Pdcd8***	NM_031356	0.4	0.41

Tissue inhibitor of metalloproteinase 3	***Timp3***	NM_012886	2.08	2.14
Proteolysis				
Angiotensin 1 converting enzyme 1	***Ace***	NM_012544	0.2	0.09
A disintegrin and metalloprotease domain 7	***Adam7***	NM_020301	0.29	0.25
A disintegrin and metalloproteinase domain 9	***Adam9***	NM_001014772	0.48	0.45
Complement factor D (adipsin)	***Cfd***	NM_001077642	3.09	5.12
Calpain 10	***Capn10***	NM_031673	0.36	0.32
Cathepsin C	***Ctsc***	NM_017097	4.25	5.58
Aminopeptidase A	***Enpep***	NM_022251	2.03	2.03
Matrix metalloproteinase 11	***Mmp11***	NM_012980	2.47	3
Nerve growth factor, gamma	***Ngfg***	NM_031523	2.73	2.67
Transmembrane protein 27	***Tmem27***	NM_020976	3.97	3.07
Proprotein convertase subtilisin/kexin type3	***Pcsk3***	NM_019331	0.26	0.21
Plasminogen activator, urokinase	***Plau***	NM_013085	2.05	3.16
Protein C	***Proc***	NM_012803	0.49	0.37
Sperm associated antigen 5	*Spag5*	NM_001044224	0.08	
Spermatogenesis				
Follistatin-like 3	*Fstl3*	NM_053629	0.34	
Guanidinoacetate methyltransferase	***Gamt***	NM_012793	0.49	0.43
Gamma-glutamyltransferase 1	***Ggt1***	NM_053840	0.2	0.18
C-kit receptor tyrosine kinase	***Kit***	NM_022264	2.13	2.45
Putative homeodomain transcription factor 1	***Phtf1***	XM_001073852	0.49	0.45
Protein phosphatase 2 (formerly 2A), regulatory subunit B (PR 52), beta isoform	***Pppr2b2***	NM_022209	2.25	2.99
Pituitary tumor-transforming 1	*Pttg1*	NM_022391	0.35	

DNA repair				
N-methylpurine-DNA glycosylase	***Mpg***	NM_012601	0.3	0.21
Potassium channel tetramerisation domain containing 13	*Kctd13*	NM_198736	2.39	
Others				
CD52 antigen	***Cd52***	NM_053983	0.005	0.005
Crystallin, beta A4	***Cryba4***	NM_031689	0.34	0.31
Hypothetical protein	***dd25***	NM_199403	0.49	0.33
Dynein cytoplasmic 1 intermediate chain 1	***Dync1i1***	NM_019234	0.29	0.18
Dermatopontin	***Dpt***	XM_213925	3.97	3.79
Espin	***Espn***	NM_019622	0.17	0.18
Hypothetical protein LOC313453	***RGD727788***	NM_182822	3.15	3.24
Tumor protein p53 inducible nuclear protein 2	*Trp53inp2*	XM_342551	0.45	
Surface protein MCA-32	***Mca32***	NM_021585	0.44	0.43
Microtubule-associated protein 2	*Mtap2*	NM_013066	2.63	
Mucin 1	*Muc1*	XM_342281	2.18	
N-myc downstream regulated gene 4	***Ndrg4***	NM_031967	0.49	0.42
Neuronatin	***Nnat***	NM_053601	5.06	3.86
Olfactomedin related ER localized protein	*Olfm1*	NM_053573	0.43	
Proteoglycan peptide core protein	***Pgsg***	NM_020074	0.4	0.33
Peroxisomal membrane protein 4	***Pxmp4***	NM_172223	0.41	0.35
Reticulocalbin 2	***Rcn2***	NM_017132	0.48	0.42
Secreted frizzled-related protein 2	*Sfrp2*	XM_227314	2.98	
DNA-damage-inducible transcript 4-like	*Ddit4l*	NM_080399	2.16	

Synuclein, gamma	***Sncg***	NM_031688	3.31	4.78
Testis expressed gene 101	*Tex101*	NM_139037	0.06	
Transmembrane protein with EGF-like and two Follistatin-like domains 1	***Tmeff1***	NM_023020	0.45	0.45
Testis specific X-linked gene	*Tsx*	NM_019203	0.16	

Supplemental Table 2 List of E2-regulated genes with known annotations in the initial segment and caput epididymis after regression and their classification based on biological function at: 12 h, 1 day, and 7 days.

Suppl Table 2

Common gene name	Gene symbol	GenBank accession no.	Fold change	
			Orch. vs Orch. + E2	Orch. vs Sham operated
Apoptosis			**12-h**	
Nerve growth factor receptor	***Ngfr***	NM_012610	0.4	0.3
Calcium ion binding				
S100 calcium binding protein G	*S100g*	NM_012521	0.085	
			1-d	
S100 calcium binding protein G	*S100g*	NM_012521	0.08	
Purkinje cell protein 4	*Pcp4*	NM_013002	0.33	
			7-d	
S100 calcium binding protein G	*S100g*	NM_012521	0.04	
Purkinje cell protein 4	*Pcp4*	NM_013002	0.26	
Transport				
Albumin	***Alb***	NM_134326	2.63	6.4
Hemoglobin alpha, adult chain 1	*Hba-a1*	NM_013096	2.88	
Hemoglobin beta chain complex	*Hbb*	NM_033234	4.69	
Rhesus blood group-associated C glycoprotein	***Rhcg***	NM_183053	0.48	0.12

CONNECTING TEXT

Thus far, the studies presented clearly determined that androgen, in particular, DHT induces the remodeling of a regressed androgen-deprived tissue by a cascade of gene modulation. Interestingly, there are few genes that were regulated by E2, while many were affected (both up and down) by DHT. IGF family genes were found to be among the first affected genes in the early response pathway activated by DHT. Epidermal growth factor (EGF) and IGF1 apparently played an important role in the pathway due to their function in regulation and expression of many other genes. What remained unclear was the more rapid action of DHT and signaling pathways that may act upstream of transactivation of genes regulated by DHT in the epididymis, at the cellular level. Therefore, the elucidation of potential DHT signaling mechanisms in the mouse proximal caput (PC-1) epididymal cell line formed the basis of the final objective. Two main potential pathways, MAPK/ERK and AKT, were chosen to identify the rapid non-genomic action of DHT, for the first time, in the immortalized PC-1 epididymal cell line which retains their androgen responsiveness. These two pathways were chosen based on increasing evidence for their involvement in DHT-activated signaling in other reproductive cells such as prostate cancer cells and Sertoli cells.

CHAPTER 4

Androgens Activate Mitogen-Activated Protein Kinase via Epidermal Growth Factor Receptor/Insulin-Like Growth Factor 1 Receptor in the Mouse Proximal Caput Epididymidis-1 (PC-1) Cell Line

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Abstract

Androgens are the primary regulators of epididymal structure and functions. In the classical view of androgen action, binding of androgen to the intracellular androgen receptor (AR) produces the receptor-steroid complex that has high affinity for DNA response elements and regulates the transcription of target genes. In this study, we demonstrate that in epididymal cells, dihydrotestosterone (DHT) can cause an alternative and rapid response that is independent of AR-DNA interactions and is mediated by activation of signaling pathways through the androgen receptor. We examined changes in AKT and ERK1/2 activation at early time points after DHT supplementation in the mouse proximal caput epididymidis-1 (PC-1) cell line. DHT had no significant effect on AKT activation at any time point. However, DHT activated the ERK pathway as early as at 1 min, the pathway remained activated at 10 min, but activation was not sustained until 15 min. Interestingly, ERK activation was blocked by hydroxyflutamide (HF), indicating that early ERK activation was an androgen receptor mediated response in PC-1 cell lines. DHT activated SRC kinase and this activation was required for the ERK response. EGFR and IGF1R were downstream of SRC and these two receptors together, contributed to enhance ERK and CREB phosphorylation. We postulate that this rapid action of androgen may ultimately act to modulate the transcription of genes regulated by AR in the nucleus. These results support the hypothesis that DHT can activate a pathway involving the sequential activation of SRC, MEK, ERK1/2, and CREB through the EGFR/IGF1R in epididymal cells.

Introduction

The epididymis plays an important role in male fertility because it is the site where spermatozoa are matured and stored. Maturation of spermatozoa, including the acquisition of motility and capability to fertilize an oocyte, occurs during transit through the unique and dynamic microenvironment of the epididymal lumen. The specificity of the microenvironment is primarily under the influence of androgens that ultimately regulate epididymal cell structure and function (1-4). Principal cells are the major cell type of this tissue (5) and are particularly sensitive to androgen removal (6;7). The major circulating androgen, testosterone is metabolized by 5α-reductases into the potent androgen 5α-dihydrotestosterone (8-10). Androgens mediate their effects by diffusing through the plasma membrane and binding to intracellular androgen receptors (AR). The AR functions as a ligand-inducible transcription factor modulating transcription of target genes (11;12). In addition to this slow genomic action of androgens, several lines of evidence suggest that androgens, like progestins and estrogens, can exert rapid non-genomic effects (13;14).

Unlike genomic effects that take hours or days to exert their action, non-genomic effects are activated within seconds or minutes (13-18). The extracellular signal-regulated protein kinases (ERK1/2), members of the MAPK-superfamily, are activated in response to androgens. These protein kinases play a key role in cell growth, differentiation and function at both the transcriptional and post-transcriptional level, by phosphorylating a range of proteins, including nuclear transcription factors, cytoskeletal proteins, other protein kinases, and receptors for hormones and growth factors (18-20). Androgens can also activate the phosphatidylinositol-3 kinase (PI-

3K) and AKT pathway rapidly in a ligand-binding independent manner (21-23). It is unclear, however, whether those two pathways, ERK1/2 and AKT, are activated in epididymal cells in response to DHT.

Recent studies have confirmed that the non-genomic action of androgens depends on the ability of the AR to interact with the steroid receptor co-activator (SRC) (12); activation of PI-3K by SRC-family kinases has also been established (24). The androgen-AR complex interacts with the SH3 domain of SRC kinase and leads to a rapid activation of Raf-1 and MEK, and as a result, the ERK pathway; thus, the SRC-MAPK is one of the major routes in signal transduction (25-28). Furthermore, SRC is also able to activate EGFR directly or indirectly and subsequently the MAPK pathway by phosphorylating tyrosine residues on the cytoplasmic face of EGFR (29); however, the role of IGF1R in mediating the rapid action of androgen through ERK pathway activation remains to be elucidated. Activation of SRC by androgen occurs only in the cell membrane, without the interaction of EGFR with AR, whereas EGFR, estrogen receptor, and SRC make a complex upon estrogen stimulation (30).

In Sertoli cells, the rapid action of androgen leads to activation of transcription factors such as cAMP response element-binding protein (CREB); the latter is an essential factor required for Sertoli cells to support spermatogenesis (25;31). Similarly, a recent study has shown that androgens up-regulate the IGF1R by inducing CREB activation, through activation of the c-SRC/ERK pathway in prostate cancer cells (32;33), indicating the role of non-genomic action of androgens in regulation of gene transcription.

While several studies have established different pathways mediated by a non-genomic action of androgens, in this study we present an integrated model of an extranuclear, AR-mediated, rapid MAPK activation pathway, that acts through the activation of EGFR/IGF1R signaling in epididymal cell lines. The mouse PC-1 cell line was derived from primary culture of epididymal cells from transgenic mice harboring a temperature-sensitive simian virus 40 large T antigen. It is a pure population of epithelium-derived caput principal cells (34).

Matherials and Methods

Cell Culture and Treatments

The mouse proximal caput epididymidis PC-1 cell line (kindly provided by Dr. M.-C. Orgebin-Crist, Vanderbilt University) was grown in Iscove modified Dulbecco medium (without phenol red) supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 4 mM glutamine, 0.7% penicillin-streptomycin (25000 U penicillin sodium, 25 mg streptomycin sulfate), and 1 nM 5α-DHT. All cell culture products were purchased from Wisent (St Bruno, QC, Canada) and DHT was from Steraloids Inc (Newport, RI). PC-1 cells were cultured at 33°C with 5% CO_2. Two passages before the experimental procedure, FBS was replaced by charcoal-filtered FBS, and the latter was used for the duration of the experiments. The cells were seeded onto flasks for 2, 4, and 6 d in the media described above and subjected to vehicle as control or to different DHT concentration. Media were changed every 24 h. Viability of the cells was determined by using trypan blue. Total RNA of the cells was then extracted. To assess the

signaling activation, cells were seeded for 2 d in the media described above. Proteins from the cells were extracted at various time points after changing the media. In some cases, cells were pretreated for 30 min with the signaling pathways inhibitors prior to being stimulated with DHT for 5 min.

Inhibitors employed included hydroxyflutamide (Toronto Research Chemicals Inc, ON, Canada), tyrphostin I-OMe-AG 538 (T7697, Sigma, St Louis, Missouri), tyrphostin AG 1478 (9842, Cell Signaling Technology, Beverly, MA), and Y294002, PD98059, and PP2 were obtained from EMD biochemicals (San Diego, CA).

RNA Extraction and DNase Treatment

Each experimental group was comprised of five replicates. Total RNA was extracted and DNase treated using the RNeasy Mini kit (Qiagen, Mississauga, ON, Canada) according to the manufacturer's instructions. RNA concentrations were assessed by OD determination at 260 nm (DU7 spectrophotometer, Beckman, Montreal, Quebec, Canada). The RNA used for microarray analysis had an A260/A280 ratio greater than 1.8. To verify the quality of each sample, 2 μg RNA was run on a denaturing gel containing 1% agarose.

Quantitative Real-Time PCR

Quantitative real-time PCR was done using the Light-Cycler system (Roche Diagnostics, Laval, QC, Canada) with the Quantitect One-Step SYBR green RT-PCR kit (Qiagen), according to the manufacturer's protocol. The specific primer sequences for *Ar*, *Igf-1* and *cyclophilin A (Ppia)* were designed using Primer3

software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi/). *Cyclophilin A* was chosen as the endogenous control as its mRNA levels did not change with androgen manipulation (35). The primer sequences are as follows: *cyclophilin A*: forward primer, 5'-GTGGTCTTTGGGAAGGTGAA-3'; reverse primer, 5'-GTTGTCCACAGTCGGAGATG-3' (NM-008907). *Ar*: forward primer, 5'-CAGGCAAAAGCACTGAAGAGA-3'; reverse primer, 5'-GAGCCAGCGGAAAGTTGTAG-3' (NM-013476). *Igf-1*: forward primer, 5'-GGCATTGTGGATGAGTGTTG-3; reverse primer, 5'-GTCTTGGGCATGTCAGTGTG-3' (NM-178866). The qRT- PCR results were confirmed by melting-curve analysis. All standards and samples were assayed in duplicate. Average values for the target gene RNA concentrations of each sample were normalized to the average value for *cyclophilin* RNA concentration in the same sample.

Western Blot Analysis

Whole-cell extracts for each group were collected into lysis buffer containing protease and phosphatase inhibitors using the cellular fractionation kit (Cat. No: 40010) from Active Motif (Carlsbad, CA), according to the manufacturer's protocol. We evaluated total protein concentration by the Bradford method, using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Protein samples (15 µg per group) were boiled with loading buffer for 5 minutes and fractionated by SDS-PAGE using 10% acrylamide gels. Prestained precision standards (Bio-Rad Laboratories) were used as molecular weight markers. The fractionated proteins were transferred

to a Hybond-P membrane (Amersham Biosciences UK, Buckinghamshire, UK). They were incubated with primary antibodies (1:1000; unless stated otherwise), followed by horseradish peroxidase conjugated second antibodies (1:10,000; Amersham Pharmacia Biotech UK).

The primary antibodies employed were against total MAP kinase 1/2 (ERK 1/2), CREB, and phospho-CREB (Ser^{133}) (nos. 06-182, 06-863, and 06-519, Millipore Corporation, Temecula, CA), phospho-44/42 MAPK (ERK1/2) (Thr^{202}/Tyr^{204}), total AKT (pan), and phospho-AKT (Ser^{473}) (nos. 9106, 4691, and 4060, Cell Signaling Technology, Beverly, MA), a polyclonal antibody against phospho-SRC [pY^{418}] (44660G, Invitrogen Corporation, Cammarilla, CA), a monoclonal antibody against c-SRC (B-12) (sc-8056, Santa Cruz Biotechnology, Inc, CA). The levels of P-ERK, P-AKT, P-SRC, and P-CREB were normalized to overall ERK, AKT, SRC, and CREB expression levels, respectively. Actin (1:4000, sc-1616) was used as second loading control and was detected using a secondary, donkey anti-goat IgG-horseradish peroxidase antibody (1:10,000, sc-2056). Western blots were visualized with the Enhanced Chemiluminescence Plus kit and Hyperfilm Enhanced Chemiluminescence (Amersham Biosciences UK). Quantification of Western blot data was done by line densitometry using a ChemiImager 4000 imaging system with AlphaEase (version 5.5 software; Alpha Innotech, San Leandro, CA).

Statistical Analysis

Statistical analysis was done using t-test or one-way ANOVA followed by the multiple comparisons Dunnett's test. Data were expressed as mean +/- SEM. Values of $p \leq 0.05$ were regarded as statistically significant.

Results

Cell Viability Is Not Affected by Treatment

The effects of androgen withdrawal and treatment on cell viability were determined using the trypan blue staining technique. Cells did proliferate; the number of cells had doubled after 2 d and was increasing more slowly after 4 d (Suppl. Fig. 1). Very few dead cells were observed within 6 days, although significant cell death was observed by 8 d after androgen withdrawal (data not shown).

Ar and Igf1Genes Are Expressed in PC-1 Cell Line

The mRNA expression of *Ar* and *Igf1* genes was determined by qRT-PCR at 2, 4, and 6 d in androgen deprived cells before and after DHT treatment. DHT had no significant effect on *Ar* expression in cells compared to that of vehicle-treated cells (Suppl. Fig. 2). In comparison, *Igf1* expression was significantly increased at 2, 4, and 6 days in DHT-treated cells compared to the control (Suppl. Fig. 2).

Activation of AKT and ERK1/2 by DHT

To assess whether DHT can enhance the activation of the AKT or the ERK pathway, we stimulated cells with DHT (0.1, 1, or 10 nM) for 10 min, 30 min, or 45 min. The activities of AKT and ERK were measured relative to total AKT and ERK. DHT had no significant effect on AKT phosphorylation compared to control at any time point (Fig. 1). In contrast, DHT (0.1 and 1 nM) significantly increased ERK phosphorylation at 10 min; this activation was not sustained at 30 and 45 min (Fig. 2a). To determine whether this activation occurred earlier then 10 min, ERK activation by DHT (0.1 and 1 nM) was investigated at 1, 3, and 5 min. DHT significantly increased ERK phosphorylation levels compared to control at all time points (Fig. 2b). Similarly, AKT activation in response to DHT was also measured at earlier and later time points, but no significant changes were observed (data not shown). DHT did not alter total expression of AKT or ERK relative to actin.

ERK1/2 Is Activated by DHT via an Androgen Receptor Mediated Mechanism

To determine whether ERK was activated by DHT through an androgen receptor mediated mechanism, cells were pretreated with hydroxyflutamide (HF), a non-steroidal and reversible AR antagonist, before stimulation with DHT (0.1, 1, or 10 nM). Pretreatment with HF repressed ERK phosphorylation significantly in DHT-treated (0.1 and 1 nM) cells compared to control. Interestingly, the repression of ERK phosphorylation by HF was partially reversed at higher DHT concentrations (Fig. 3). These data suggest that there is a basal level of activation for ERK that is not blocked by an AR antagonist and not due to an effect of DHT.

SRC kinase Is Activated by DHT and Is Required for ERK1/2 Activation

We investigated the activation of c-SRC by DHT and found that DHT (1 nM) significantly increased SRC phosphorylation compared to the control (Fig. 4). Although it is possible that the activity of other SRC family members may be regulated by DHT, this result indicates that androgen stimulation results in a rapid activation of SRC in PC-1 cells. To assess whether SRC activation was required for DHT-mediated induction of ERK activation, cells were pretreated with PP2 before stimulation with DHT (1 nM). ERK phosphorylation was significantly repressed by inhibition of SRC activation (Fig. 5). The inhibition of ERK phosphorylation by PP2 was comparable to that of cells pretreated with MEK inhibitor, PD98059. In addition, ERK activation was significantly repressed by PI3 kinase inhibitor, LY294002 (Fig. 5). These results suggest that DHT-dependent activation of ERK requires the activated SRC kinase, as well as PI3 kinase and MEK, the kinase that phosphorylates ERK. P-ERK was not significantly decreased with the lower concentration of inhibitors (2.5 μM LY and PD, and 0.5 μM PP2) (data not shown).

DHT Activates the ERK Pathway via IGF1R and EGFR

To determine whether ERK is activated by DHT via IGF1R and EGFR, cells were pretreated with IGF1R inhibitor, EGFR inhibitor, and the combination of both inhibitors before stimulation with DHT (1nM). ERK phosphorylation was significantly repressed by 25 μM IGF1R inhibitor or 1 μM EGFR inhibitor. Interestingly, using lower concentrations of the two inhibitors in combination, 10 μM IGF1R inhibitor and

50 nM EGFR inhibitor, ERK activation was significantly blocked, although at these concentrations each alone had no effect on the level of P-ERK (Fig. 6). These results suggest that DHT mediates induction of ERK activation via IGF1R and EGFR and indicate that there is cross talk between these two receptors.

SRC Acts Upstream of IGF1R and EGFR

To test whether the SRC kinase required for ERK phosphorylation was acting upstream of IGF1R and EGFR, cells were pretreated with inhibitors of IGF1R and EGFR alone or a combination of the two inhibitors prior to stimulation with DHT (1 nM), then SRC activation was assessed. The latter was not affected by IGF1R and EGFR inhibitors at any concentrations that repressed ERK phosphorylation (Suppl. Fig. 3), suggesting that IGF1R and EGFR act downstream of SRC.

Activation of ERK1/2 by DHT Leads to CREB Phosphorylation

To ensure that increased ERK phosphorylation resulted in an increased activation of CREB, we tested whether there was an increase in the phosphorylation of CREB was as a result of treatment with DHT and, if so, whether this increase was repressed by ERK inhibition. Cells were treated with vehicle or DHT (0.1 nM or 1 nM) or were pretreated with a combination of IGF1R and EGFR inhibitors, before stimulation with DHT; P-CREB was assessed. DHT (1 nM) significantly increased CREB phosphorylation (Fig. 7a), but this activation was partially repressed in cells pretreated with IGF1R and EGFR inhibitors (Fig. 7b). These results suggest that the activated ERK can stimulate transcription factors such as CREB.

Discussion

The results from these studies suggest that there is a mechanism for androgen action in epididymal epithelial cells that utilizes rapid activation of the MAP kinase pathway via IGF1R and EGFR and activation of CREB transcription factor. DHT increased IGF1 expression compared to the control, without having a mitogenic effect in the epididymal cells or killing them. Similar results have been observed in studies of breast cancer and prostate cancer cells, in which androgen activates the ERK pathway within minutes (36;37). Androgen induced-phosphorylation of ERK1/2 occurred within 1 min of androgen stimulation and was sustained for 10 min. However, AKT phosphorylation was not altered upon androgen administration. Activation of ERK within minutes was repressed by reducing the activity of the androgen receptor, suggesting that this action occurred through an androgen receptor and was not mediated via binding of their cognate nuclear receptors to DNA, but rather through direct stimulation of cell signaling pathways. A schematic representation of the proposed pathway described in these studies is presented in Figure 8.

The mechanism by which the androgen-AR complex initiates signaling in epididymal cells is not known. Recently, a population of AR has been localized to the plasma membrane in Sertoli cells where testosterone rapidly and transiently promotes AR localization to the plasma membrane (25). AR has also been localized to the plasma membrane in *Xenopus* oocytes and in hypothalamic cell lines (23;38). Association of AR with caveolin-containing membrane fractions is believed to facilitate interactions with their cognate ligands and shift the complex near

membrane-associated signaling molecules, such as SRC kinase, PI3 kinase, or G proteins (39;40).

The non-receptor tyrosine kinase SRC is a major factor in signal transduction (41-43). We have shown that SRC is activated by DHT at 5 min and that it is required for ERK activation. The essential role of SRC kinase in the nongenomic action of androgen receptors was demonstrated in studies on embryonic fibroblasts derived from SRC-/- mice in which the cells do not show rapid activation of the MAPK pathway in response to ligand binding to AR (26). In LNCaP prostate cells, androgen triggers the direct association of the proline-rich region of AR and the SH3 domain of SRC, causing the activation of SRC (44); the latter is known to rapidly induce the formation of Shc/Grb2/SOS complexes, leading to the activation of Ras and subsequently stimulation of a Raf MAPK kinase kinase, the initial member of the MAPK pathway (45). Activation of PI3 kinase by SRC kinase has been shown (24). Our data indicate that inhibition of PI3 kinase significantly reduces ERK1/2 activation.

The IGF and EGF ligands are mitogenic for both normal and tumorogenic mammary epithelial cells *in vitro* (46). The IGF1 and EGF receptors share a common molecular structure, with a cytoplasmic tail containing tyrosine residues; activation of these receptors ultimately leads to the activation of the PI3k and MAPK signal transduction pathways (47-49). SRC is capable of directly activating EGFR by phosphorylating tyrosine residues on the cytoplasmic face of EGFR and subsequently the MAPK pathway (50;51). Our finding identified the essential role of IGF1 and EGF receptors in DHT-induced ERK1/2 activation in epididymal epithelial

cells. Using a combination of specific IGF1R and EGFR inhibitors, we observed a synergistic effect on blocking DHT-mediated phosphorylation of ERK. This effect was associated with an increased activity of the CREB transcription factor. CREB bound to cAMP response element motifs (TGACGTA) (52;53) in gene promoters is able to associate with the binding protein coactivator that facilitates the recruitment of RNA polymerase to the transcription initiation site (54;55). For instance, CREB phosphorylation has been linked to the activation of numerous Sertoli cell genes that contribute to germ cell development and survival (56), and up-regulation of IGF1R in prostate cancer cells (33).

Taken together, these data identify a rapid signaling pathway that is activated by DHT stimulation in epididymal epithelial cells. This represents the first study in cells of the epididymis to report the upstream signaling components for ERK activation through the androgen receptor. Targeting this signaling pathway should be considered as a means of regulating androgen action, and hence function, in this tissue.

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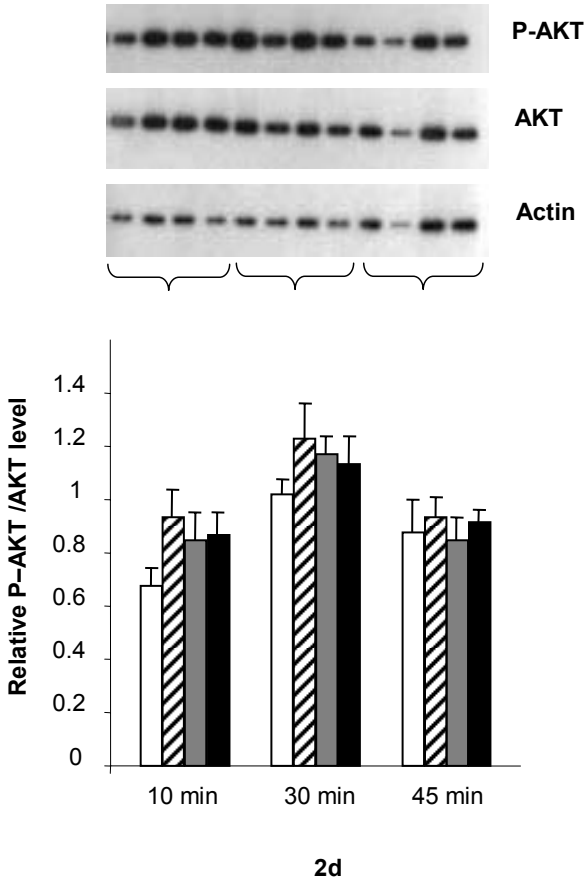
Figure 1. Effect of DHT on AKT activation. Cells were treated with vehicle (□) or DHT (0.1 nM▨ ,1 nM ■, 10 nM ■) for 10 min, 30 min, or 45 min. AKT phosphorylation was analyzed by Western blot. Each bar represents the mean of 5 replicates +/- SEM.

Figure 1


P-AKT
AKT
Actin
Relative P–AKT /AKT level
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1.2
1
0.8
0.6
0.4
0.2
0
10 min
30 min
45 min
2d

Figure 2. DHT elicited an increase in phospho-ERK1/2. a) Cells were treated with vehicle (□) or DHT (0.1 nM ▨ ,1 nM ▩, 10 nM ■) for 10, 30, or 45 min. **b**) Cells were treated with vehicle (□) or DHT (0.1 nM ▨ ,1 nM ▩) for 1, 3, 5 or 15 min. ERK phosphorylation was analyzed by Western blot. (*) Indicates that values were significantly different from vehicle-treated controls ($p \leq 0.05$). Each bar represents the mean of 5 replicates +/- SEM.

Figure 2

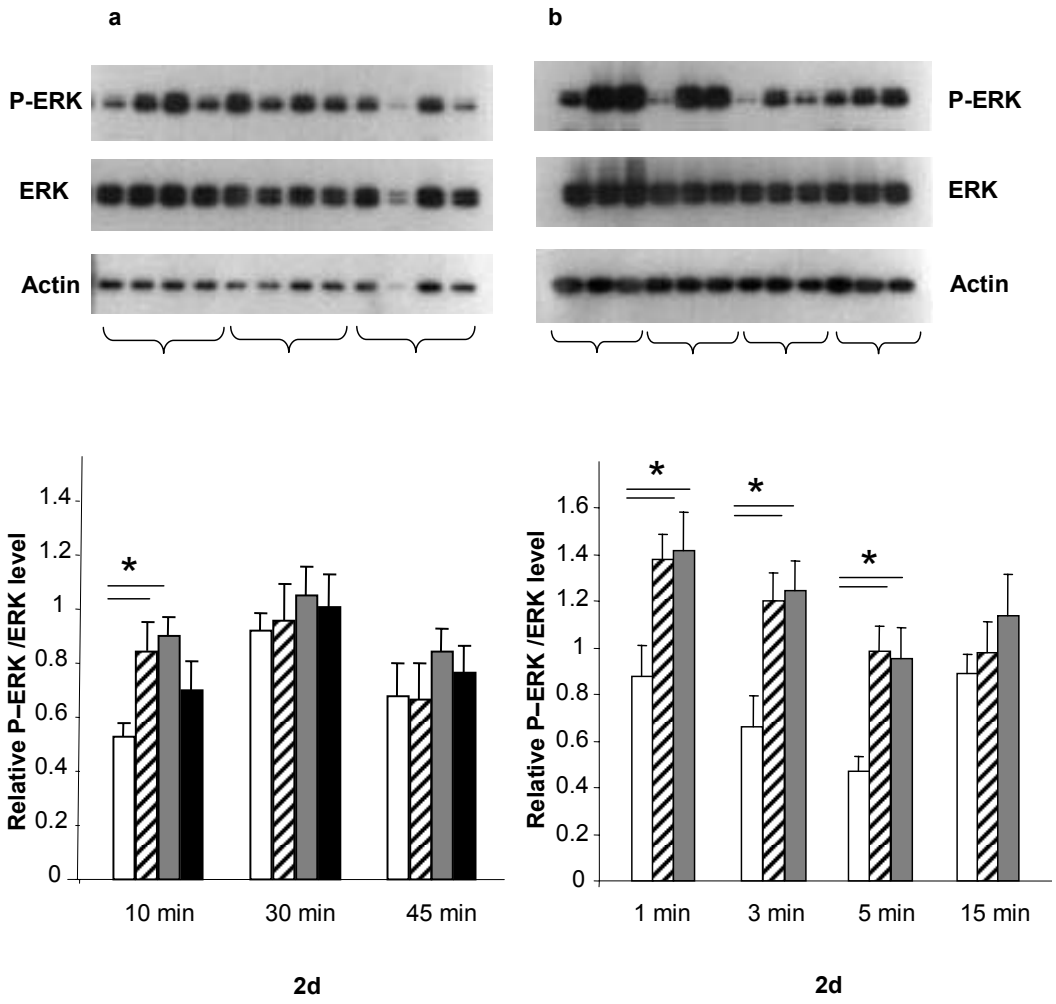

a
b
P-ERK
ERK
Actin
P-ERK
ERK
Actin
Relative P–ERK /ERK level
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
10 min
30 min
45 min
2d
Relative P–ERK /ERK level
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
*
1 min
3 min
5 min
15 min
2d

Figure 3. Hydroxyflutamide (HF) blocked DHT-induced ERK1/2 phosphorylation. Cells were pretreated for 30 min with Hydroxyflutamide (10 μM) before stimulation for 5 min with vehicle (□) or DHT (1 nM ■). ERK phosphorylation is shown by Western blot. (*) Indicates that P-ERK was significantly repressed by HF compared to control and this effect was reversed by increasing the concentration of DHT ($p \leq 0.05$). Each bar represents the mean of 5 replicates +/- SEM.

Figure 3

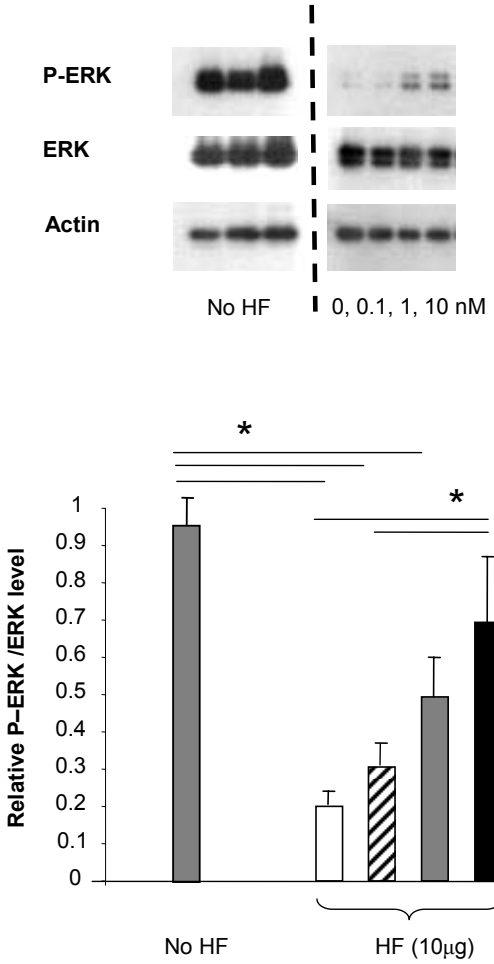

P-ERK
ERK
Actin
No HF
0, 0.1, 1, 10 nM
*
*
Relative P–ERK /ERK level
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
No HF
HF (10μg)

Figure 4. DHT induced SRC kinase activation. Cells were treated with vehicle □ or DHT (0.1 nM ▨, 1nM ■, and 10 nM ■) for 5 min. Relative expression of SRC phosphorylation was analyzed by Western blot. 1 nM DHT significantly increased P-SRC compared to control ($p \leq 0.05$). Each bar represents the mean of 4 replicates +/- SEM.

Figure 4

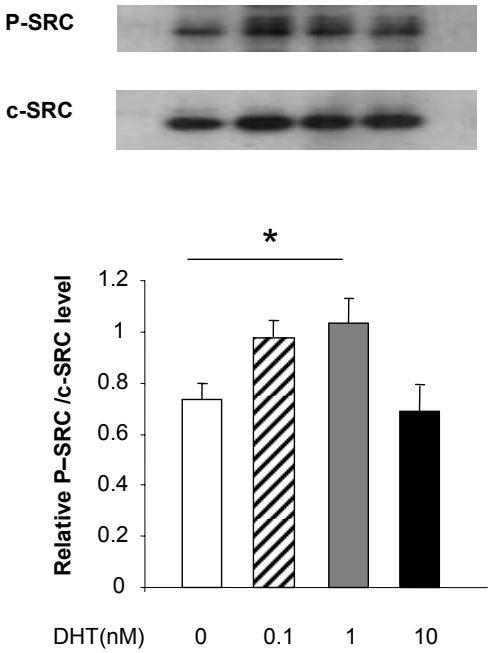

P-SRC
c-SRC
*
Relative P–SRC /c-SRC level
1.2
1
0.8
0.6
0.4
0.2
0
DHT(nM)
0
0.1
1
10

Figure 5. DHT-mediated induction of ERK1/2 required SRC kinase. Cells were pretreated for 30 min with SRC inhibitor PP2 (1, 5, 10 μM), MEK inhibitor PD98059 (5, 10, 20 μM), or PI-3K inhibitor LY294002 (5, 10, 25 μM) before stimulation for 5 min with vehicle and DHT (1 nM). ERK phosphorylation was analyzed by Western blot. The letter b indicates that values were significantly different compared to control value (a) ($p \leq 0.05$). Each bar represents the mean of 4 replicates +/- SEM.

Figure 5

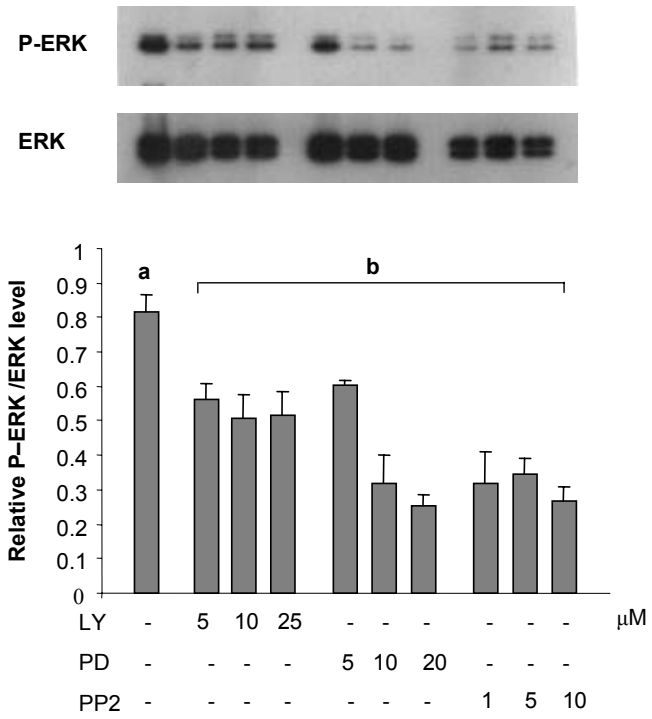

P-ERK
ERK
Relative P–ERK /ERK level
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
a
b
LY - 5 10 25 - - - - - - μM
PD - - - - 5 10 20 - - -
PP2 - - - - - - - 1 5 10

Figure 6. IGF-1R and EGFR inhibitors blocked P-ERK1/2. Cells were pretreated for 30 min with Tyrphostin I-OMe-AG 538 (5, 10, and 25 M) or Tyrphostin AG 1478 (25, 50, 150 nM, and 1 μM) before stimulation for 5 min with vehicle or DHT (1 nM). ERK phosphorylation was analyzed by Western blot. The letter b indicates that values were significantly different compared to control values (a) ($p \leq 0.05$). Each bar represents the mean of 5 replicates +/- SEM.

Figure 6

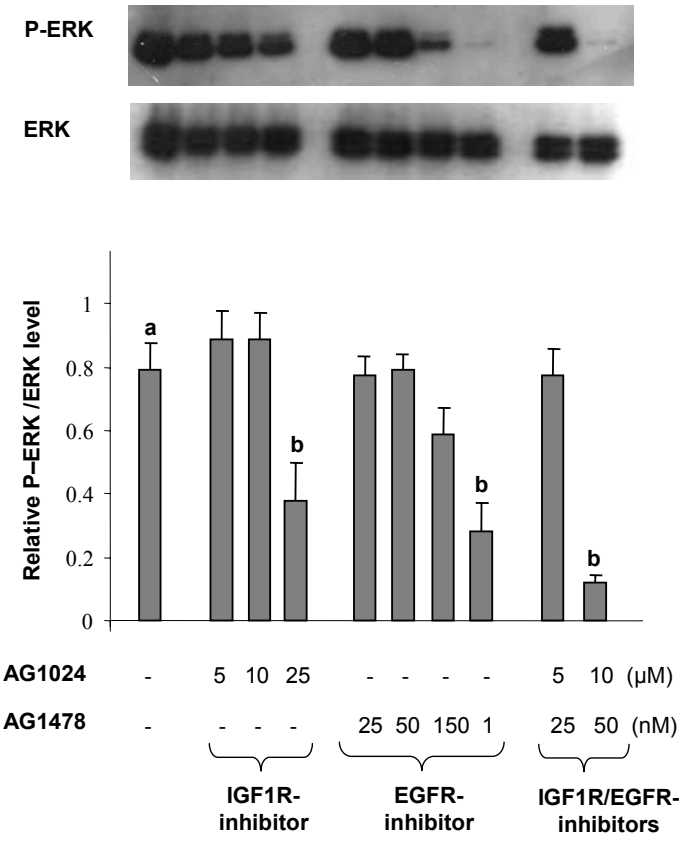

P-ERK
ERK
Relative P–ERK /ERK level
1
0.8
0.6
0.4
0.2
0
a
b
b
b
AG1024 - 5 10 25 - - - - 5 10 (μM)
AG1478 - - - - 25 50 150 1 25 50 (nM)
IGF1R-inhibitor
EGFR-inhibitor
IGF1R/EGFR-inhibitors

Figure 7. DHT induced CREB activation. a) Cells were treated with vehicle or DHT (0.1 nM ▨, and 1 nM ■) for 5 min. CREB phosphorylation was analyzed by Western blot. 1 nM DHT significantly increased P-CREB compared to control ($p \leq 0.05$). Each bar represents the mean of 4 replicates +/- SEM. **b)** Cells were pretreated for 30 min with IGF1R and EGFR inhibitors before stimulation for 5 min with vehicle and DHT (1 nM) for 5 min. Inhibitors partially repressed P-CREB. The figure shown is representative of three experiments.

Figure 7

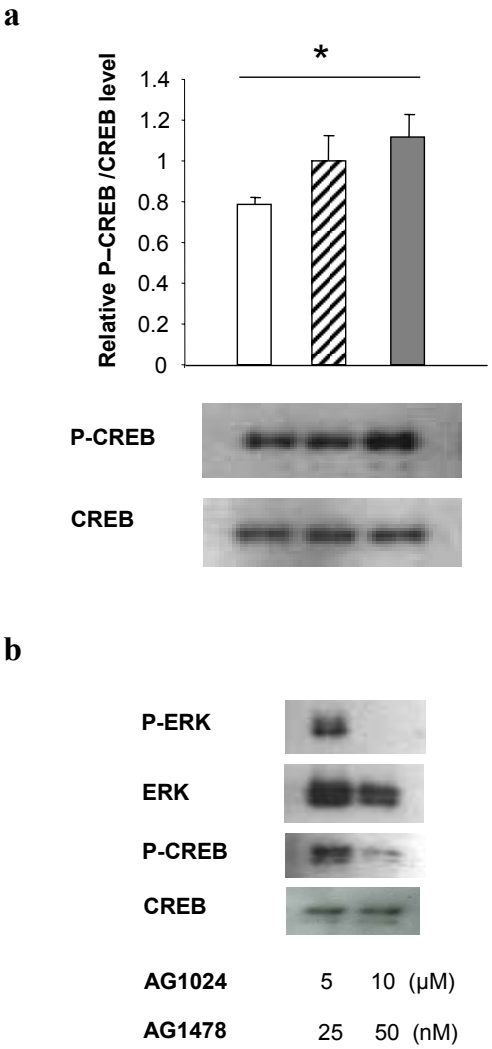
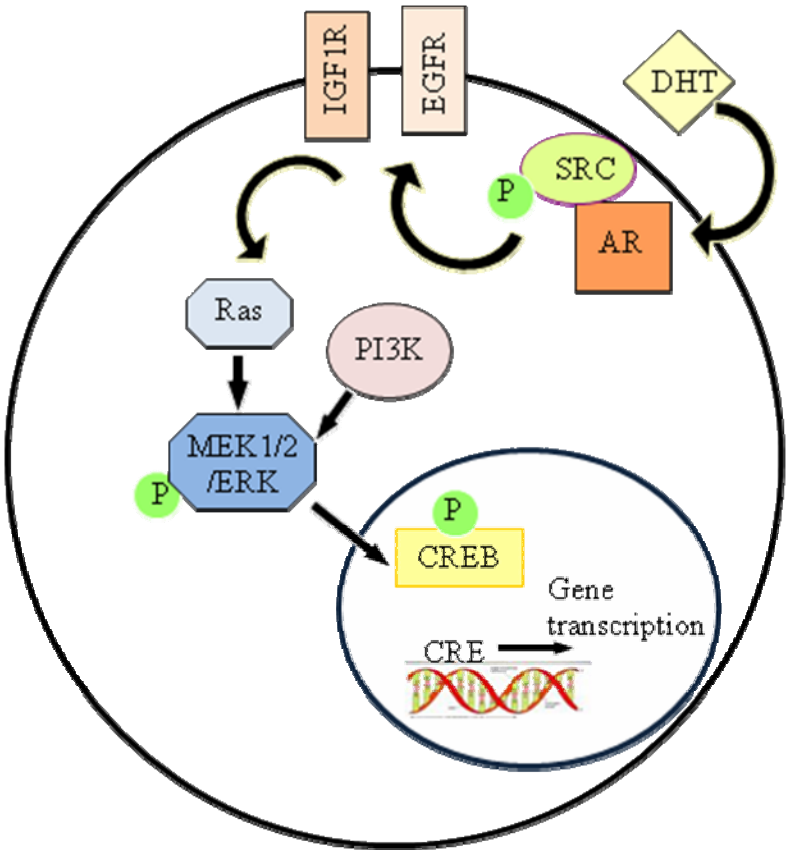

a
*
Relative P–CREB /CREB level
1.4
1.2
1
0.8
0.6
0.4
0.2
0
P-CREB
CREB
b
P-ERK
ERK
P-CREB
CREB
AG1024 5 10 (μM)
AG1478 25 50 (nM)

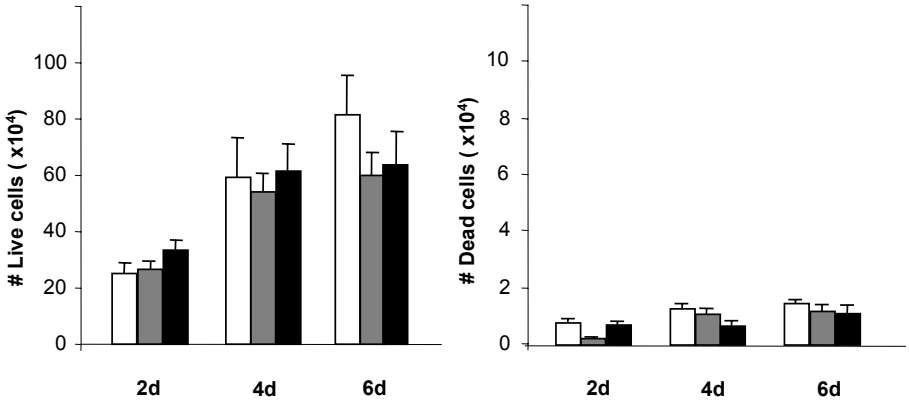
Figure 8. Signaling pathway activated by DHT in epididymal epithelial cells. DHT bound to the AR can activate a linear pathway involving the sequential activation of SRC, MEK, ERK1/2, and CREB through the EGFR/IGF1R in epididymal epithelial cells.

Figure 8


IGF1R
EGFR
DHT
SRC
P
AR
Ras
PI3K
MEK1/2
/ERK
P
P
CREB
Gene
transcription
CRE

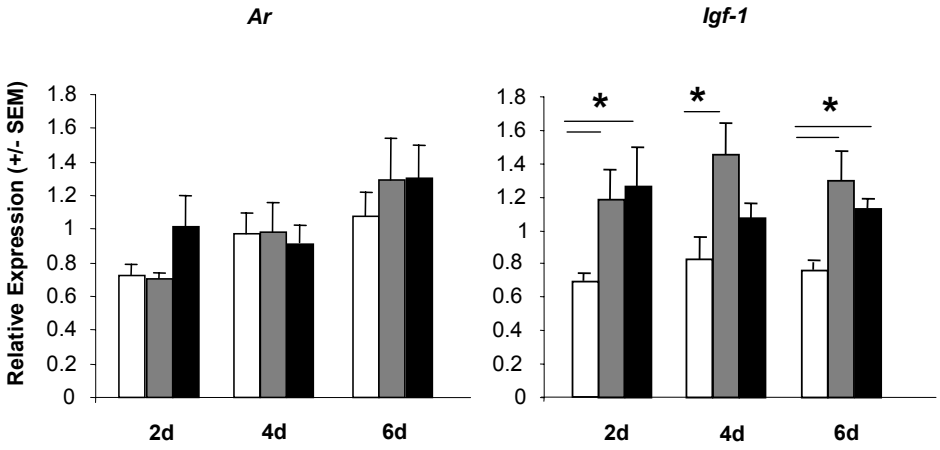
Supplemental Figure 1. Effect of androgen withdrawal and supplementation on viability of PC-1 cells. Cells were treated with vehicle (□) or DHT (1 nM ▩, 10 nM ■) for 2 d, 4 d, or 6 d. Each bar represents the mean of 5 observations +/- SEM.

Suppl Figure 1



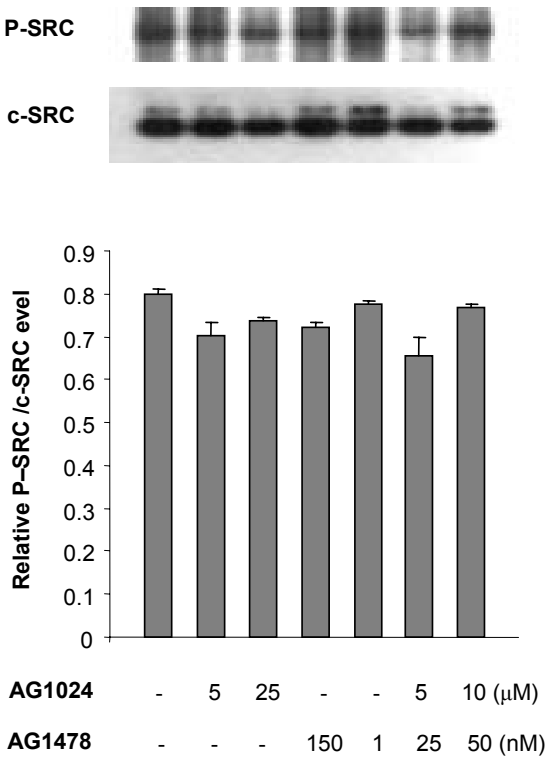
Supplemental Figure 2. Analysis of the effect of DHT on Igf-1 and Ar mRNA expression level by real-time PCR. Cells were treated with vehicle (□) or DHT (1 nM ■, 10 nM ■) for 2 d, 4 d, or 6 d. Relative expression of Igf-1 gene was significantly greater at 2, 4 and 6 d compared to the controls ($p \leq 0.05$). Each bar represents the mean of 5 replicates +/- SEM.

Suppl Figure 2



Supplemental Figure 3. SRC acted upstream of IGF-1R and EGFR. Cells were pretreated for 30 min with IGF1R or/and EGFR inhibitors before stimulation for 5 min with vehicle and DHT (1 nM) for 5 min. SRC phosphorylation is shown by Western blot. Each bar represents the mean of 4 replicates +/- SEM. No changes in protein expression were observed between the groups treated with inhibitor compared to control.

Suppl Figure 3



CHAPTER 5

Discussion

Given the fact that the male reproductive system is androgen dependent (1-3), manipulation or alteration of androgen status has been an important methodological approach for studying this complex system. In the present thesis, such an approach has been used to exclusively focus on epididymal androgen dependency. Over the past decades, the epididymis has been found to be an attractive target for the development of male contraception as well as for identifying underlying causes of male infertility. Sperm mature and gain the capacity for fertilization via interactions with the epididymal epithelium during their transit through this tissue (4-6). These interactions under the control of androgen, presumably involve the activation of signaling cascades in the epithelial cells leading to gene expression, and subsequently to secretion of proteins and small molecules into the epididymal lumen that result in creating an established structure with a unique microenvironment, which are important for producing functional sperm (7). Thus, the androgen alteration approach has been used throughout this thesis by complete hormonal removal and subsequent androgen replacement. This chapter will discuss the significance of some of the key findings presented in this thesis. In addition, future research directions and clinical applications will be discussed that will not only serve to expand our current findings, but also to provide novel insights into the mechanisms of androgen action in the epididymis that will lay the foundation for new therapeutic approaches targeted at the epididymis.

1. Androgen Manipulation as an Experimental Approach to Study the Androgen Action in the Epididymis

Androgens are the primary regulators of epididymal structure and functions. Androgen replacement following androgen deprivation of the epididymis has been used in this thesis, to identify the role of androgen in establishing the integrity of epididymal processes. Androgen deprivation can be achieved by several methods, including treatment with the androgen antagonists (e.g., flutamide and cyproterone acetate) (8;9) and GnRH antagonists (10;11), or is derived from androgen ablation. The latter is achieved predominantly by bilateral orchidectomy (removal of the testes) (12) and to a lesser extent by hypophysectomy (removal of the pituitary) (13) which is a much more severe form of hormonal withdrawal. Among several options, orchidectomy is the most routine and almost complete androgen ablation method because it abolishes both circulating and luminal androgenic support to the epididymis by removing the primary site of testosterone biosynthesis without having any side effects at the level of other hormones. For instance, the circulating androgen concentration decreases to less than 10% of intact control levels within 2 hours following orchidectomy (14). In addition, using this kind of approach has been used in several studies to elucidate the region specific characteristics of the epididymis (15).

Structurally, orchidectomy causes a time dependent decrease in rat epididymis weight that reaches approximately 70% by 4 weeks post-orchidectomy. This weight loss is associated with cell cytoplasmic shrinkage, apoptotic cell death, as well as loss of sperm and fluid input from the testis. Regressive changes occur

predominantly in principal cells indicating that this cell type is particularly sensitive to androgens, while other epithelial cell types appear less affected by orchidectomy (16).

Functionally, orchidectomy induces a dramatic change in gene expression that is ultimately crucial for sperm maturation and motility. For instance, important epithelial processes such as the transport of ions and small organic molecules, metabolic functions and protein and lipid secretion, processing and degradation have been shown to be affected by orchidectomy (16).

Results obtained by orchidectomy have enhanced understanding of androgen-dependent and of segment-specific epididymal structure and function. However, these have not demonstrated conclusively the action of androgen, but rather the involvement of either or both androgen and factors that originate from the testis. Therefore, in several studies androgen has been replaced following orchidectomy in order to resolve this issue. While some groups have re-administered testosterone, others replaced dihydrotestosterone (DHT). Even though both androgens act via the same intracellular receptor, several lines of evidence indicate that they have unique roles (17). Their differential roles during development are best characterized. Testosterone is essential for the development of the Wolffian-duct structures, the pubertal and post pubertal increase in muscle mass and the development of male sex behavior, while DHT is involved in the development of the prostate, external urogenitalia from urogenital sinus and the tubercle, as well as sex maturation at puberty (17-19). In addition, several studies have demonstrated that testosterone and DHT also carry out unique roles in adult tissues. For instance, in

the prostate particularly, the effects of orchidectomy and 5α-reductase inhibitor treatment on differential expression of androgen-regulated genes have been identified (20;21). In this thesis, testosterone was employed for structural and morphological studies. However, the main androgen acting on the epididymal function, based on evidences from the literature, is DHT rather than testosterone (22;23), DHT has been used to determine the role of androgen at the molecular and cellular levels of the epididymal epithelium. In addition, using DHT rather than testosterone ensured us that the effects observed are mediated via the androgen receptor, as opposed to estrogen.

To elucidate the mechanism of androgen action at the cellular level, using epididymal epithelial cells in isolation, the immortalized caput epididymal cell line (PC-1) was employed in this thesis. Several caput epididymal cell lines were generated for various purposes. One of the examples of caput epididymal cell lines currently available is mE-Cap cells that have been used to characterize the regulation of PEA3 mRNA in the epididymis, in which it is regulated via protein kinase A and Erk signaling cascades (24). DC-2 cells are another caput epididymal cell line that has been used to identify regulatory elements of the DNA sequence responsible for lipocalin 5 gene expression (25). In this thesis, PC-1 cells were used to identify the signaling cascades activated by DHT. In accordance with the other two objectives, cells first were deprived of DHT supplemented in their routine regime, and of all endogenous steroids, by replacing regular fetal bovine serum (FBS) with charcoal-filtered FBS. These cells were subsequently treated with DHT.

In the present thesis, this experimental approach was used and sought to answer three main questions: 1) What are the consequences of testosterone replacement on the structure and cell renewal of the regressed epididymis? 2) What are the impacts of DHT/E2 stimulation in the sequence of gene activation or suppression on the regressed epididymis? 3) What are the signaling transduction pathways activated by DHT in epididymal principal cells?

Resolving these questions, together, provide novel insights into the mechanisms of androgen regulation in the epididymis. They also provide better understanding of the way the epididymis is normally sustained, and to some extend of pathological conditions, such as hypogonadism and andropause which are associated with a marked decline in androgen production.

2. Androgen Action in the Epididymis

2.1. Testosterone Action on the Epididymal Structure

It is well known how androgen withdrawal by orchidectomy causes a dramatic alteration in the epididymal structure. While changes to the epididymal appearance and weight have been described, ultrastructural organization and shape of the cellular organelles of the principal cells also undergo many changes, as revealed by using electron microscopy (26).

In chapter 2 of this thesis, the role of androgen in maintaining the structure of the epididymis was extensively investigated. Our model was a Sprague-Dawley rat that is the most studied and understood animal model in male reproductive biology. Once the epididymis was regressed, testosterone administration had the ability to

remarkably restore most of the histological features of the epithelium. The size of the tubules in terms of the epithelial cell heights and lumen diameters were mostly restored, and the mitotic rate reached the control levels, becoming even higher than that of the control values in a region-specific and time-dependent manner. This study conclusively showed that testosterone triggers a restoration of the morphological changes induced by orchidectomy. However, it follows to extend this work by monitoring the ultrastructural organization of the epithelial cells, in particular the principal cells. In the pilot study, I found evidence that DHT, an active metabolite of testosterone, is able to reverse a number of changes incurred by orchidectomy in the cellular organelles of the principal cells. For instance, DHT caused a decrease in lysosome content, an increase in rough endoplasmic reticulum, and an increase in the volume of the Golgi cisternae in the regressed epididymal epithelial cells. However, more analysis and replicates need to be carried out.

2.2. DHT/E2 Action on the Epididymal Functions

The development and advancement of gene array technology has provided tremendous opportunities for large-scale gene expression analysis in all areas of research including epididymal physiology. While a number of studies have been attempted to identify the epididymal gene expression in response to hormone, little is known about the way androgen action is initiated in the regressed tissue. The study described in chapter 3 of this thesis assessed how the triggering of androgen action is mediated by the time dependent, sequential activation and repression of gene families in the regressed epididymis. Importantly, differential gene expression was

demonstrated in response to each of the hormones, DHT or E2, which mimics luminal concentration in the intact rat epididymis. In addition, using the Pathway Studio software made it feasible to visualize the direct linkage between genes obtained by microarray analysis and to improve data interpretation. Our model was the brown Norway rat that has been used as the basis for the rat genome project, because it is an inbred rat and low variation in gene expression is observed between replicates. In the regressed epididymis, the genes exhibiting changes in expression following DHT treatment were classified based on their biological functions. They were involved in various functions, including in signal transduction, metabolic processes, transport, regulation of cell proliferation, cell-cell signaling, cell adhesion, regulation of transcription, proteolysis, immune response, and spermatogenesis.

Several genes in the solute carrier family (Slc) are affected by DHT after androgen deprivation; they play an important role in secretion of osmolytes under the control of androgen and regulation of the epididymal volume. In some species and more recently in four transgenic mice models, epididymal defects resulting in swollen spermatozoa have been associated with male infertility (27). These spermatozoa are unable to regulate their volume under the hypotonic conditions, presumably because of the blockage in the secretion of osmolytes by the epididymal epithelium. Given that also several epididymal enzymes and transporters responsible for osmolyte secretion are under the control of androgen (28;29), a better understanding of androgen action in this tissue can provide therapeutics avenues for this disorder.

Cystein-rich secretory protein 1 (Crisp1) is an androgen-regulated secretory protein of the rat epididymis, in which DHT treatment for 7 days reversed the suppression of expression caused by orchidectomy to the control levels. Crisp1 binds to the post-acrosomal region of the sperm head; therefore, it is one of the sperm surface proteins thought to be involved in the fusion of sperm and egg plasma membranes (30). As discussed in chapter 3, many genes identified to be regulated under the control of androgen, might prove to be potential targets for the development of drugs that will regulate epididymal functions for purposes of controlling male fertility.

In addition to the critical role of DHT, one cannot conclude the tissue's functions and regulation without considering the effect of estradiol. The importance of estradiol and the presence of the estrogen receptors in the epididymis were confirmed following the generation of estrogen receptor α (ERα) knockout mice, which are infertile (31). Also, two different aromatase deficient mouse lines have been generated by disrupting the cyp19 gene (32;33). These mice show different phenotypes, with defects in sexual behavior or impaired spermatogenesis. In gene expression profiling results, as discussed in the chapter 3, few genes were identified that responded to estradiol, while the expression of a number of genes was regulated by DHT. Expression of these genes, for the first time, was reported to be regulated by estradiol in the epididymis. This study can be extended further, as discussed later in section 3.1. of this chapter.

2.3. DHT Action on Rapid Signaling Pathways Activation in the Epididymis

In chapter 3, we identified the specific cascade of gene modulation that causes the remodeling of a regressed androgen-deprived tissue in response to hormone treatment; Igf-1and Egf genes possessed an integral role. With the advent of immortalized epididymal cell lines, it has become possible to elucidate the rapid action of androgen in a pure population of principal cells (PC1). Importantly, the PC-1 cell line retains several characteristics of principal cells in the *in vivo* system such as principal cell- and caput-specific gene expression and androgen responsiveness (34). Additionally, we found that DHT increased the Igf-1 expression in androgen-deprived PC-1 cells, consistent with our *in vivo* results. What remained unclear was the identity of signaling pathways involved in mediating the effects of DHT on epididymal gene expression and function, which formed the basis of our third and final objective.

As discussed in chapter 4 of this thesis, the MAPK/Erk pathway was activated in response to DHT via an androgen receptor-mediated mechanism. The IGF1R and EGFR were found to be upstream of Erk pathway, since inhibiting these two receptors blocked Erk activation. Subsequently, this activated pathway leads to activation of transcription factors such as CREB. It may also act to modulate transcriptional activity of genes regulated by AR in the nucleus. A study by Belfiore et al. is an example of gene transcription activity following signaling transduction pathway activation in response to DHT (35;36); IGF-1R expression is up-regulated by DHT in prostate cancer cells via CREB phosphorylation, which is required for CREB-binding protein recruitment and CREB-mediated transactivation of gene

transcription. Moreover, the CREB-binding site has been identified at the 5'-untranslated region fragment of IGF-1R promoter, and thus CREB silencing abrogated the IGF-1R up-regulation. In the epididymis, further investigation needs to be done to confirm that this rapid signaling pathway activated by androgen can modulate transcriptional activity of gene regulated by AR in the nucleus.

While several studies have shown that the rapid action of androgen can be AR-independent, as using AR-antagonist is not able to inhibit the fast activation of Erk1/2 and that activation is Ca^{+2} –dependent (37), our results demonstrated the dependency of Erk1/2 phosphorylation on AR. Interestingly, most tumors in androgen-independent prostate cancer are associated with AR overexpression. Increasing the expression of specific coactivators such as glyceraldehydes-3-phosphate dehydrogenase (GAPDH) (38) or Twist1(39) which bind to AR and enhance transcriptional activity of AR. The fact that the level of AR has not changed in the epididymal cells under different androgenic conditions could be an explanation of tumor resistance characteristic seen in the epididymis.

3. Future Directions

This thesis has provided novel insights of selective aspects of the mechanisms by which androgens exert their action to maintain epididymal cell integrity. It lays the basis for further studies on the regulation of genes that respond to various androgenic conditions and characterization of their functions in the epididymis, as well as opens new avenues for future studies on the role of androgen

in induction of rapid signaling transduction that may lead to gene transcriptional activities.

3.1. Functional Characterization of Genes Regulated by DHT or E2 in the Epididymis

The function of genes regulated by DHT or E2 in the epididymis, as discussed in chapter 3, can be explored and verified by different techniques.

With the advent of transgenic technology to generate spatially restricted targeted gene disruptions, it has become possible to resolve the function of related genes. For instance, bone morphogenetic proteins (Bmp) are intercellular signaling molecules with various roles during development. Targeted inactivation of several genes in this family has revealed that all play roles in maintaining epididymal integrity and in spermatogenesis (40-42). Bmp4 is one of these genes identified in our gene array results as being regulated by DHT, although there are many genes for which knockout models have not yet been developed.

Gene silencing agents, such as RNAi (43;44) and ribozyme (45), to manipulate gene expression have also become useful tools to detect functions of epididymal genes. This type of study can be done in both *in vitro* and *in vivo* conditions. Ribozymes are RNA molecules with catalytic activity, almost like proteins. By using gene specific promoters to drive the expression of highly specific ribozymes in a spatially regulated manner, it is possible to carry out conditional gene ablation studies. For delivery, electroporation can be used as a non-viral technique with several advantages to viral strategies (46). This technique is being used in the

last decade as a method to deliver genes for therapeutic purposes in the treatment of diseases such as cancer.

Among the many genes regulated by DHT, IGF1 and its family were particularly notable. We found that the decline in IGF1 expression relative to control after epididymal regression was prevented by DHT. In contrast, DHT caused a suppression of Igfbp-3, the most prevalent of the six IGFBPs (47). In addition, IGF1 and EGF were found to play central roles in the early pathway activated by DHT. Understanding the physiological role of IGF1 has been a focus of interest of many research groups for more than a decade, due to the generation of adult mice with a homozygous null mutation of the Igf1 gene (48). Interestingly, these mice were infertile dwarfs with reduced testosterone production, and consequently reduced spermatogenesis and reproductive organ size. The epididymal phenotype of Igf-1 null mice is more severe in the distal regions of the tissue. Given our finding that regulation and expression of many other genes were linked to the Igf-1gene, this study can be extended by elucidating the genomic profile of androgen responsive genes in Igf-1 null mice, in particular the distal regions of the epididymis. Therefore, the ability of the epididymis to illustrate a normal response to orchidectomy in the absence of Igf-1, and in the presence or absence of androgen will be identified.

It has been shown that IGF1 and EGF play synergistic roles in pathological processes, such as carcinogenesis and wound healing (49). In addition, IGF1/EGF-mediated regulation of IGFBP3 via two signaling pathways, Ras/Raf/MAPK or PI3K/AKT/ mTOR pathway, has been identified in several cells, including prostate (50), esophageal epithelial (51), and chondrocyte cells (52). However, the underlying

mechanisms regulating IGFBP3 expression and its functional role in epididymal cell growth remain to be elucidated. This could be done using a siRNA approach in the immortalized epididymal PC-1 cell line, followed by the assessment of cell viability and appearance, as well as the expression of key genes involved in androgen action (e.g., androgen receptor, 5α-reductases), and other members of the IGF family. Therefore, in regards to following-up our findings in chapter 3, several experiments can be done to prove an in depth understanding of the role of these growth factors in the epididymis under the control of androgen.

To elucidate the role of genes regulated by E2 in the epididymis, a complementary approach can be used by assessing the expression of these genes in the aromatase deficient mice or in mice treated with aromatase inhibitors, before and after treatment with estradiol. Any changes in the expression of these genes could be the consequence of the result found in the aromatase deficient mice as they displayed an impairment of spermatogenesis.

In addition to using gene arrays to examine gene expression in the epididymis, several studies revealed regionalized proteins in the intact tissue in different species (53-55). However, proteomic profiling of the epididymis in adult regressed tissue and in response to DHT or E2 is yet to be conducted. Both transcriptomic and proteomic approaches provide a dramatic advance to our understanding of the role of the epididymis in the male reproductive system and in sperm maturation which is fundamental for the survival of all mammalian species. It is likely the most efficacious post-testicular contraceptive will be comprised of a

combination of compounds targeting the expression of various epididymal-specific proteins that are crucial for sperm function (56).

3.2. Consequences of Androgen action in Signaling Transduction Activation

In chapter four of this thesis, we established the potential signaling pathway activated by DHT. To follow-up these studies, various approaches can be undertaken to identify the consequences of signaling pathway activation and its role in regulating gene expression, including Igf1 in PC-1 cells under various androgenic conditions. The level of Igf1up-regulation induced by androgen could be assessed upon CREB silencing, particularly as we have identified the potential conserved CREB responsive elements in the upstream region of the Igf1 promoter sequence. However, functional analysis of this sequence needs to be undertaken. Additionally, the occupancy of the Igf1 promoter by DHT-activated CREB can be evaluated by using the DNA affinity precipitation assay, or CREB occupancy on Igf1 promoter can be analyzed by chromatin immunoprecipitation.

Moreover, global gene expression of PC-1 cells can be examined following treating cells with IGF1R and EGFR inhibitors that selectively block their kinase activity, under different androgenic conditions. An alternate approach is to assess the consequences of inhibiting IGF1R expression using siRNA and compare these two approaches. Despite the numerous potential applications of the epididymal cell lines, they could never truly represent the complex regionalized diversity of the epididymis as a whole. For this reason, the effects of using two inhibitors of IGF1R and EGFR would also need to be investigated in the animal model, using

orchidectomized rats treated with either DHT or vehicle. These gene inactivation and inhibitors studies should further resolve the role of growth factor receptors in mediating the effects of DHT on signaling pathway activation and in modulating androgen action.

A study by Lattouf et al. is an example of evidence to support the crucial role of growth factors in reproductive tissues (57). In this case, in an androgen-resistant human prostate cancer, the androgen receptor is stimulated for sustained growth. This stimulation could take place via ligand binding, through binding of alternate molecules such as estrogens, or activation through peptide growth factors, including insulin-like growth factor, or human epidermal growth factor receptor 2 would be possible. The activation takes place through MAPK or AKT pathways. Therefore, inhibitors of these cascades seem to be promising candidates to achieve the growth inhibition in prostate cancer. Determining the roles of growth factors and growth factor signaling in the development, regulation, and pathogenesis of the male reproductive system, particularly of the prostate has become an intriguing area of research in this field. Similarly, a better understanding of these growth factors and the way they mediate rapid action of androgen will have implication in the maintenance of epididymal integrity and related pathological conditions.

3.3. Developing Novel Tools for *in vitro* Studies

All the region-specific epididymal cell lines published to date are derived from the caput epididymidis and are essentially principal cells.

Even given the fact that the majority of epididymal cell types are principal cells, and the caput epididymidis is very active in synthesis and secretion of proteins, one cannot exclude the important role of other regions and cell types for epididymal functions. Thus, the immortalized cell lines need to be developed for each cell type and different regions. Using the region- and cell type-specific cell lines, various aspects of androgen action can be identified, including the AR signaling cascade and non-classical modes of androgen action, as well as the regulatory regions of androgen-regulated genes in the different cell types of the different regions of the epididymis. In addition, generating them should make it possible to elucidate the interactions between different cell types by using co-culture systems.

4. Final Conclusions

Regionalized diversity of the epididymis, a hallmark feature of this tissue, and its ability to produce functional sperm, makes it an interesting and unique reproductive target to study the role of androgen action. In this thesis, androgen replacement following an androgen deprivation approach has been used, to address three different questions directed toward gaining better insights into the role of androgens in maintaining key features and functions of epididymal epithelial cells. Collectively, the studies presented contribute greatly to our understanding of cascade of events associated with initiation and sequence of androgen action in the epididymis. From the clinical perspective, by increasing the consequences of androgen ablation that occurs with advancing age as well as in some pathological conditions, an in depth understanding of how androgen action is mediated is essential. Additionally, a greater knowledge of the role of androgens in maintaining the unique microenvironment of the epididymis, which are crucial for sperm maturation, would allow further informed diagnoses of the underlying epididymal causes of male infertility and indicate potential treatments of this pathology. Therefore, the epididymis could also be an extremely promising target for male contraceptive approaches; these should be highly specific and have a rapid onset of action compared to testis-based or steroid hormonal contraceptive approaches.

References

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LIST OF ORIGINAL CONTRIBUTIONS

1. Determined the lumen diameter and epithelial cell height in the regressed rat epididymis, before and after testosterone replacement.
2. Determined the number of epithelial cells that immunostained positively for cell proliferation markers (PCNA and BrdU) in all regions of the regressed rat epididymis, before and after testosterone replacement.
3. Identified proliferating activity in all cell types of the intact and regressed rat epididymis in response to testosterone.
4. Determined the impact of dividing cells on the relative cell populations after testosterone replacement in the regressed rat epididymis.
5. Identified weights of sex accessory tissues, including the epididymis in the orchidectomized and regressed tissues, before and after dihydrotestosterone (DHT) or estradiol (E2) replacement.
6. Determined the sequence of gene activation and suppression in the regressed epididymis upon re-administration of the two active metabolites of T (DHT or E2).
7. Determined the impact of DHT or E2 treatment on gene expression in the regressed rat epididymis at different time and classified those genes based on their biological function.
8. Designed rat insulin-like growth factor 1(Igf1) primer for qRT-PCR and quantified its mRNA expression in the regressed epididymis, before and after DHT replacement at different time points.
9. Confirmed, at the protein level, IGF1 is an androgen-regulated gene in the epididymis.

10. Visualized direct relationships between genes differentially affected by DHT administration after 12 h and 1 d in the regressed rat epididymis.
11. Identified genes involved in the common pathway, with potential roles in cell survival, cell proliferation, differentiation, secretion, and motility in the regressed epididymis in response to DHT.
12. Found many of the genes affected by DHT to be functionally linked by direct interactions to IGF1/EGF.
13. Found the IGF1/EGF family to play a central role in restoring epididymal function and integrity.
14. Elucidated the signaling pathway activated by DHT in the mouse proximal caput epididymidis (PC-1) cell line, which has never been previously explored *in vivo* or in epididymal cell lines.
15. Determined the consequences of androgen withdrawal and DHT replacement on the number of live and dead cells in PC-1 cells.
16. Determined the consequences of DHT treatment on Igf1 and androgen receptor expression in androgen deprived PC-1 cells.
17. Identified ERK pathway, but not AKT to be a rapid pathway activated in PC-1 cells in response to DHT.
18. Found the activated ERK pathway to be mediated by androgen receptor.
19. Identified the IGF1R and EGFR to be the upstream of ERK pathway and downstream of SRC kinase in PC-1 cells.
20. Determined the consequence of activated ERK pathway induced by DHT in activation of transcription factors such as CREB.