Apoptosis and Cell Survival in the Epididymis after Androgen Withdrawal

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ABSTRACT

Androgens regulate many reproductive and non-reproductive functions. Dysregulation of androgen responses can lead to different pathologies. There is, therefore, a need to better understand the molecular mechanisms underlying androgen actions. We focus on the epididymis, an androgen-dependent tissue responsible for the proper maturation and storage of spermatozoa. Unlike the response of other hormone-dependent tissues, there is little apoptosis in the epididymis after androgen withdrawal. Hence, our overall objective is to understand the molecular mechanisms involved in the resistance of the epididymis to apoptosis triggered by androgen withdrawal. We hypothesize that androgen withdrawal triggers the activation of a series of specific survival signaling pathways that act to help protect the epididymis against high levels of apoptosis.

The first objective was to identify the apoptotic and cell survival genes activated after androgen withdrawal and/or replacement in the epididymis using apoptosis-focused arrays. The expression of apoptotic and cell survival genes changed in a region-specific manner and putative androgen-response elements were identified in the promoter region of affected genes. Changes in expression for *Bmf*, *Mcl1*, *Tnfrsf11b*, and *Rad52* were further characterized.

The second objective was to determine the involvement of the IGF1 signaling pathway in the response of the epididymis to androgen withdrawal. In the different epididymal regions, *Igf1*, *Igf1r*, *insulin-degrading enzyme*, *Igfbp3*, and *Birc5* were differentially regulated after androgen withdrawal. This study indicated that members of the IGF1 signaling pathway participate in the response of the epididymis to androgen withdrawal.

The third objective was to assess the effects of androgen withdrawal on the PC-1 and DC-3 mouse epididymal cell lines. Androgen withdrawal did not decrease PC-1 and DC-3 cell survival, which mimicked the *in vivo* situation. For the markers studied, DC-3 cells seemed more sensitive to androgens than PC-1 cells.

Together, the three objectives of this thesis increase our understanding of androgen regulation of apoptotic and cell survival genes in the epididymis as well

as of the molecular mechanisms underlying epididymal resistance to apoptosis triggered by androgen withdrawal.

ABRÉGÉ

Les androgènes régulent plusieurs fonctions reproductives et non-reproductives. La dérégulation des réponses aux androgènes peut causer différentes pathologies. Il y a donc un besoin de mieux comprendre les méchanismes moléculaires sous-jacents aux actions des androgènes. Nous nous concentrons sur l'épididyme, un tissue dépendant des androgènes qui est responsable de la maturation appropriée et du stockage des spermatozoïdes. Contrairement à la réponse d'autres tissues dépendant des androgènes, il y a très peu d'apoptose dans l'épididyme après le retrait des androgènes. Alors, notre objectif général est de comprendre les méchanismes moléculaires impliqués dans la résistance de l'épididyme à l'apoptose stimulée par le retrait des androgènes. Nous avons posé l'hypothèse que le retrait des androgènes stimule l'activation d'une série de chemins de signalisation spécifiques de survie qui agissent pour aider à protéger l'épididyme contre des niveaux élevés d'apoptose.

Le premier objectif a été d'identifier les gènes d'apoptose et de survie celluaire activés après le retrait des androgènes et/ou leur remplacement dans l'épididyme en utilisant des micropuces spécifiques à l'apoptose. L'expression des gènes d'apoptose et de survie cellulaire a changé de manière spécifique à chaque région et des éléments de réponse aux androgènes possibles on été identifés dans la région promoteuse des gènes affectés. Les changements d'expression de *Bmf*, *Mcl1*, *Tnfrsf11b* et *Rad52* ont été charactérisés plus en détails.

Le second objectif a été de déterminer l'implication du chemin de signalisation du facteur IGF1 dans la réponse de l'épididyme au retrait des androgènes. Dans les différentes régions épididymales, *Igf1*, *Igf1r*, l'*enzyme de dégradation de l'insuline*, *Igfbp3* et *Birc5* ont été régulés différemment après le retrait des androgènes. Cette étude a indiqué que les membres du chemin de signalisation du facteur IGF1 participent à la réponse de l'épididyme au retrait des androgènes.

Le troisième objectif a été d'évaluer les effets du retrait des androgènes sur les lignées cellulaires épididymales de souris PC-1 and DC-3. Le retrait des androgènes n'a pas diminué la survie cellulaire de PC-1 and DC-3 ce qui a ressemblé à la situation *in vivo*. Pour les marqueurs étudiés, les cellules DC-3 ont semblé plus sensibles aux androgènes que les cellules PC-1.

L'ensemble des données des trois objectifs de cette thèse augmente notre compréhension de la régulation par les androgènes des gènes d'apoptose et de survie cellulaire aussi bien que des méchanismes moléculaires sous-jacents à la résistance épididymale à l'apoptose stimulée par le retrait des androgènes.

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LIST OF ABBREVIATIONS

17β-HSD	17 β-hydroxysteroid dehydrogenase
3β-HSD	3β-hydroxysteroid dehydrogenase
9-cis-RA	9-cis-retinoic acid
ABP	androgen binding protein
Acta1	actin, alpha 1, skeletal muscle
ADAM	a desintegrin and metalloprotease
Adam7	ADAM metallopeptidase domain 7
Ahr	aryl hydrocarbon receptor
AIDS	acquired immune deficiency syndrome
Akt1	thymoma viral protooncogene 1
ALS	acid-labile subunit
APAF1	apoptotic protease activating factor-1
AR	androgen receptor
ARE	androgen response element
Armcx3	armadillo repeat containing, X-linked 3
Atf4	activating transcription factor 4
ATPase	adenosine triphosphatase
Bcl2	B cell leukaemia/lymphoma 2
BH	BCL2 homology
BIR	baculovirus IAP repeat
BIRC5	baculoviral IAP repeat-containing 5
BMP8a	bone morphogenetic protein 8A
Bmyc	brain-expressed myelocytomatosis oncogene
BN	Brown Norway
bp	base pair
C/EBP	CCAAT/Enhancer binding protein
Ca	caput
Calcrl	calcitonin receptor-like
cAMP	cyclic adenosine monophosphate
Casp	caspase

Ccna2	cyclin A2
Cd	cauda
Cdh1	cadherin 1
Cdh2	cadherin 2
CF-FBS	charcoal-filtered fetal bovine serum
Clu	clusterin
Co	corpus
Cox1	cyclo-oxygenase 1
CRABP	cellular retinoic acid-binding protein
CRBP	cellular retinol-binding protein
CRISP	cystein-rich secretory protein
Cst11	cystatin 11
Cst12	cystatin 12
CTP/HE1/NCP2	cholesterol transfer protein
Ctsc	cathepsin C
Ctsh	cathepsin H
d	day
Dad1	defender against cell death protein 1
DBD	DNA-binding domain
DC-3	distal caput epididymis cell line 3
DcR	decoy receptor
DD	death domain
Defb11	beta-defensin 11
DHT	5α-dihydrotestosterone
DIABLO	direct IAP-binding protein with low pI
DISC	death inducing signaling complex
DR	death receptor
EDAR	ectodysplasin A receptor
EGF	epidermal growth factor
ER	estrogen receptor
E-RAPB	epididymal retinoic acid-binding protein

ERK	extracellular signal-regulated kinase
Etv5	ets variant gene 5
FADD	Fas-associated death domain protein
FBS	fetal bovine serum
FGF	fibroblast growth factor
Fgfr1	fibroblast growth factor receptor 1
FHCE	fertile human caput epididymal cell line
FHSE	fertile human corpus epididymal cell line
Figf	c-fos induced growth factor
FSH	follicle-stimulating hormone
Gapdh	glyceraldehydes-3-phosphate dehydrogenase
Gas7	growth arrest-specific 7
GF	growth factor
GGT	γ-glutamyl transpeptidase
Gj	gap junction protein
GnRH	gonadotropin-releasing hormone
GPX5	glutathione peroxidase 5
Grb2	growth factor receptor-binding protein 2
Grp	glucose-regulated protein
H	hinge region
HDL	high-density lipoprotein
hrs	hours
hsp	heat shock protein
HTRA2	high-temperature requirement serine protease A2
IAP	inhibitor of apoptosis protein
ICAD	the inhibitor of caspase-activated DNase
IDE	insulin-degrading enzyme
IGF	insulin-like growth factor
IGF1	insulin-like growth factor 1
IGF1R	insulin-like growth factor 1 receptor
IGFBP	IGF binding protein

Igfbp2	insulin-like growth factor binding protein 2
IGFBP3	insulin-like growth factor binding protein 3
IHCE	infertile human caput epididymal cell line
Ilk	integrin linked kinase
IMCE	immortalized canine epididymis
IRS	insulin receptor substrate
IS	initial segment
Itpr3	inositol 1,4,5-triphosphate receptor 3
Jund1	jun protooncogene-related gene d1
kb	kilobases
Lama5	laminin
LBD	ligand-binding domain
Lcn5	lipocalin 5
Lcn8	lipocalin 8
LDH	lactate dehydrogenase
LDL	low-density lipoprotein
LH	luteinizing hormone
Lrp2	low-density lipoprotein receptor-related protein 2
Man2	alpha-mannosidase II
MAPK	mitogen-activated protein kinase
Mcl1	myeloid cell differentiation protein 1
MEPC5	mouse epididymis caput epithelial cell line
Mgst1	microsomal glutathione S-transferase
NADPH	nicotinamide adenine dinucleotide phosphate
NAIP	neuronal apoptosis inhibitory protein
NF- κB	nuclear factor-κB
NGFR	nerve growth factor receptor
NTD	N-terminal regulatory domain
OT	oxytocin
P45017α	17α-hydroxylase cytochrome P450
P450scc	cytochrome P450 enzyme cholesterol side-chain cleavage

PARP	poly(ADP-ribose)polymerase
Pbp1	phosphatidylethanolamine binding protein 1
PC-1	proximal caput epididymis cell line 1
PDGF	platelet-derived growth factor
Pdgfc	platelet-derived growth factor, C polypeptide
PEA3	polyomavirus enhancer activator 3
PGDS	prostaglandin D2 synthase
Phgdh	3-Phosphoglycerate dehydrogenase
PI3K	phosphatidylinositol 3-kinase
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
Plau	plasminogen activator, urokinase
Pppr2b2	protein phosphatase 2, regulatory subunit B, beta isoform
pRB	retinoblastoma susceptibility protein
qRT-PCR	quantitative real-time PCR
Rab2	RAB2, member RAS oncogene family
Rad21/23b	RAD21/RAD23b homolog
Ramp3	receptor (G protein-coupled) activity modifying protein 3
RAR	retinoic acid receptor
RBP	retinoid-binding protein
RCE	rat caput epididymal cell line
RIP	receptor-interacting protein 1
Ripk1	receptor (TNFRSF)-interacting serine-threonine kinase 1
ROCK1	Rho-associated coiled-coil forming kinase 1
rT3	reverse T3
Serpinh1	serine (or cysteine) peptidase inhibitor, clade H, member 1
SHBG	sex hormone-binding globulin
SHC	Src- and collagen-homology
Slc	solute carrier family
Sod1	superoxide dismutase

SR	steroid 5 alpha-reductase
SV40LT	SV40 large T-antigen
T	testosterone
T3	3,5,5'-triiodothyronine
T4	thyroxine
TBG	thyroxine binding globulin
TEBG	testosterone-estradiol-binding globulin
TGFβ	transforming growth factor-β
TH	thyroid hormone
Thoc4	THO complex 4
Timp2	tissue inhibitor of metalloproteinase 2
TNF	tumor-necrosis factor
TNFR	tumor-necrosis factor receptor
TNFRSF	tumor necrosis factor receptor superfamily
Tnfrsf1a	tumor necrosis factor receptor 1
TR	thyroid hormone receptor
TRADD	TNFR1-associated death domain protein
TRAF	TNF-receptor-associated factor
TRAIL-R1	TNF-related apoptosis inducing ligand receptor-1
TRAMP	TNF-receptor-related apoptosis mediating protein
TRE	thyroid response element
Ts-IAP	testis-specific IAP
tsSV40LT	temperature-sensitive mutant of SV40LT
Ttf1	transcription termination factor 1
XAF-1	XIAP-interacting protein
XIAP	X-chromosome-linked IAP 5

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PREFACE

Thesis Format

This is a manuscript-based thesis, which conforms to section 1.C. of the "Thesis Preparation and Submission Guidelines" of the Faculty of Graduate Studies and Research at McGill University. This thesis is comprised of five chapters. Chapter one is a general introduction; it is a comprehensive review of the epididymis, androgens, androgen regulation of the epididymis, as well as cell survival and apoptosis. This chapter concludes with a rational for the studies presented in this thesis and the objectives of the thesis. Chapters two to four are data chapters bridged by connecting text to ensure that the thesis has continuity. Chapters two and three will be submitted for publication, whereas chapter four is a thesis chapter in the form of a manuscript. Chapter five is a discussion of the overall results and includes ideas for future studies; it is followed by a list of original contributions. References are provided at the end of each chapter. The appendices contain supplemental data that could not be included in the chapters. The ethics certificates for work on animal subjects and for the use of radioactive materials as well as the copyright agreements for the figures used in the first chapter are submitted separately.

Contributions of Authors

All the experiments and analyses described in this thesis were completed by the candidate with the exception of the rat perfusions, which were done by Ludovic Marcon and the dot blot experiments that were done by Trang Luu. Trang Luu has also helped to do some qRT-PCR and western blots.

CHAPTER 1

Introduction

1. The male reproductive system

The male reproductive system consists of a series of organs that act in a concerted manner to produce spermatozoa able to fertilize an oocyte and to deliver these spermatozoa to the female reproductive tract (1). The testes produce the gametes that are transported through a series of ducts that include in the following order, the efferent ducts, epididymis, vas deferens, and urethra inside the penis. The testes also produce androgens, a process that occurs in the interstitial Leydig cells (2). In addition, the seminal vesicles, prostate, and bulbourethral glands secrete fluids that constitute the ejaculated semen (3). This thesis focuses on the epididymis, a critical site for spermatozoa maturation (the process by which spermatozoa acquire the ability to swim, recognize, and fertilize an oocyte) and where they are stored before ejaculation (2;4-8); spermatozoa become fully capable of fertilizing an oocyte after they reside in the oviduct and become capacitated (291).

2. The epididymis

The word "epididymis" comes from the Greek *epí* for "on" and *dídymoi* for "twins" (testes) and refers to the localization of the epididymis on the surface of the testis (4). It was first described by Aristotle in *Historia Animalum* in the 4th century B.C., but it was only in 1668 that De Graaf described the first dissected human epididymides (6;9;10). Despite an early discovery, the epididymis received little attention up to the 1960's when its role as the key player in spermatozoa maturation was finally recognized (2;6;11).

2.1. Structure

Most studies done to understand the structure, function, and regulation of the epididyimis were carried out in animal models (rat, mouse, rabbit, boar, and stallion) due to the lack of available human epididymal tissue. However, the human epididymis possesses specificities compared to other mammals that will be introduced when appropriate (9).

2.1.1. Gross anatomy

The epididymis is a single highly convoluted tubule that links the efferent ducts of the testis to the vas deferens (4;6;8) (Fig. 1). The highly coiled nature of the epididymis can be illustrated with the human epididymis: only 10-12 cm in length, it contains 6-7 m of coiled tubule (9). The epididymal tubule varies in length from 1 m in mice (12), 3 m in rats (13), to up to 80 m in stallions (14). Based on structural differences, it is usually separated into four distinct regions: the initial segment (IS), caput (head, Ca), corpus (body, Co), and cauda (tail, Cd) (2;6;15) (Fig. 1); in humans, the initial segment is absent (9). Other species-specific nomenclatures have divided the epididymis in more regions or zones based on the organization of each region into lobules separated by connective tissue septa (6;16-19). These septa might participate in the lobule-specific and region-specific expression of genes and proteins (20). Developmentally, the initial segment seems to be derived from the mesonephric (Wolffian) tubule, while the remaining regions are derived from the mesonephric duct [reviewed in (2;6;21;22)]. This different developmental origin of the initial segment from the other epididymal regions might explain the differences in their regulation (6); this point will be reviewed in many sections of this thesis. In the adult, the epididymis becomes highly differentiated with a mitotic index below 0.6% in rats (23). The epididymis is further divided into three compartments: a lumen, an epithelium, and an inter-tubular compartment (Fig. 2) (2). The lumen contains spermatozoa bathing in a fluid with a composition that varies from region to region, thereby creating specialized microenvironments for the proper maturation of spermatozoa (24). These microenvironments are created by the secretion and absorption of water, ions, small organic molecules, and proteins by the epithelium (2). The composition of the epididymal epithelium will be discussed in the next section. The epithelium is surrounded by myoid cells, connective tissue, and an interstitium that contains blood vessels, lymphatics, and nerves (4). From proximal (initial segment and caput) to distal (corpus and cauda) regions, the epithelial cell height decreases and the luminal diameter increases (7).

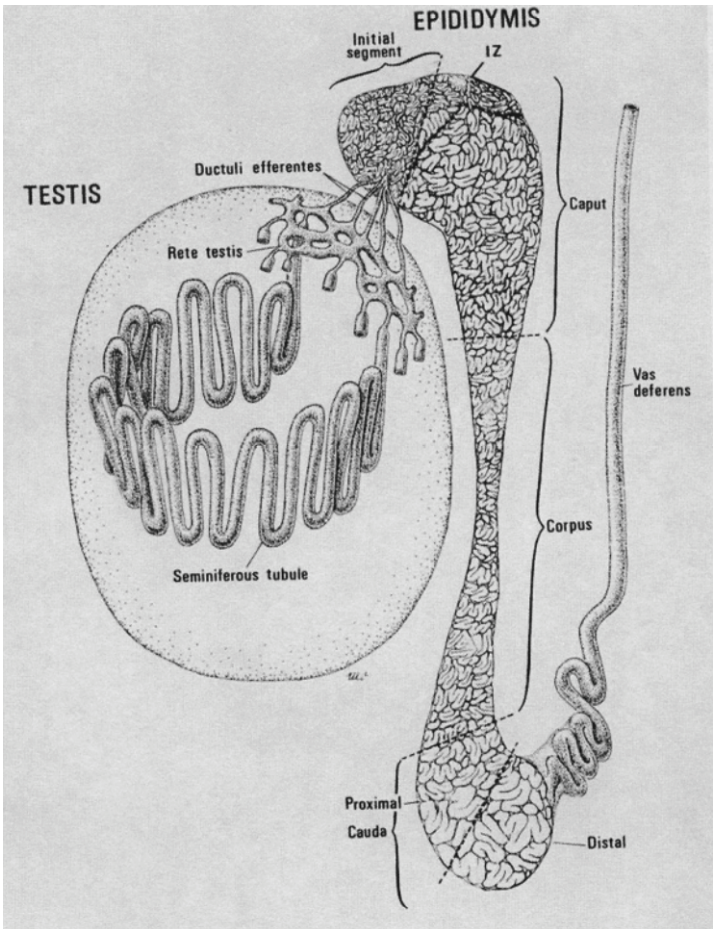

Figure 1: Diagrammatic Representation of the Testicular Excurrent Duct System

The testicular excurrent ducts of the male reproductive system conduct spermatozoa from their site of production to their site of ejaculation. Spermatozoa are produced in the seminiferous tubules and collected in the rete testis before leaving the testis through the efferent ducts. The efferent ducts converge into a single highly convoluted tubule, the epididymis. The epididymis is morphologically and functionally separated into four distinct regions: initial segment, caput, corpus, and cauda. Spermatozoa remain in the cauda epididymidis until ejaculation at which time they empty into the vas deferens.

Reproduced from reference (6).

2.1.2. Cell types of the epididymal epithelium

The epididymal epithelium is composed of six cell types: principal, basal, clear, narrow, apical, and halo cells (Fig. 2) (25). The structure, size, and number of epididymal cells are region-specific [reviewed in (2)].

Principal cells. Principal cells, as their name implies, are the major cell type present in the epididymis and, depending on the region, comprise 65% to 80% of the total cell population (2;8). Actively involved in protein synthesis, they are also the main absorptive and secretory cells of the epididymis. In fact, all proteins secreted in the lumen are synthesized by principal cells (8). Furthermore, they are the main androgen-responsive cells of the epididymis (26).

Basal cells. Basal cells are the second most common cell type of the epididymis and are located throughout the tissue (2;8). Their name derives from their localization at the basement membrane, which prevents them from having direct access to the lumen of the epididymis (6). They are not stem cells that could replenish principal cells (23). Basal cells are closely associated with principal cells and hence could regulate their functions by secretion and endocytosis of proteins (6;25). In addition, they might act as immune cells because they express macrophage antigens (27;28).

Clear cells. Clear cells are found in all epididymal regions, except initial segment (8). In immunohistochemistry, these cells do not stain after counterstaining with methylene blue hence their name. Involved in endocytosis of proteins from the lumen in a region-specific manner, they particularly clear the lumen of proteins from cytoplasmic droplets released by maturing spermatozoa. Clear cells in collaboration with narrow and apical cells are also involved in luminal acidification (6).

Narrow and apical cells. Narrow and apical cells are only found in the initial segment. They differ from one another in terms of morphological appearance, relative distribution, and protein expression. They both participate in endocytosis of proteins and in luminal acidification (8).

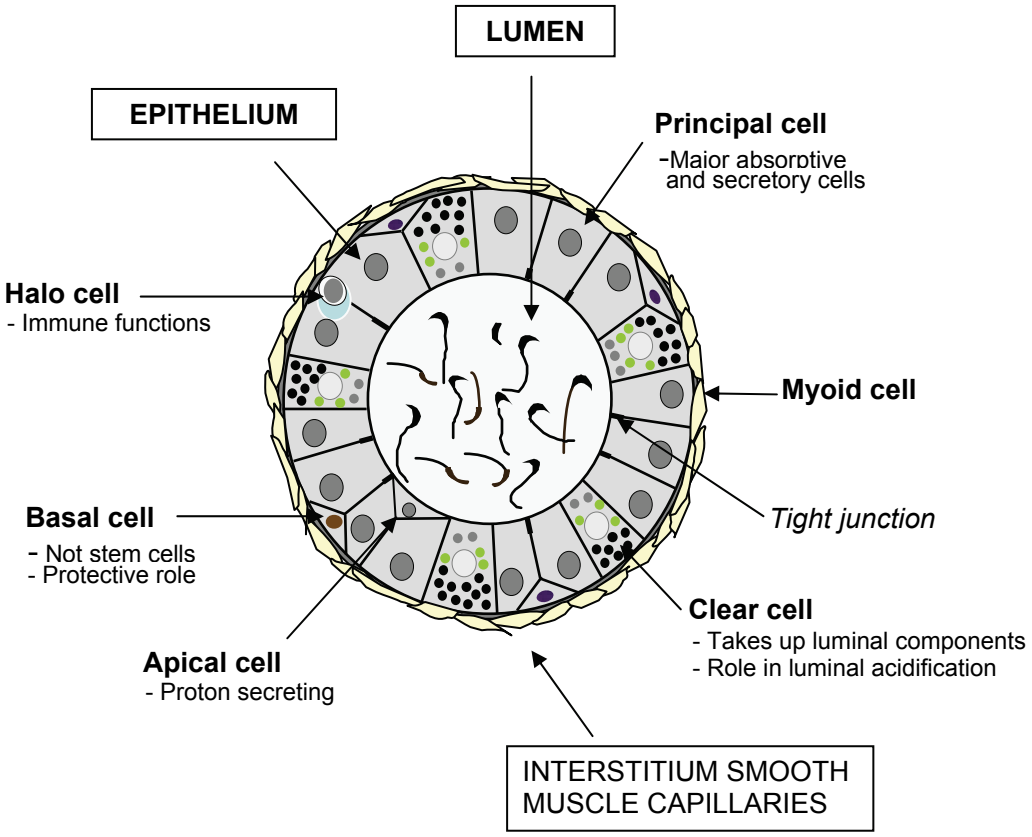

Figure 2: Schematic Diagram of the Cellular Organization in the Rat Epididymis

The epididymis is separated into three compartments: a lumen, an epithelium, and an inter-tubular compartment**.** The lumen contains maturing spermatozoa, while the epithelium is composed of six cell types. The relative position and distribution of the different cell types are illustrated. The major functions associated with each cell type are also identified.

Adapted from reference (6).

Halo cells. Halo cells are the primary immune cells of the epididymis and are present throughout the tissue at the base of the epithelium. They comprise helper T lymphocytes, cytotoxic T lymphocytes, and monocytes (8).

2.1.3. The blood-epididymis barrier

The blood-epididymis barrier, as its name implies, describes a physical division between the blood content and the luminal environment of the epididymis. The barrier is created by tight junctions located on the luminal side of adjacent principal cells (29). Communication between cells is maintained by gap junctions (6). This allows the epididymis to tightly regulate the molecules that enter the lumen in a region-specific manner thereby creating specific microenvironments along the duct (29-31). In addition, the blood-epididymis barrier protects spermatozoa, that are immunogenic, from degradation by immune cells, as well as from some toxic substances (6;7).

2.2 Functions

The epididymis participates in the transport, maturation, storage, and protection of spermatozoa (9). Each epididymal region accomplishes specific functions; the caput and corpus epididymides are involved in early and late spermatozoa maturation, respectively, while the cauda epididymidis is the main storage site for mature spermatozoa (25).

2.2.1. Spermatozoa transport

The epididymis transports spermatozoa from the testis to the vas deferens thereby allowing ejaculation (32). Once released from the testis, spermatozoa are transported to the epididymis by the movement of testicular fluid and possibly by the beat of the ciliated cells of the efferent ducts (6). In the epididymis, the epithelium is lined by immotile sterocilia and fluid flow is reduced by fluid uptake, therefore, movement down the duct is maintained by hydrostatic pressures and rhythmic muscular contractions of the smooth muscle surrounding the

epithelium (6;9). These events are controlled by adrenergic and cholinergic mechanisms (33), neuropeptides (vasopressin) (34), hormones (androgen, estrogen, and oxytocin) (34;35), prostaglandins (36), and temperature (37;38). For different species, transit time through the epididymis takes around 12 days (2). In the rat, luminal fluid goes through the initial segment/caput in 2.1 days, the corpus in 0.8 day, and the cauda in 9.8 days (24). However, transit time for the human epididymis is much faster with only 2-4 days. This raises a question on the quality of spermatozoa produced by humans (32). In general, spermatozoa move faster through the proximal regions where fluid is non-viscous and slower in the distal regions where the luminal content is more viscous (9;24).

2.2.2. Spermatozoa maturation

As spermatozoa move down the epididymis, they acquire the potential for vigorous and forward motility as well as the ability to fertilize an oocyte (capacity to undergo the acrosome reaction, binding to and penetration of the zona pellucida, binding to and fusion with the zona-free vitellus, and syngamy) (4). This maturation process is active and occurs in multiple steps; the epididymis secrete specific proteins leading to morphological and biochemical changes in the spermatozoa (2;25). The site in the epididymis where spermatozoa acquire their fertilizing ability varies from species to species, but in general this ability is only gained after passage through the proximal epididymis (6). Changes allowing spermatozoon-oocyte fusion usually occur in the proximal regions, whereas changes allowing spermatozoon-zona binding occur in the distal regions (4). Morphological changes in spermatozoa include changes in the dimension and appearance of the acrosome and nucleus, chromatin condensation, migration and removal of the cytoplasmic droplet, and structural changes in intracellular organelles [reviewed in (39)]. The plasma membrane proteins of spermatozoa are also reorganized, modified (through phosphorylation, deglycosylation, and proteolytic processing), and renewed (25). In addition, the methylation status of some spermatogenesis-specific genes has been shown to be modified after

epididymal transit (40). The maturation of spermatozoa in the epididymis is regulated by 5α-dihydrotestosterone (DHT), a metabolite of testosterone (T) (41).

2.2.3. Spermatozoa storage

The cauda region is the major storage site of spermatozoa. In fact, 50% to 80% of spermatozoa present in the excurrent ducts are found in the cauda epididymidis. Spermatozoa can be stored for periods longer than 30 days and remain fertile (6). Compared to other mammals that can store three- to five-fold more spermatozoa in the cauda than the daily production, the human epididymis has a very limited storage capacity (6;42). To store spermatozoa, the cauda epididymidis needs to maintain them in a quiescent state. This is achieved through (i) the lowering of luminal sodium ion concentration thereby preventing proton efflux and a rise in intracellular pH that triggers motility; (ii) a high concentration of spermatozoa and secretion of a viscous mucoprotein (immobilin in rodents) that restrict movement; and (iii) the secretion of proteins preventing inappropriate acrosome reaction (4).

2.2.4. Spermatozoa protection

As stated in section 2.1.3., the blood-epididymis barrier protects spermatozoa from immune cells and some xenobiotics by preventing their access to the lumen. In addition, the epithelium secretes specific proteins that protect spermatozoa from microbes and radical oxygen species; spermatozoa are highly susceptible to oxidative damage. Antimicrobial defenses are ensured by defensins or defensin-like proteins. Protection from oxidative stress is achieved through the use of many antioxidant enzymes such as superoxide dismutase, γ-glutamyl transpeptidase, glutathione peroxidases, and glutathione transferases (6;9).

2.3. Organ and cell culture

The goal of i*n vitro* methods to study the functions and regulations of tissues is to permit the precise manipulation of the environment in which the tissue or cells reside. This would allow measurements of end points, for example

phosphorylation of proteins, that could not be done *in vivo*. Attempts at developing an *in vitro* culture system of epididymal cells have been under way since 1972 (43) and included the development of methods to culture epididymal tubules (organ culture) and primary epithelial cells (2). However, it is only in 2001 that the first immortalized epididymal cell line was generated (44).

2.3.1. Organ culture

Using organ culture to study the epididymis has two advantages. First, hormone-dependent tissues, such as the epididymis, retain their hormone responsiveness *in vitro,* thereby allowing for the study of the effects of a single hormone or several compounds on the epididymis. Second, the histological architecture is preserved permitting the maintenance of functions, such as sperm maturation, that would be lost in an isolated cell system (2). Using static and continuous flow organ culture, it has been shown that sperm maturation is dependent on DHT (45), and that the action of DHT is mediated through the synthesis of RNA and proteins (46), some of which are potentially important for sperm maturation (47-49). However, organ cultures can only be maintained for a few days, which limit their usefulness (2).

2.3.2. Primary epithelial cell culture

Primary cell cultures provide simplified model systems to obtain specific information on the activity and functions of individual cell types under defined conditions and possess a longer lifespan than organ cultures (2). In culture, epididymal cells flatten, form monolayers, and maintain some of their *in vivo* structural features such as surface microvilli, prominent Golgi apparatus, abundant rough and smooth endoplasmic reticula, lipid droplets, and multivesicular bodies (50-52). They also maintain some *in vivo* functions including ion secretion and reabsorption (53-55), testosterone metabolism (50;56), expression of epididymal genes (57;58), and protein secretion (59-62). Studies using primary epididymal cell cultures have allowed researchers to reach important conclusions such as the requirement of factors found in the rete testis

fluid to maintain the conversion of T to DHT (56). Nonetheless, primary cell cultures have limitations: they cannot be maintained indefinitely in culture, they divide very slowly, and they lose their differentiated phenotype after a few passages (51;56;63). In addition, results obtained with these cultures are variable and they are often contaminated with fibroblasts (2).

2.3.3. Immortalized cell lines

Unlike primary epithelial cell cultures, immortalized cell lines are homogeneous cell populations that will grow indefinitely in culture. Therefore, they are useful tools to generate reproducible results. Furthermore, they reduce the need for new material and hence the need to euthanize animals on a regular basis and the consequent costs (64). However, the different cell types forming the epididymal epithelium, the high proportion of connective tissue, the highly differentiated state, and the slow proliferation of the epididymal epithelium have rendered very challenging the immortalization of these cells (23;64;65). Most epididymal cell lines generated so far have used caput epididymides because it is the region with the most active protein secretion and hence is a good region to study epididymal functions (65). Nevertheless, immortalized epididymal cells are transformed and hence have lost the expression of certain epididymal markers and some of their differentiated state features.

Generation of immortalized epididymal cell lines has relied on spontaneous transformation or the use of the simian virus 40 large T-antigen (SV40LT) both *in vitro* and *in vivo* (64). The SV40LT is an immortalizing gene that can convert on its own primary cells into transformed cells (66). Although the exact mechanisms for immortalization and transformation by SV40LT are still unknown, it is established that SV40LT binds and inactivates p53 and retinoblastoma susceptibility protein (pRB), two tumor-suppressor genes, inhibiting apoptosis and allowing re-entry into the cell cycle, respectively (67;68). In addition, p53-independent pathways have been reported (67;68). Figure 3 illustrates the chronological development of immortalized epididymal cell lines (69).

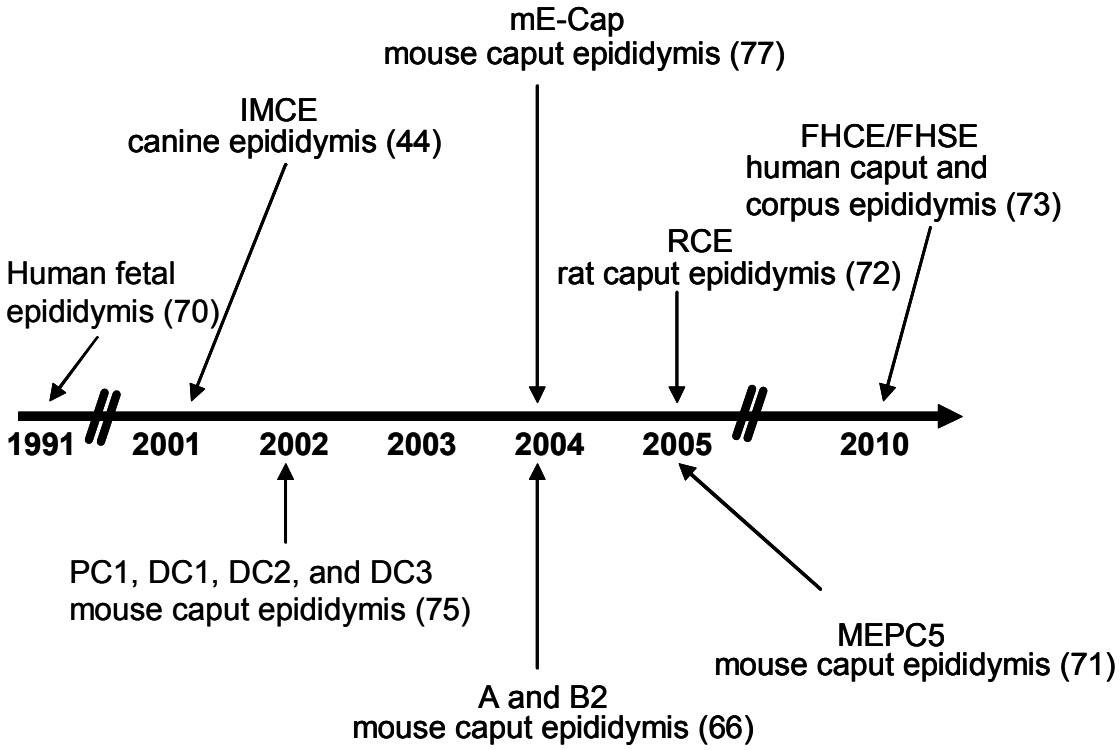

Figure 3: Chronological Order of the Development of Epididymal Epithelial Cell Lines

The immortalized epithelial cell lines of the epididymis are illustrated in the chronological order of their development. Adapted from reference (45).

2.3.3.1. Spontaneously immortalized cell lines

Spontaneously immortalized cell lines are derived from cells that were not immortalized using transforming oncogenes; they are selected for their ability to proliferate and maintain their phenotype in culture over long periods of time. Two spontaneously immortalized cell lines, cell lines A and B2, were generated using primary cultures of mouse caput epididymidis. These cell lines are composed of a homogeneous epithelial cell population, maintain some of the characteristic features of *in vivo* epididymal epithelial cells such as polarization, and express epididymis-specific genes. However, they are not androgen-responsive (65).

2.3.3.2. *In vitro* immortalization of epididymal epithelial cells

Epididymal cell lines have been generated by transfecting primary cultures of epithelial cells with an SV40LT plasmid. The first epididymal cell line originates from human fetal epididymis, but the cells lose their epididymal characteristics over time (70).

The first immortalized cell lines generated from a differentiated adult epididymis were obtained from the canine epididymis (IMCE) (44). They are all of epithelial origin, retain some epididymal-specific gene expression, and maintain the expression of the androgen receptor (AR) mRNA and protein. However, known androgen-regulated genes do not respond to androgen stimulation suggesting that the cells may have partially lost their differentiated phenotype (44;64).

The next two generated cell lines were derived from mouse (mouse epididymis caput epithelial cell line; MEPC5) (71) and rat (rat caput epididymal cell line; RCE) (72) epididymides. The MEPC5 cell line is a conditionally immortalized cell line established using the temperature-sensitive mutant of SV40LT (tsSV40LT) (71). This mutant contains a single nucleotide mutation, which produces a product that functions at the permissive temperature of 33^0C, but is rapidly degraded at the nonpermissive temperature of 39^0C. This allows to turn on or off cell proliferation by culturing cells at either 33^0C or 39^0C (64). The MEPC5 cells express some epididymis-specific genes and maintain polarity and

gap junctions (71). The RCE cell line is the only rat epididymal cell line available to date. These cells are mostly composed of principal cells with some clear cells. They retain many characteristics of cells *in vivo* such as polarity and expression of tight and adhering junctions. In addition, they express many epididymis-specific genes as well as the AR. However, they are not fully responsive to androgen stimulation (72).

Recently, human epididymal cell lines have been derived from one fertile (73) and one azoospermic (absence of spermatozoa in the ejaculate) (74) patient, by transforming cells from each with SV40LT. Using epididymal tissue from a fertile patient, Dube et al. (73) have developed four cell lines originating from the caput epididymidis (FHCE1-4 for fertile human caput epididymal cell line) and one originating from the corpus epididymidis (FHSE1 for fertile human corpus epididymal cell line); they have been unsuccessful at creating a cauda epididymal cell line. These cell lines comprise homogeneous cell populations of principal cells that have maintained characteristics of *in vivo* cells. In addition, they express mRNA and proteins of adhering and tight junctions. However, only three cell lines express the AR (FHCE1-3) and they have a very slow doubling time of 13 to 20 days (73). The five cell lines derived from the caput epididymidis of an azoospermic patient are called infertile human caput epididymal cell lines (IHCE1-5). They comprise homogeneous populations of epithelial cells that resemble structurally *in vivo* principal cells and express some epididymal markers and jucntional proteins. However, only IHCE1-2 express AR and they have a slow doubling time of 7 to 11 days (74).

2.3.3.3. *In vivo* immortalization of epididymal epithelial cells

Two different transgenic mouse models have been used to create immortalized epididymal cells. In the first approach, Araki et al. (75) have used transgenic mice constitutively expressing tsSV40LT (76). These mice do not express SV40LT at the nonpermissive temperature of the body; this prevents the formation of tumors. Immortalization is achieved when the cells are isolated and cultured at 33^0C (64;76). They have generated four stable cell lines from the

proximal caput (PC-1) and three cell lines from the distal caput (DC-1, DC-2, and DC-3) epididymides that maintain morphological characteristics of *in vivo* epithelial cells. These cells also express some epididymal-specific markers and are androgen-responsive (64;75). In the second approach, Sipila et al. (77) have used transgenic mice expressing SV40LT only in the caput epididymis through the use of a 5.0-kb mouse glutathione peroxidase 5 promoter (Gpx5-Tag) (78). They have generated eighteen epithelial cell lines, named mE-Cap11-28, that express some epididymal-specific genes. However, they express low levels of AR, and hence are not androgen-responsive (77).

2.4. Gene expression

The molecular mechanisms responsible for the creation of specific microenvironments along the epididymal duct have been investigated by many groups. These groups have looked at the tissue-, region-, and cell-specific gene expression patterns of the epididymis; the region-specific gene expression is a hallmark of the epididymis. These groups have looked at overall gene expression in the human (79-84), rat (85-89), mouse (88-93), and boar (94) epididymides and their regulation under different pathological (81) and experimental conditions (95-99). A few of the generated databases are available online:

- Mammalian Reproductive Genetics Database (mouse and rat transcriptomes): http://mrg.genetics.washington.edu/ (89;100)
- Mouse epdididymis transcriptome : http://www.wsu.edu/~griswold/microarray/epididymis_dht/ (99) and http://www.ttuhsc.edu/cbb/faculty/cornwall/Nelson%5Csupplemental%20data.html (90)
- Human epididymis transcriptome: http://www.scbit.org/human_epididymis_transcriptomes (82-84) and http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi (GSE7808) (80)

2.4.1. Tissue- and region-specific gene expressions

According to Turner et al. (101), there are, in the mouse epididymis, 307 genes that are considered epididymis-selective (mean expression of that gene in any region is at least three-fold higher than the mean expression in any other tissues) and 75 genes that are considered epididymis-specific (present in the epididymis but never detected in other tissues). Some epididymal specific genes include the antioxidant enzyme *glutathione peroxidase 5* (*Gpx5*) (102), the protease inhibitor *cystatin-related epididymal spermatogenic* (*cystatin 11*, *Cst11*) (103), the antimicrobial peptide *beta-defensin 11* (*Defb11*) (104), and the transporters *lipocalin 5* and *8* (*Lcn5, Lcn8*) (101;105) . In the human, the sperm protein P34H is exclusively expressed in the epididymis (106).

Most genes are more highly expressed or enriched in the proximal regions, the regions most active in terms of protein synthesis and secretion (2). In fact, Hsia and Cornwall (90) have identified 53 genes that are at least 2.5 times more highly expressed in the initial segment than in the other regions. They include the protease inhibitor *cystatin 12* (*Cst12*) (107), the transcription factor *ets variant gene 5* (*Etv5*) (108), the endopeptidase *cathepsin H* (*Ctsh*) (109), the antioxidant enzyme *microsomal glutathione S-transferase* (*Mgst1*) (110), and the tumor suppressors *armadillo repeat containing, X-linked 3* (*Armcx3*) (111) and *brain-expressed myelocytomatosis oncogene* (*Bmyc*) (112). In addition, Sipila et al. (93) have identified 235 genes only expressed in the initial segment, whereas Chauvin and Griswold (99) have identified 162, 55, and 133 genes enriched in the caput, corpus, and cauda, respectively. Hence, genes expressed in a region-specific manner belong to different gene families and include modifying enzymes [*α-mannosidase* (113)] and growth factors [*bone morphogenetic protein 8A* (*Bmp8a*) (114)]. They can also be intracellular proteins including transcription factors [*CCAAT/Enhancer binding protein* (*C/EBP*) (115)] and kinases [*A-raf serine threonine kinase* (116)]. Tissue- and region-specific gene expressions are reviewed in (117).

2.4.2. Cell-specific gene expression

In the epididymis, some genes are only expressed in a particular cell type. Principal cells exclusively express *a desintegrin and metalloprotease 7* (*Adam7*) (118), *low-density lipoprotein receptor-related protein 2* (*Lrp2*) (119), and *cadherin 1* (*Cdh1*) (120), whereas basal cells express *cyclo-oxygenase 1* (*Cox1*) (121) and *superoxide dismutase* (*Sod1*) (122); clear cells specifically express *alpha-mannosidase II* (*Man2*) (123). Furthermore, some of the genes are expressed in a "checkerboard-like" pattern where some principal cells intensely or faintly express the gene and others do not (124); these genes include *clusterin* (*Clu*) (125) and *phosphatidylethanolamine binding protein 1* (*Pbp1*) (126).

2.5. Protein expression

The epididymis secretes and absorbs proteins in a sequential and region-specific manner to create specific microenvironments for the maturation of spermatozoa. In humans, region-specificity is achieved by the modulation of protein secretion and not by the presence or absence of region-specific proteins (127). In order to identify the proteins responsible for the maturation process, proteomics studies have been done in different species including human (127), rat (128), mouse (129), rhesus monkey (130), boar (131), bull (132), stallion (131), dog (132), hamster (133), guinea pig (133), rabbit (133), sheep (131), and platypus (134). However, no more than 10% of the secreted proteins have been identified so far (131). Most of the proteins that enter the lumen of the epididymis from the rete testis are absorbed in the proximal caput epididymidis. Therefore, most of the proteins identified are secreted in the epididymis with the exception of some blood proteins (albumin and transferrrin) (131;133). In fact, secreted proteins represent 20% to 60% of total protein synthesis in the corpus-cauda and caput, respectively (133). In addition, new proteins are mostly synthesized in the proximal caput with, for example in the boar, 107 new secreted proteins in the caput, but only 13 and 5 new secreted proteins in the corpus and cauda epididymidis, respectively (133). Protein concentrations in epididymal fluid vary from region to region: from 2-4 mg/ml in initial segment, a maximum of 50-60

mg/ml in the distal caput, to 20-30 mg/ml in the cauda; these changes follow the decrease in luminal water content from proximal to distal epididymis (131). More than 60-80% of the total protein is composed of only 15-20 proteins, which include lactoferrin, procathepsin D, NCP2 (HE1, CTP, cholesterol transfer protein), glutathione peroxidase 5 (GPx5), beta-N-acetyl-hexominidase, mannosidase, galactosidase, prostaglandin D2 synthase (PGDS), clusterin, cystein-rich secretory protein (CRISP), and epididymal retinoic acid-binding protein (E-RAPB) (131) [each protein is reviewed in (133)]. Secreted proteins can be metabolic enzymes (lactate dehydrogenase (LDH), pyruvate kinase, enolase) or enzymes involved in protection against peroxidation (glutathione S-transferase P, thioredoxin peroxidase, and superoxide dismutase) (127). Although most of the secreted proteins are not epididymis-specific, their secreted isoforms are epididymis-specific due to their high degree of glycosylation and sulfation (133). The roles of the identified proteins in epididymal spermatozoa maturation are still unknown (132).

2.6. Diseases of the epididymis

2.6.1. Epididymitis

Epididymitis, an inflammation of the epididymis, is the most common pathology of the epididymis and affects sexually active men. It is mostly caused by infection with *Chlamydia trachomatis*, *E. coli* or *Neisseria gonorrhoeae* (135).

2.6.2. Cancer

Primary tumors of the epididymis are extremely rare, less than 40 cases have been reported in the literature since 1916. Most epididymal tumors are benign; there are only 24 reported cases of malignant tumors and they include both primary and metastatic tumors (136;137).

3. Regulation of epididymal functions

The epididymis depends on hormones, vitamins, testicular factors, and growth factors for the regulation of its structure, functions, and gene and protein expressions.

3.1. Hormones and vitamins

Androgens are steroid hormones that are the primary regulators of epididymal functions and hence will be dealt with in greater details in section 4. Estrogens are also steroid hormones that play a role in the epididymis (6). The role of other steroid hormones (glucorticoids, mineralocorticoids, and progestagens) are still poorly understood (138). In addition, other hormones such as oxytocin and thyroid hormones as well as vitamins such as retinoids (vitamin A) play important roles in the epididymis (6).

3.1.1. Estrogens

The main estrogen in men is estradiol that arises from the irreversible conversion of testosterone by cytochrome P450 aromatase (139). In the epididymis, conversion of testosterone to estradiol can occur in the lumen and/or epithelium. In the lumen, cytochrome P450 aromatase activity is found in spermatozoa (6), whereas in the epithelium, cytochrome P450 aromatase activity is found in principal cells (140;141). In the mouse, estradiol has been reported to increase the rate of spermatozoa transport through the epididymis (35). This process is mediated by the activation of the RhoA/ROCK (Rho-associated coiled-coil forming kinase 1) signaling pathway that increases calcium sensitivity of the contractile apparatus independently of intracellular calcium levels (142).

Estrogens act through the estrogen receptor (ER), which exists in two isoforms (α and β). ERα and β are functionally distinct receptors that have specific ligand binding domains (143). Expression of ERs in the epididymis is developmentally-regulated (144) and there are cellular and regional differences in their expressions. In fact, in the initial segment, narrow, apical, and some basal cells express ERα; in the caput, principal and clear cells stain positively, whereas

in the distal regions, only clear cells express it. In contrast, ERβ is present in the entire epididymis with stronger staining observed in the distal regions (6). The importance of ERα in epididymal function has been demonstrated using the ERα knockout mouse, which is infertile. The infertility is due to back-pressure atrophy of the seminiferous tubules caused by the inability of the efferent ducts and initial segment to reabsorb the large volume of fluid secreted by the testis. Hence, estrogens control fluid reabsorption in the initial segment of the epididymis (145). In addition, ERα knockout mice produce abnormal spermatozoa due to the exposure of spermatozoa to an epididymal luminal environment with increased pH and decreased osmolality (146;147). The role of ERβ in epididymal function is still unknown because the ERβ knockout mouse is fertile and has normal testes and epididymides (148).

In the epididymis, estradiol controls expression of genes involved in apoptosis [*nerve growth factor receptor* (*Ngfr*)], calcium ion binding [*S100 calcium binding protein G* (*S100g*)], transport [*albumin* (*Alb*) and *rhesus blood group-associated C glycoprotein* (*Rhcg*)] (149), and solute transport [*cystic fibrosis transmembrane regulator homolog* (*Cftr*) and *solute carrier 26, member 3* (*Slc26a3*)] (150).

3.1.2. Oxytocin

Oxytocin is a neurohypophysial hormone secreted by the hypothalamus and stored in the posterior pituitary until its release into the circulation. Traditionally considered a "female hormone" because of its role in parturition and milk ejection, oxytocin plays important roles in the epididymis (151). Oxytocin receptors localize to peritubular cells as well as to principal and basal cells in a region- and species-specific manner (6). Oxytocin plays two important roles in the epididymis. First, it stimulates basal contractility of the duct therefore promoting transport of spermatozoa through the epididymis (152). This activity is regulated in part by estrogens that increase gene and protein expressions of the oxytocin receptor (153). Second, oxytocin promotes formation of DHT by stimulating 5α-reductase activity in the initial segment (154); 5α-reductase (Srd5a) converts T to

DHT and exists as two isoforms, Srd5a type 1 and type 2 (6). The mechanism underlying the stimulation of Srd5a activity by oxytocin is still unknown, but is believed to involve phosphorylation of the enzyme by a tyrosine kinase (155).

3.1.3. Thyroid hormones

Thyroid hormones, the pro-hormone thyroxine (T4) and the active hormone 3,5,5'-triiodothyronine (T3), produced by the thyroid gland, are essential for normal development, growth, and metabolism (156). They also play crucial roles in sexual maturation and reproductive function (157). Thyroid hormones have been shown to affect the epididymis. In fact, hyperthyroidism changes the activity of different glycosidases (158). On the other hand, hypothyroidism causes morphological changes in the caput and cauda epididymidis with a decrease in the number of epithelial cells (159) and an increased expression of thyroid hormone receptor α1 ($TR_{\alpha1}$) and $TR_{\beta1}$ at the mRNA and protein levels (160). In addition, sperm recovered from the cauda of hypothyroid rats are less motile (161). Gestational-onset hypothyroidism causes decreased secretory activity of the epididymis, decreased Srd5a activity and decreased expression of the androgen receptor (AR) protein (162). The epididymis also expresses the highest level of type I deioidinase of all male reproductive tissues; its activity and mRNA expression are regulated by estradiol (163).

3.1.4. Retinoids

Retinoids (vitamin A) are highly potent molecules that control a wide range of biological processes during development and in the adult (164). Due to their hydrophobic nature, retinoids are bound to chaperones to ensure proper storage, transport, and uptake by tissues. There are two extracellular retinoid-binding proteins (RBP) [retinol-binding proteins and epididymal retinoic acid-binding protein (E-RABP)] and four intracellular RBPs [cellular retinol-binding protein (CRBP) I and II, and cellular retinoic acid-binding protein (CRABP) I and II]. Retinoids act by binding to two classes of nuclear retinoic acid receptors, RAR and RXR, each of which consists of three receptor subtypes α, β, and γ.

RARs and RXRs form heterodimers that bind retinoic acid response elements in the promoter region of target genes (165).

Most components of the retinoid signaling pathway have been identified in the epididymis. In fact, the epididymis expresses retinol, retinyl ester, all-*trans* retinoic acid, and 9-*cis*-retinoic acid (9-*cis*-RA) in a region-specific manner. In addition, the epididymis expresses RARα, β, and γ, as well as CRBPs, CRABPs, and E-RABP (6). The importance of retinoids in the maintenance of epididymal structure can be seen through vitamin A deficiency and RARα and/or γ knockout mice. Indeed, vitamin A deficiency causes benign changes in the epithelial lining (squamous metaplasia) of the epididymis (6). In addition, RARα knockout mice show loss of organization of the columnar epithelium lining of the cauda, vacuolization, and transformation by squamous metaplasia. This causes blockage or rupture of the duct, inflammation, and infertility (166;167). The RARα/γ double-null mutants show severe abnormality in development (dysplasia) or complete failure of development during embryonic growth (agenesis) (168).

3.2. Testicular factors

Epididymal functions are also regulated by factors coming from the testis through the lumen. These testicular factors regulate gene expression of the epididymis in a paracrine manner termed “lumicrine” because it occurs in a duct/tubal system (169). Lumicrine regulation occurs not only between the testis and epididymis, but also between epididymal regions. The initial segment is the region most sensitive to the regulation by testicular factors, but there could be more distal effects that need to be investigated. In fact, testicular factors are important for maintaining initial segment morphology (170), region-specific gene expression (117), as well as protein synthesis and secretion (171). Although the identity of most testicular factors is still unknown, candidates include steroids such as androgens and estrogens and nonsteroidal proteins such as the androgen binding protein (ABP), the basic fibroblast growth factor (FGF2), and spermatozoa or spermatozoa-associated factors (6).

The ABP, as its name implies, acts as a carrier of T (6). It is synthesized by Sertoli cells of the testis and more than 80% of it reaches the epididymis (172). Principal cells also synthesize and secrete ABP (173). Where needed, T is released from ABP and converted to the more potent DHT by Srd5a (6). ABP has also been suggested to regulate Srd5a (174), to play a role in protein synthesis in the caput (171), and to enhance the conversion of T to DHT in cultures of epididymal principal cells (174).

FGFs play a role in mitogenesis, differentiation, migration, cell survival, and male reproduction, acting in a paracrine or endocrine manner (175). FGFs activate cell membrane FGF receptors (FGFRs) with tyrosine kinase activity leading to the activation of the extracellular signal-regulated kinases (ERKs). ERKs, in turn, phosphorylate downstream targets to mediate specific cell responses (176). Although three soluble FGFs (2, 4, and 8) have been identified in testicular luminal fluid, FGF2 is the only one with a functional role in the epididymis; it regulates the mRNA expression of *γ-glutamyl transpeptidase IV*, a gene highly expressed in the initial segment (6). Gene expression analysis has identified *Fgfr1-4* and *Fgf1, 2, 7, 9* and *12* as being expressed in a region-specific manner in the epididymis (176;177). In addition, FGFR-1 has been localized to principal cells in the initial segment (178).

The presence of spermatozoa has been postulated to regulate initial segment function, not by themselves, but through the ligands they might carry (179). In fact, testicular spermatozoa express growth factor (GF) receptors (180). It is possible that once in the initial segment, these GFs dissociate from the sperm surface and become available to bind their receptors on epididymal cell surfaces (6).

4. Androgens

Androgens are nonaromatized C19 steroids that play important roles in different physiological processes (181). The two main androgens that act on male reproductive tissues are T and the more potent DHT (182). In males, the Leydig cells of the testis produce more than 95% of total circulating T, the remainder being produced by the cortical cells of the adrenal glands (183;184).

4.1. Steroidogenesis, metabolism, and regulation

This section provides a brief overview of the production, secretion, and metabolism of T, as well as the regulation of T biosynthesis by the hypothalamus-pituitary axis.

4.1.1. Steroidogenesis in Leydig cells

Cholesterol is the precursor of androgens and other steroids. In Leydig cells, cholesterol either enters the cells from plasma carried by low-density lipoprotein (LDL) or high-density lipoprotein (HDL), or is synthesized *de novo* from lipid droplets (185). Once the Leydig cells are stimulated by LH to synthesize T, cholesterol is transported to the mitochondria where it is converted to pregnenolone by the cytochrome P450 enzyme cholesterol side-chain cleavage (P450scc), which is the rate-limiting step in the biosynthesis of T. Pregnenolone then diffuses across the mitochondria membrane to the smooth endoplasmic reticulum where it is converted to T following a series of enzymatic reactions catalyzed by 17α-hydroxylase cytochrome P450 (P45017α), 3β-hydroxysteroid dehydrogenase (3β-HSD), and 17 β-hydroxysteroid dehydrogenase (17β-HSD) (186;187) (Fig. 4).

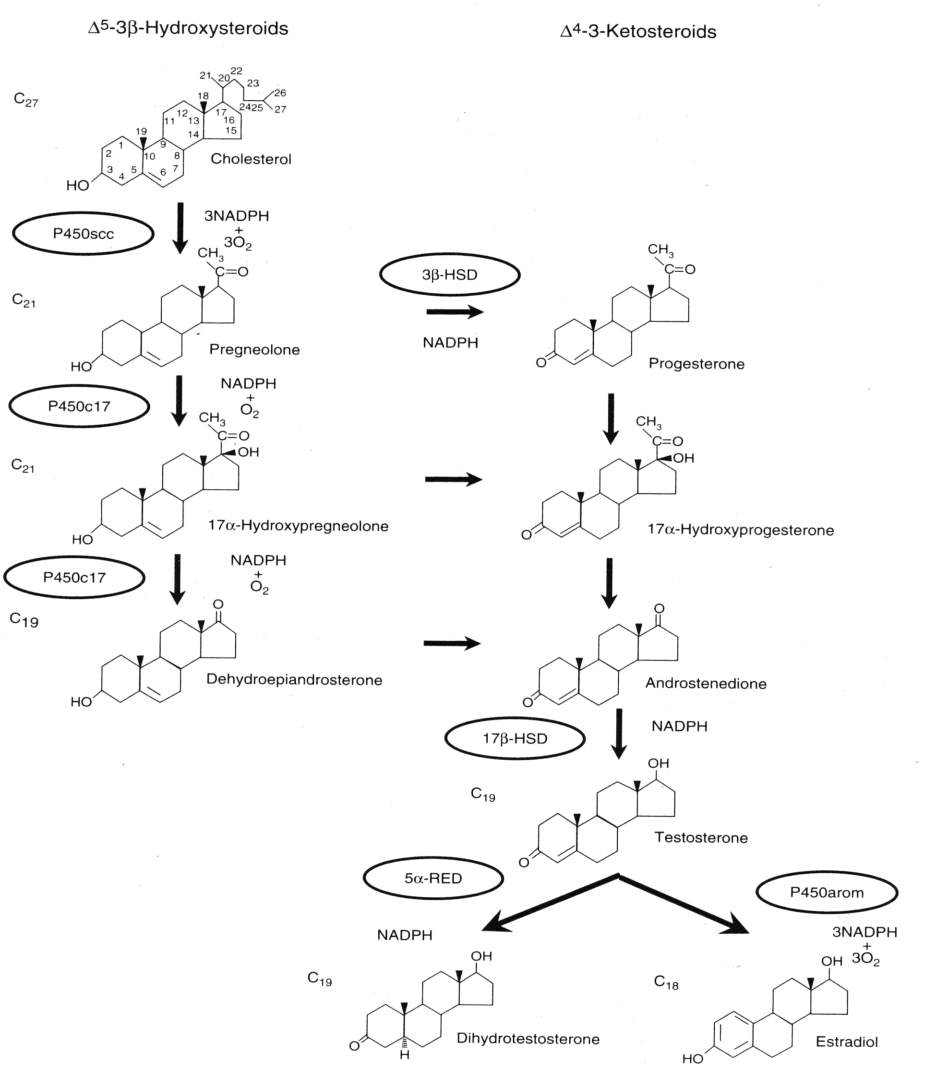

Figure 4: Steroid Biosynthetic Pathways in Leydig Cells

Testosterone is synthesized from cholesterol in Leydig cells through a series of enzymatic reactions. The designations Δ4 and Δ5 refer to the localization of the double bond in the steroid. P450scc is localized in the mitochondria, whereas all the other enzymes are localized in the smooth endoplasmic reticulum. The abbdreviations are as follows: P450scc: cytochrome P450 enzyme cholesterol side-chain cleavage; P450c17: 17α-hydroxylase cytochrome P450; 3β-HSD: 3β-hydroxysteroid dehydrogenase; 17β-HSD: 17 β-hydroxysteroid dehydrogenase; 5α-RED: 5α-reductase; P450arom: cytochrome P450 aromatase; NADPH: nicotinamide adenine dinucleotide phosphate.

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4.1.2. Secretion, transport, and metabolism

After synthesis, T leaves the Leydig cells by passive diffusion. In blood, only 2% of total T circulates freely, the majority is bound to albumin or a carrier protein called sex hormone-binding globulin (SHBG) or testosterone-estradiol-binding globulin (TEBG) (184;186). SHBG is a glycoprotein that shares the same amino acid sequence with ABP, the only difference being the types of oligosaccharides associated with them (188). Although albumin has a low binding affinity for T, the high concentration of albumin, as compared to SHBG, in blood results in the approximate same proportion of T bound to albumin and SHBG. Once it reaches its target tissue, T dissociates from albumin and enters the cell by diffusion where it can exert its biological effects. Testosterone can directly mediate its effects or be converted to its more potent metabolite DHT by Srd5a or to estradiol by cytochrome P450 aromatase, thereby triggering different biological responses (184) (Fig. 5). If T does not reach its target tissue, it is metabolized in the liver through oxidation by 17β-HSDs, reduction through 3α-HSDs, followed by glucuronidation and renal excretion (183) (Fig. 6).

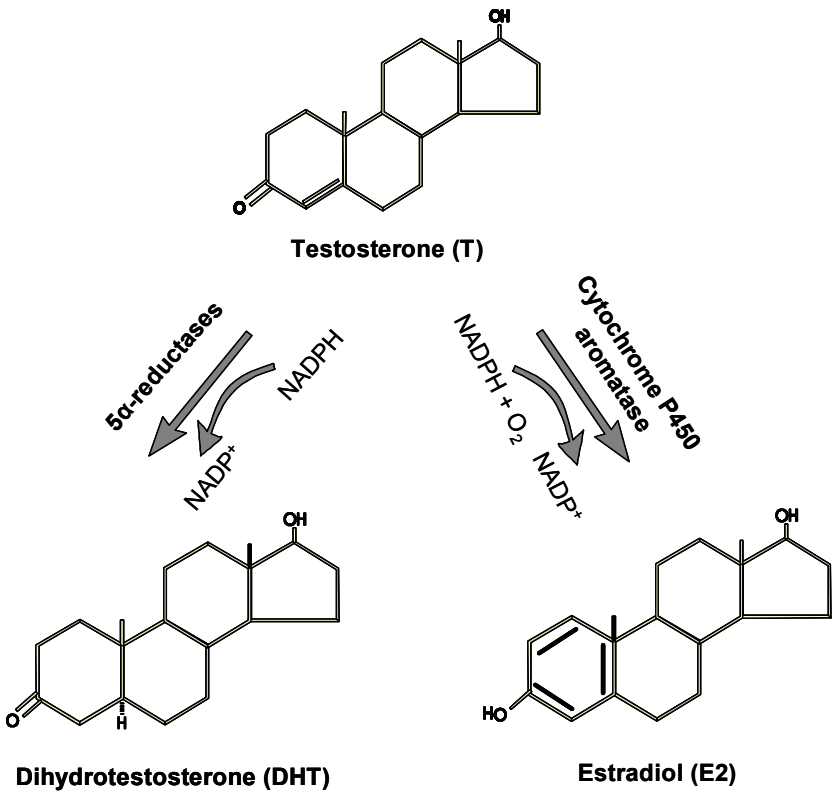

Figure 5: Active Metabolites of Testosterone

Testosterone (T) can be reduced to dihydrotesterone (DHT) or aromatized to estradiol (E2) in peripheral tissues.

Adapted from reference (45).

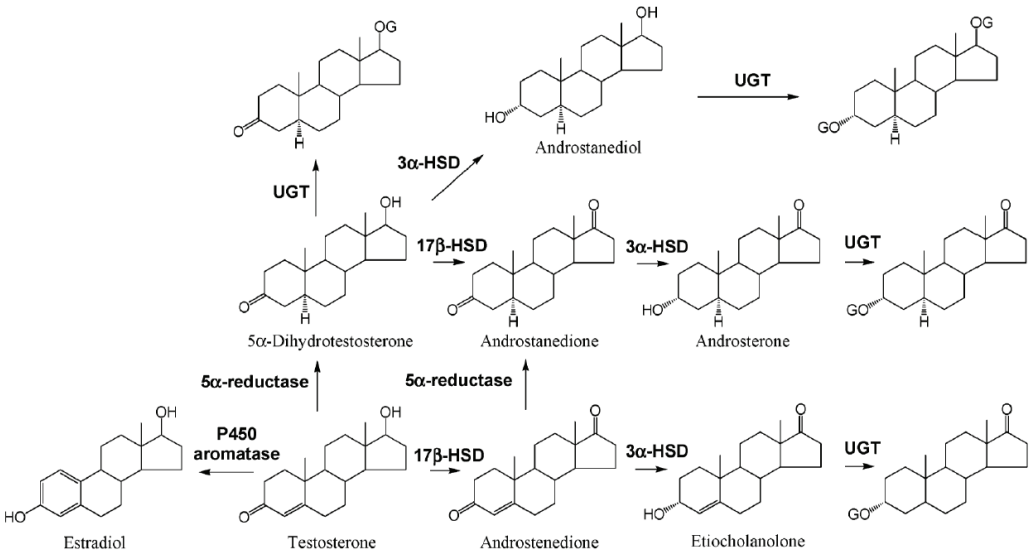

Figure 6: Testosterone Metabolism

In target tissues, testosterone can be irreversibly converted to dihydrotestosterone or estradiol. Testosterone or dihydrotestosterone can be metabolized through oxidation by 17β-HSDs, reduction through 3α-HSDs, followed by glucuronidation and renal excretion. Abbreviations are as follow: 3α-HSD: 3α-hydroxysteroid dehydrogenase; 17β-HSD: 17 β-hydroxysteroid dehydrogenase; G: glucuronide; HSD: hydroxyl steroid dehydrogenase; UGT: UDP-glucuronosyltransferase.

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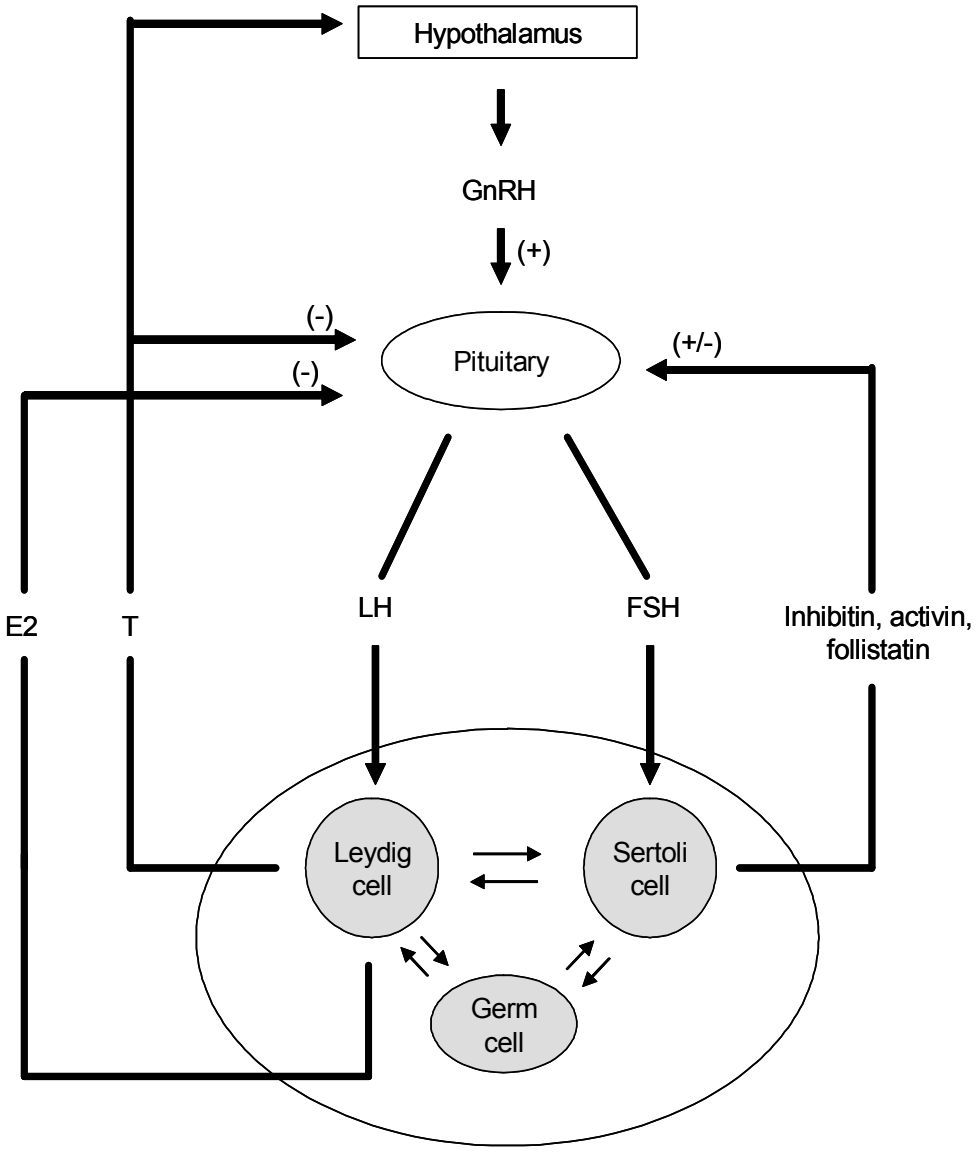
4.1.3. The hypothalamus-pituitary axis

In males, steroidogenesis is controlled by luteinizing hormone (LH), whereas spermatogenesis is under the control of both T and follicle-stimulating hormone (FSH) (189). The relative role of FSH in the regulation of spermatogenesis is beyond the scope of this section but is reviewed in references (190-192). The gonadotropins LH and FSH are produced and secreted by the anterior pituitary in response to stimulation by hypothalamic gonadotropin-releasing hormone (GnRH). Secretion of GnRH is pulsatile and hence gonadotropin release also occurs in pulses resulting in a series of peaks and troughs in the circulation (183). LH binds to LH receptors present on the surface of Leydig cells, thereby activating adenylate cyclase and stimulating production of cyclic adenosine monophosphate (cAMP) that activates protein kinases. This in turn stimulates the transport of cholesterol to the mitochondria to initiate T production. LH is also necessary to maintain the expression of the T biosynthetic enzymes. Once T is secreted, LH receptors are internalized and degraded (186;187).

Regulation of T production occurs through a negative feedback mechanism. Secreted T inhibits GnRH release from the hypothalamus, as well as LH and FSH release from the pituitary. Leydig cells also produce E2 that inhibits LH stimulation of T biosynthesis. This regulation occurs through AR and ER present in the hypothalamus and the pituitary. Sertoli cells also regulate FSH release by secreting inhibins, activins, and follistatin (193) (Fig. 7).

Figure 7: Feedback Mechanisms in the Hypothalamus-Pituitary-Testis Axis. The production of testosterone is regulated by the hypothalamus and pituitary. (+) indicates positive feedback, and (-) indicates negative feedback. Abbreviations are as follows: GnRH: gonadotropin-releasing hormone; LH: luteinizing hormone; FSH: follicle-stimulating hormone; T: testosterone; E2: estradiol.
Reproduced from reference (45).


Hypothalamus
GnRH
(+)
(-)
(-)
Pituitary
(+/-)
E2
T
LH
FSH
Inhibitin, activin, follistatin
Leydig cell
Sertoli cell
Germ cell

4.2. Mechanisms of androgen action

Androgens predominantly act by binding to AR leading to the transcription of target genes. There is also increasing evidence that androgens can have rapid nongenomic effects.

4.2.1. Androgen receptor (AR)

The AR belongs to the steroid and nuclear receptor superfamily that are ligand-inducible transcription factors (194). Located on the X chromosome, the AR gene encodes a multidomain receptor that includes an N-terminal regulatory domain (NTD), a DNA-binding domain (DBD), a small hinge region (H), and a ligand-binding domain (LBD) (194;195). The NTD mediates the majority of AR transcriptional activity and AR binding to co-regulators. Co-regulators affect ligand selectivity and DNA-binding capacity of AR in a positive (co-activators) or negative (co-repressors) manner (194). Co-activators and co-repressors are reviewed in (196) and (197), respectively. More than 70 AR co-regulators have been identified (198). The DBD is not only responsible for the binding of AR to specific androgen response elements (AREs) in the promoter of target genes, but also for the dimerization between AR monomers (194). The H region contains the nuclear localization signal and sites for phosphorylation, acetylation, and degradation, whereas the LBD mediates high affinity binding to androgenic ligands (194). Mutations in AR cause a spectrum of disorders of androgen insensitivity syndrome, prostate cancer, and feminization [reviewed in (198)].

4.2.2. Genomic androgen action

In target cells, the inactive AR is in the cytoplasm where it is bound by heat shock proteins (hsps) that maintain it in a hormone-binding state (199). Upon binding of T or DHT, AR goes through a series of conformational changes that include dissociation of hsps, homodimerization, phosphorylation, and translocation to the nucleus through the NLS (183). DHT has a two- to three-fold higher affinity for AR and a five-fold slower dissociation rate than T making it a more potent ligand than T (200). Once in the nucleus, AR binds to the AREs,

which are found as direct (5'-TGTTCTNNNTGTTCT-3') and inverted (5'-GGTACANNNTGTTCT-3') repeats. While the direct repeat is specific for the AR, the inverted repeat can be recognized by glucocorticoid, mineralocorticoid, and progesterone receptors (201). AR then recruits other transcription co-regulators that can (i) directly regulate transcription through physical interaction with general transcription factors and RNA polymerase II; (ii) covalently modify histone tails; and (iii) remodel the chromatin structure (194). Binding of T and DHT to the AR elicits the transcription of different sets of genes (202). Physiological effects of genomic androgen action take place 6h to 24h after stimulation because they require *de novo* protein synthesis (203) (Fig. 8).

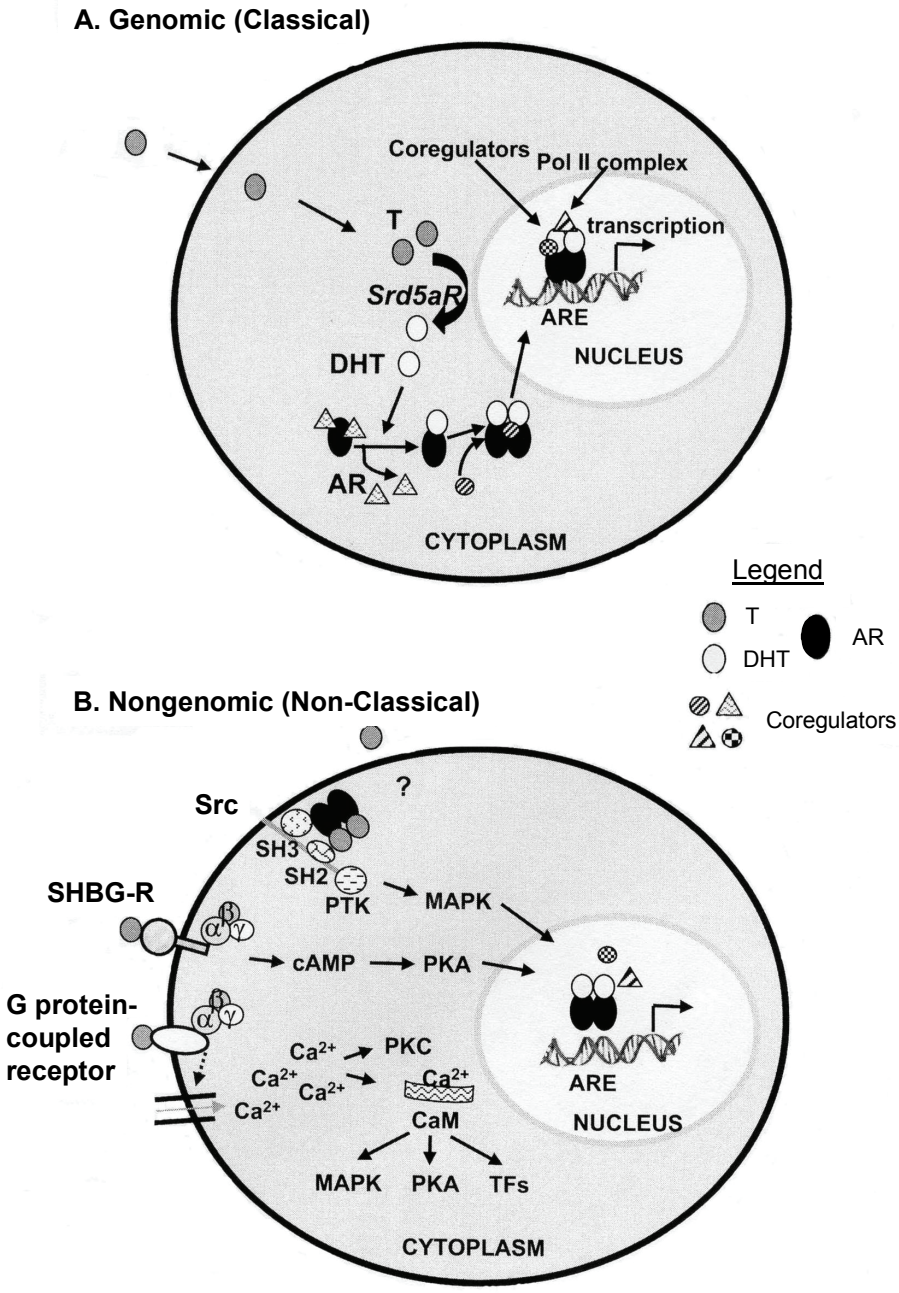
4.2.3. Non-genomic androgen action

Androgens can also trigger rapid responses, within seconds or minutes, that occur too quickly to be accounted for by effects on transcription (204). For example, application of T to a rat hypothalamus triggers neuron firing within seconds (205), whereas application of T to rat anterior pituitary tissues stimulates release of prolactin within 5 min (206). These effects are not prevented by inhibiting transcription or translation (204). They occur through the rapid induction of signaling cascades that include increase in free intracellular calcium as well as activation of protein kinase A (PKA), protein kinase C (PKC), and mitogen-activated protein kinases (MAPKs). These effects appear to be mediated by membrane-bound receptors, although no membrane-associated AR has been isolated so far (207;208). In addition, T can induce cAMP and PKA by binding to SHBG associated to its receptor on the cell surface; SHBG normally binds T in the circulation (reviewed in section 4.1.2.). The induction of these signaling cascades may ultimately regulate the transcriptional activity of AR or other transcription factors. T and DHT can also influence membrane fluidity by interacting with membrane phospholipids (208) (Fig. 8).

Figure 8: Mechanisms of Androgen Action

Androgens predominantly act by binding to AR leading to the transcription of target genes, a mode of action referred to as genomic (classical) (A). They can also trigger rapid responses through the activation of second messengers in a nongenomic (non-classical) manner (B). Nongenomic modes of action can occur through stimulation of intracellular tyrosine kinase c-steroid receptor co-activator (Src) and sex hormone binding globulin receptor (SHBG-R) leading to the activation of protein kinase A (PKA) and mitogen-activated protein kinases (MAPKs). In addition, androgens may bind a plasma membrane G protein-coupled receptor or an uncharacterized membrane-bound AR causing an increase in free intracellular calcium and activation of protein kinase C (PKC), PKA, and MAPKs. The induction of these signaling cascades may ultimately regulate the transcriptional activity of AR or other transcription factors. Abbreviations are as follows: ARE: androgen response element; cAMP: cyclic AMP; CaM: calmodulin; Pol II complex: RNA polymerase II complex; PTK: phosphotyrosine kinase; SH2: Src homology 2 domain; SH3: Src homology 3 domain; Srd5aR: steroid 5α-reductase; T: testosterone; DHT: dihydrotestosterone; TFs: transcription factors.

Adapted from reference (208).


A. Genomic (Classical)
Coregulators
Pol II complex
transcription
T
Srd5aR
ARE
NUCLEUS
DHT
AR
CYTOPLASM
Legend
T
AR
DHT
Coregulators
B. Nongenomic (Non-Classical)
?
Src
SH3
SH2
PTK
MAPK
SHBG-R
cAMP
PKA
G protein-coupled receptor
Ca2+
PKC
Ca2+
Ca2+
Ca2+
Ca2+
ARE
NUCLEUS
CaM
MAPK
PKA
TFs
CYTOPLASM

4.3. Androgen action in peripheral tissues

In the male, T plays crucial roles in sexual differentiation *in utero* and in the development of secondary sexual characteristics at puberty (186). In the adult, T maintains secondary sex organs and regulates male sexual behavior and spermatogenesis. It also acts on muscle to maintain muscle mass and strength as well as on bone to regulate bone mineral density. In addition, T acts on the central nervous system and cardiovascular system. The tissue-specific effects of androgens can be attributed to the expression of specific co-regulators as well as the presence of metabolizing enzymes. As mentioned previously in section 4.2.1, co-regulators affect ligand selectivity and DNA-binding capacity of AR in a positive (co-activators) or negative (co-repressors) manner (194). Metabolizing enzymes determine not only which androgen acts on the target tissue but also the duration of the response. In the muscle, where Srd5a activity is low, T directly acts on the AR, whereas in some brain nuclei, T is aromatized to estradiol to exert its effects. In addition, in tissues such as the epididymis, prostate, seminal vesicles, and skin, T is converted to DHT by Srd5a (6). Enzymes such as 17β-HSD2 and 3α-HSDs regulate intracellular androgen concentration and activation of AR; 17β-HSD2 inactivates T, DHT, and estradiol, whereas 3α-HSDs specifically inactivate DHT (184).

4.4. Androgen action in the epididymis

Although few studies have focused specifically on the mechanisms of androgen action in the epididymis, it is highly probable that many of the characteristics and modes of action of AR resemble those in other tissues, while some are likely to be specific to the epididymis (6). In the epididymis, AR is expressed in a cell-specific manner throughout the duct with a slight decline in mRNA and protein expressions from caput to cauda (209;210).

Androgens, in particular T and DHT, regulate epididymal structure, gene expression, and survival. In order to study androgen action in the epididymis, different approaches have been used that include treatment with AR antagonists to inhibit androgen action (211-213), treatment with SR inhibitors to distinguish the

effects of T from DHT (214;215), treatment with GnRH antagonists to inhibit T biosynthesis (216;217), removal of both testes by bilateral orchidectomy (26;85;218-222), and efferent duct ligation (219;223-226). Bilateral orchidectomy causes not only a loss of androgens, but also of testicular factors, whereas efferent duct ligation only removes testicular factors and maintains serum T. In order to assess the direct effects of T and DHT on epididymal functions, T replacement is often used (227-230).

4.4.1. Structure

After orchidectomy, epididymal weight decreases to 25% of control over a two week period, followed by a further 5% in the subsequent two weeks. This decrease in weight is not as marked as other reproductive tissues such as the prostate; prostate weight decreases to 10% of control by four weeks after orchidectomy. Unlike other reproductive tissues, T replacement, even at supraphysiological levels, restores epididymal weight to only 50% of control. This partial rescue is due to the loss of luminal fluid and spermatozoa that, in the rat, make up half of the epididymal weight (231;232). Orchidectomy also causes major morphological changes to the epididymal epithelium that include a decrease in luminal diameter and epithelial cell height as well as an increase in intertubular stroma (219). Principal cells are the most affected cells after orchidectomy indicating that they are particularly sensitive to androgen levels (26). In an androgen-deprived state, principal cells have compromised secretory function marked by the disappearance of the endoplasmic reticulum and of vesicles from the cell apex; they also undergo loss of apical microvilli from their surface, vacuolization, lysosome accumulation, and increased endocytosis (26;222;233). In that state, AR activity is decreased, while Srd5a activity becomes undetectable, suggesting that androgen action is compromised (226;231). In addition, after orchidectomy, total epididymal protein, RNA, and DNA content are decreased, whereas DNA concentration is increased (234). DNA concentration increases because there is a decrease in cell volume, which is the principal mechanism by which the epithelium regresses after orchidectomy (6). Restoration of T to serum

T levels reverses regressive changes in the caput, corpus, and cauda epididymides observed after orchidectomy, but not in the initial segment, even when supraphysiological levels are administered (231).

In the adult rat epididymis, the mitotic rate is not affected by the constant androgen stimulation as opposed to other androgen-dependent tissues such as the prostate and seminal vesicles (23;235). This may be due to the presence of antiproliferative signals that prevent cellular proliferation in response to androgen stimulation. In fact, the proximal caput highly expresses a potential antiproliferative protein B-myc. B-myc is a transcription factor that inhibits cellular proliferation; its expression depends on androgens and testicular factors (112;236). However, in the regressed rat epididymis, T supplementation increases mitotic rate in all epididymal regions (237).

4.4.2. Gene expression

Androgens regulate many epididymal functions that include intermediate metabolism, ion transport, synthesis and secretion of many epididymal proteins, as well as the activity of some enzymes. Androgens also regulate transport, maturation, and storage of spermatozoa (6). This dependence on androgens for epididymal functions is associated with the control of gene transcription by androgens. Although many transcripts have been shown to be androgen-regulated (89;99;150), only a few have been shown to contain functional AREs in their promoter region. These genes include *Gpx5* (238;239), *lipocalin 5* (240), *reproductive homeobox 5* (241), and *Crisp1* (242). Some genes such as *Ar* (243), *Gpx3* (244), and *carbonic anhydrase IV* (245) are regulated by androgens. Genes having a level of expression that does not return to control levels after T replacement are regulated by testicular factors. These genes include *γ-glutamyl transpeptidase* (*GGT*) (246), *polyomavirus enhancer activator 3* (*PEA3*) (247), *Gpx5* (248), and *Srd5a* (249).

Many groups have used gene array technology to identify androgen-regulated genes in the epididymis (85;89;99;150;250;251). These studies have allowed not only to confirm the androgen-dependence of previously characterized

genes, but also to identify novel genes and overall patterns of gene expression after orchidectomy. They have shown that genes belonging to different functional families are regulated by androgens. These functional families include solute carrier family (*Slc1a5*, *Slc12a3*, *Slc15a2*, *Slc22a5*, and *Slc9a2*), heat shock proteins [*Hsp47*, *Hsp27, glucose-regulated protein 97* and *78* (*Grp97* and *Grp78*], gap junction proteins (*Gja1*, *Gja4*, and *Gjb3*), proteins regulating cell growth, cell proliferation, and apoptosis [*aryl hydrocarbon receptor* (*Ahr*), *plasminogen activator urokinase* (*Plau*), *platelet-derived growth factor, C polypeptide* (*Pdgfc*), and *c-fos induced growth factor* (*Figf*)], proteins involved in signal pathway and signal transduction [*receptor (G protein-coupled) activity modifying protein 3* (*Ramp3*), *calcitonin receptor-like* (*Calcrl*), and *inositol 1,4,5-triphosphate receptor 3* (*Itpr3*)], proteolytic and peptidolytic enzymes [*Adam7*, *Adam9*, and cathepsin C (*Ctsc*)], and proteins regulating development [*actin, alpha 1, skeletal muscle* (*Acta1*), *growth arrest-specific 7* (*Gas7*), and *protein phosphatase 2, regulatory subunit B, beta isoform* (*Pppr2b2*)] (85;150;251). In addition, in the PC-1 mouse epididymis cell line, genes involved in apoptosis [*thymoma viral protooncogene 1* (*Akt1*) and *caspase 1* (*Casp1*)], cell adhesion [*cadherin 2* (*Cdh2*) and *laminin, α5* (*Lama5*)], cell signaling [*fibroblast growth factor receptor 1* (*Fgfr1*) and *integrin linked kinase* (*Ilk*)], cell cycle [*cyclin A2* (*Ccna2*) and *jun protooncogene-related gene d1* (*Jund1*)], cell proliferation [*insulin-like growth factor binding protein 2* (*Igfbp2*)], DNA repair [*RAD21 homolog* (*Rad21*) and *RAD23b homolog* (*Rad23b*)], metabolism [*glyceraldehyde-3-phosphate dehydrogenase* (*Gapdh*) and *tissue inhibitor of metalloproteinase 2* (*Timp2*)], enzyme activity [*3-phosphoglycerate dehydrogenase* (*Phgdh*)], protein folding [*serine (or cysteine) peptidase inhibitor, clade H, member 1* (*Serpinh1*)], mRNA processing [*THO complex 4* (*Thoc4*)], transcription [*transcription termination factor 1* (*Ttf1*) and *activating transcription factor 4* (*Atf4*)], and protein transport [*RAB2, member RAS oncogene family* (*Rab2*)], have been shown to be androgen-regulated (250).

5. Apoptosis and cell survival

Apoptosis or programmed cell death is a locally and temporally defined process of self-destruction. The term apoptosis comes from the Greek “falling of leaves from a tree in the fall” and refers to the life and death cycle of life (252). Apoptosis plays an important role in development and morphogenesis to control cell number and to remove damaged, infected or mutated cells (253). Many diseases are correlated with misregulated apoptotis. In fact, too much apoptosis is associated with neurodegenerative diseases such as Parkinson’s and Alzheimer’s diseases, spinal muscular atrophy, and AIDS, whereas too little apoptosis is observed in cancer or autoimmune diseases such as diabetes type I (252).

Apoptosis is initiated by specific signals and characterized by chromatin condensation, nuclear fragmentation, cytoplasmic shrinkage, membrane blebbing and formation of apoptotic bodies. The latter are removed by macrophages therefore preventing inflammation at the site (252). Apoptosis can be initiated through two specific signaling pathways, the extrinsic and intrinsic pathways, is accomplished through the activation of caspases, and is regulated at different levels.

5.1. Apoptotic and cell survival pathways

5.1.1. Caspases

Caspases are cysteine-dependent aspartate-specific proteases that are synthesized as pro-enzymes; their activation requires proteolysis by other caspases. Caspases involved in apoptosis are divided into two subgroups depending on their function; (i) the initiator caspases that initiate the apoptotic pathway and include caspase-2, -8, -9, and -10; and (ii) the effector or executioner caspases that degrade cellular targets and include caspase-3, -6, and -7. Initiator caspases can be activated either by the extrinsic pathway such as caspase-8 and -10 or by the intrinsic pathway such as caspase-9. On the other hand, executioner caspases are activated by their cleavage by initiator caspases (254). Once activated, executioner caspases cleave cellular substrates causing all the

morphological changes occurring during apoptosis. Targets of caspase cleavage include many important cellular substrates such as the inhibitor of caspase-activated DNase (ICAD), ROCK1, the DNA repair enzyme poly(ADP-ribose)polymerase (PARP), actin, lamin, cell cycle regulators [retinoblastoma protein (pRB)], transcription factors (NF-κB), and cell signaling proteins [Raf, protein kinase B (PKB)] (252;255) [reviewed in (256)].

5.1.2. Extrinsic pathway

The extrinsic pathway is initiated by the binding of tumor-necrosis factor (TNF) ligands to specific cell-death receptors, the tumor-necrosis factor receptors (TNFRs), on the surface of cells (257). Eight members of the TNFR family contain a death domain (DD) that allow them to participate in apoptosis and include FAS [Apo-1, CD95 or death receptor-1 (DR1)], TNF-R1 (DR2), DR3 [TNF-receptor-related apoptosis mediating protein (TRAMP) or Apo-3], DR4 [TNF-related apoptosis inducing ligand receptor-1 (TRAIL-R1) or DR4], DR5 (TRAIL-R2 or Apo-2), DR6, ectodysplasin A receptor (EDAR), and nerve growth factor receptor (NGFR). There are also four decoy receptors to which ligands bind, but that do not lead to signal activation therefore inhibiting apoptotic signaling; these receptors are decoy receptor-1 (DcR1), DcR2, DcR3, and osteoprotegerin (255;258). TNFRs have no enzymatic activity of their own; hence they rely on adapter proteins to transmit the signal, thereby allowing for specific responses (259). The five adaptor proteins that bind to the receptors and one another to transduce signaling are TNFR1-associated death domain protein (TRADD), receptor-interacting protein (RIP), Fas-associated death domain protein (FADD), TNF-receptor-associated factor (TRAF), and CASP2 and RIPK1 domain containing adaptor with death domain (CRADD) (260). They form a death inducing signaling complex (DISC) that recruits the initiator pro-caspase 8 leading to its processing into caspase-8. Caspase-8 then activates the executioner caspase-3 (252).

5.1.3. Intrinsic pathway

The intrinsic or mitochondrial pathway is a stress-induced response of the cell to stressors such as UV- and γ-radiations, genotoxic and cytotoxic drugs, oxidative free radicals, and cytokine and growth factor deprivation (261). These stimuli lead to the release of cytochrome c from the mitochondria into the cytoplasm, where it binds the apoptotic protease activating factor-1 (APAF-1). This binding triggers the formation of the apoptosome that recruits pro-caspase 9 to activate it into caspase-9. Caspase-9 in turn activates caspase-3 (252).

The B cell leukaemia/lymphoma 2 (Bcl2) family of proteins regulate the activation of the intrinsic pathway by either controlling cytochrome c release or formation of the apoptosome. They are categorized into three subfamilies according to their function and structure; (i) the anti-apoptotic Bcl2 subfamily that contains four Bcl2 homology (BH) domains (BH1-4) and include Bcl2, Bcl2l1 (Bcl-x_L), Bcl2l2 (Bcl-w), Bcl2l10 (Bcl-B/Diva/Boo), Mcl1, and Bcl2a1a (A1); (ii) the pro-apoptotic Bax subfamily with three BH domains (BH1-3) that include Bax, Bak1, and Bok; and (iii) the pro-apoptotic BH3-only domain subfamily that include Bik, Bad, Bid, Blk, Hrk, Bcl2l11, and Bnip3. The members of the Bcl2 and Bax subfamilies are anchored into the mitochondrial membrane, whereas the members of the BH3-only subfamily act as ligands that associate with the membrane-anchored proteins (254). The fate of the cell is determined by the interactions between the pro- and anti-apoptotic members (262).

5.1.4. Regulation of apoptosis

Tight regulation of apoptotis is required to prevent inadequate activation. Although activation of caspases is a committed step toward apoptosis, there are proteins that inhibit caspase enzymatic activity, the inhibitor of apoptosis proteins (IAPs). There are seven mammalian IAPs that are characterized by the presence of one or more copies of the baculovirus IAP repeat (BIR) motif; they are cellular IAP-1 (cIAP-1, BIRC2), cIAP-2 (BIRC3), X-chromosome-linked IAP (XIAP), neuronal apoptosis inhibitory protein (NAIP), survivin (BIRC5), livin (BIRC7), and testis-specific IAP (ts-IAP, BIRC8) (263). IAPs are in turn negatively

regulated by two mitochondrial proteins, DIABLO (direct IAP-binding protein with low pI) and HTRA2 (high-temperature requirement serine protease A2), and the nuclear protein XIAP-interacting protein (XAF-1). DIABLO and HTRA2 are released from the mitochondria into the cytoplasm during apoptosis and bind to IAPs preventing their inhibition of caspase activity. On the other hand, XAF-1 not only directly inhibits IAP activity, but also sequesters them away from the cytoplasm into the nucleus (264).

5.2. Growth factor survival pathways

Growth factors interact with their respective cell surface receptors to regulate cell growth, metabolism, differentiation, cell death, and survival (265). In the epididymis, many growth factors are expressed. They include epidermal growth factor (EGF) (266), basic fibroblast growth factor (FGF2) (177), vascular endothelial growth factor A (VEGFA) (267), nerve growth factor (NGF) (268), platelet-derived growth factor (PDGF) (269), transforming growth factor-β (TGFβ) (270), insulin-like growth factor (IGF) (271), erythropoietin (272), and hepatocyte growth factor (273). Previously, Henderson and Robaire (250) have shown that treatment with PNU157706, a dual Srd5a inhibitor, decreases *Igf1* expression in the distal epididymal regions. In addition, Hamzeh and Robaire (149) have identified *Igf1* and *Igbp3* as two genes changing early after androgen withdrawal. As these data suggest a potential involvement of the IGF1 signaling pathway in the response of the epididymis to androgen withdrawal, this section focuses on IGF1.

IGF1 binding to the extracellular domain of IGF1R triggers autophosphorylation and tyrosine phosphorylation of IGF1R substrates, which include insulin receptor substrate (IRS)-1 or -2, Src- and collagen-homology (SHC) proteins, and growth factor receptor-binding protein 2 (Grb2). These phosphorylated proteins then activate the Ras/Raf/MAPK pathway or the phosphatidylinositol 3-kinase (PI3K)/Akt pathway. Activation of the MAPK pathway leads to cell proliferation, whereas the PI3K/Akt pathway regulates the anti-apoptotic responses. Activation of the PI3K/Akt pathway leads to the

activation of the anti-apoptotic proteins Bcl2 and Bcl2l1 and inhibition of the pro-apoptotic proteins Bax, Bad, Bcl2l1, and caspase-9. Furthermore, it activates nuclear factor-κB (NF-κB) transcriptional activity leading to transcription of survival genes (274;275). IGF1 bioavailability is regulated by IGF binding proteins (IGFBPs). The most abundant one, IGFBP3 forms a ternary complex with IGF1 and acid-labile subunit (ALS) that prolongs IGF1 half-life and prevents it from reaching its receptor (275). In addition, IGFBP3 may exert IGF1-independent effects that can promote both cell death and cell survival [reviewed in (276)]. Once internalized, IGF1 is degraded by the insulin-degrading enzyme (IDE) leading to signal termination (277). Many interactions exist between the AR and the IGF1 signaling pathway [reviewed in (278)]. In fact, IGF1 (279) and IGFBP3 (280) have functional AREs in their promoter region leading to transcriptional regulation, whereas AR up-regulates IGF1R expression (279). Furthermore, activation of IGF1 and the PI3K signaling cascade leads to increased AR expression (281), whereas interaction between IDE and AR enhances AR DNA binding (282;283).

6. Cell survival and apoptosis in the epididymis

During postnatal development in the mouse, orchidectomy causes low levels of apoptosis in all regions of the epididymis; this can be prevented by administration of T (220). This illustrates the dependence of the epididymis on androgens and testicular factors for its growth and differentiation (6). In the adult rat, although after orchidectomy there are varying degrees of apoptosis observed in each region, at the peak of the response, the number of apoptotic cells averages only 1 cell per tubule. The low rate of observed apoptosis can be prevented by administration of T in all regions, except the initial segment. In addition, efferent duct ligation causes the same extent of apoptosis in the initial segment as orchidectomy; the caput is less affected than in orchidectomized rats, whereas the corpus and cauda are not affected. Principal cells are the only cell type affected (229). Investigations on the molecular mechanisms underlying the apoptosis observed after orchidectomy have shown that it is independent of p53 (284;285)

and Fos (FBJ osteosarcoma oncogene) (286). The tumour-suppressor Tp53 is a central sensor of cellular stress and its activation leads to apoptosis (287), whereas Fos combines with Jun family members to form the AP-1 transcription factor that plays a role in proliferation, differentiation, and apoptosis (288). Conflicting reports exist on its dependence on Fas with one group showing that it is (289) and the other one showing that it is not (290). In addition, Bcl2, an anti-apoptotic protein, is suppressed after orchidectomy (289).

At the transcriptional level, two pro-apoptotic genes [*defender against cell death protein 1* (*Dad1*) and *tumor necrosis factor receptor 1* (*Tnfrsf1a*)] and an anti-apoptotic gene [*myeloid cell differentiation protein 1* (*Mcl1*)] have been shown to be affected after orchidectomy in the epididymis (85).

7. Formulation of project

Androgens are steroid hormones that predominantly act by binding to AR leading to the transcription of genes thereby regulating many reproductive and non-reproductive functions. Dysregulation of androgen functions can lead to male infertility, alopecia, benign prostatic hyperplasia, and prostate cancer. There is, therefore, a need to better understand the molecular mechanisms underlying androgen actions. This thesis focuses on the epididymis, the tissue where spermatozoa acquire their fertilizing ability and where they are stored before ejaculation. The epididymis depends on androgens and testicular factors to maintain its functions. However, unlike other hormone-dependent tissues such as the prostate, the epididymis is particularly resistant to tumor formation. In addition, after androgen withdrawal by orchidectomy, there is little apoptosis with less than 1% of the cells lost; this is in sharp contrast with the prostate where 80% of the cells are lost by apoptosis within 10 days of surgery.

The overall goal of this thesis is to understand the molecular mechanisms involved in the resistance of the epididymis to apoptosis triggered by androgen withdrawal using *in vivo* and *in vitro* systems. I hypothesize that androgen withdrawal triggers the activation of a series of specific survival signaling pathways that act to help protect the epididymis against high levels of apoptosis.

Despite numerous studies on the overall gene expression in the epididymis of different species and under different pathological and experimental conditions, little is known about the apoptotic and cell survival genes activated or repressed after orchidectomy. Chapter 2 of this thesis identifies the apoptotic and survival genes activated early after orchidectomy with or without testosterone replacement in the rat epididymis. Using gene array technology, bioinformatics, and molecular biology tools, androgen-regulated genes belonging to the major apoptotic and cell survival gene families were identified in the different regions of the epididymis.

The IGF1 signaling pathway and BIRC5 are important regulators of cell survival. They are both expressed in the epididymis and regulated by androgens. In addition, IGF1 is a central regulator of the response of the mouse PC-1 epididymal cell line to androgen withdrawal. Chapter 3 explores the effects of orchidectomy on the IGF1 signaling pathway and BIRC5 in the rat epididymis. Changes in mRNA and protein expression of IGF1, IGF1R, and BIRC5 were assessed by quantitative real-time PCR (qRT-PCR) and western blots. In addition, changes in mRNA expression of two upstream regulators of IGF1 signaling, *Igfbp3* and *insulin-degrading enzyme*, as well as of *Bax* and *Diablo*, two pro-apoptotic genes acting downstream of the signaling cascade were assessed by qRT-PCR.

Most of the work on the effects of androgen withdrawal on the epididymis has been done *in vivo*. However, in order to determine the specific signaling cascades triggered after androgen withdrawal in the epididymis, *in vitro* cell model systems are necessary. The PC-1 and DC-3 mouse epididymal cell lines are androgen-dependent cells and offer a potential useful tool to answer specific questions about androgen regulation of the epididymis. Chapter 4 assesses the effects of androgen withdrawal on the PC-1 and DC-3 mouse epididymal cell lines by determining their viability after androgen treatment or withdrawal. Furthermore, changes in transcript expression for *Igf1*, *Igf1r,* and *Birc5* were determined by qRT-PCR.

Together, these studies will provide novel insights into androgen regulation of apoptotic and survival genes in the epididymis, as well as into the

molecular mechanisms underlying epididymal resistance to apoptosis triggered by androgen withdrawal.

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CHAPTER 2

Identification of Apoptosis and Cell Survival Genes Regulated by Androgens in the Rat Epididymis

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1. Abstract

The epididymis is an androgen-dependent tissue that is responsible for the maturation and storage of spermatozoa. Androgen withdrawal by orchidectomy causes a region-specific and time-dependent wave of apoptosis along the tissue, but the absolute number of apoptotic cells is limited. To investigate the early gene expression response of survival and apoptosis genes after the withdrawal and/or immediate replacement of androgen on the different regions of the epididymis, we used apoptosis-focused arrays. We assessed changes in gene expression at 0.5 and 1 day after orchidectomy with or without testosterone (T) replacement and selected genes (*Bmf*, *Mcl1*, *Rad52*, and *Tnfrsf11b*) were analyzed by qRT-PCR. Pathway analysis was used to identify direct regulatory relationships between the androgen receptor (AR), T and the affected genes; promoter sequence analysis was also undertaken to identify putative androgen response elements (AREs). Changes in protein levels and immunolocalization of TNFRSF11B were also determined. We uncovered androgen-regulated apoptotic and cell survival genes; some of these genes could be directly regulated by AR through putative AREs. We also found that *Bmf*, *Mcl1*, *Rad52*, and *Tnfrsf11b* were repressed by T. TNFRSF11B showed a region-specific localization in the cytoplasm of principal cells. These results suggest that androgens regulate the expression of apoptotic and cell survival genes in a region-specific manner in the epididymis.

2. Introduction

The epididymis, a highly convoluted tubule that links the efferent ducts of the testis to the vas deferens, provides optimal microenvironments for the proper maturation and storage of spermatozoa (1). Based on morphological and functional differences, the epididymis is divided into four regions: initial segment (IS), caput (Ca), corpus (Co), and cauda (Cd); its epithelium comprises four major cell types (principal, basal, halo, and clear cells) (2;3). Androgens, in particular dihydrotestosterone (DHT), the 5α –reduced metabolite of testosterone (T), regulate epididymal structure and functions (4;5). Furthermore, testicular factors such as basic fibroblast growth factor (bFGF2) (6) and androgen binding protein (7) regulate protein secretions in the proximal regions of the epididymis (8;9).

Androgen withdrawal by orchidectomy causes a decrease in epididymal weight due to the loss of spermatozoa and luminal fluid as well as a decrease in epididymal cell height (10;11). In addition, it has been demonstrated that, in the rat, androgen withdrawal by orchidectomy causes a region-specific and time-dependent wave of apoptosis, although at the peak of the response, the number of apoptotic cells averages only 1 cell per tubule (12). Previous studies on the molecular mechanisms explaining the apoptosis triggered by androgen withdrawal in the epididymis have determined that it is p53-independent (13;14), but conflicting reports exist on its Fas-dependence (15;16). Ezer and Robaire (17) have also identified two pro-apoptotic genes [*defender against cell death protein 1* (*Dad1*) and *tumor necrosis factor receptor 1* (*Tnfrsf1a*)] and an anti-apoptotic gene [*myeloid cell differentiation protein 1* (*Mcl1*)] as being regulated by androgens in the epididymis. However, the underlying pro- and anti-apoptotic pathways triggered by androgen withdrawal in the epididymis are still unknown.

Numerous studies have been undertaken to assess changes in overall transcription in the human (18-20), rat (17;21), and mouse (22;23) epididymides under different pathological (18;20) and experimental (19;24-27) conditions. In the present study, we investigated early changes in the expression of apoptotic and cell survival genes in the four epididymal regions after androgen withdrawal. We also determined which affected genes could be rescued by immediate

replacement with testosterone. We used apoptosis-focused arrays and qRT-PCR to determine changes in gene expression at 0.5 and 1 day after androgen withdrawal and replacement. We found region-specific changes in the expression of apoptotic and cell survival genes and uncovered genes potentially regulated by androgens through putative androgen response elements.

3. Materials and Methods

3.1. Chemicals

T (4-Androsten-17β-ol-3-one) was purchased from Steraloids Inc. (Newport, RI). Sodium azide was bought from Fisher Scientific Company (Nepean, ON). Medical adhesive (Silicone type A, cat. no. 891) and tubing (cat. no. 602-305) to make the polydimethylsiloxane (Silastic) implants were purchased from Dow Corning Silicones (Midland, MI). Bovine serum albumin (Fraction V) (BSA), NP-40 substitute and sodium deoxycholate/DOC were obtained from Sigma-Aldrich Canada Ltd. (Oakville, ON). NaCl, SDS, and TRIS were bought from Invitrogen Canada Inc. (Burlington, ON). Normal saline (0.9% w/v NaCl in water), Bestatin, PMSF, leupeptin, and aprotinin were bought from Roche Applied Science (Laval, QC). Ketamine was bought from Bioniche (Belleville, ON), acepromazine from Wyeth-Ayerst (St-Laurent, QC), xylazine from Novopharm (Montreal, QC), and buprenorphine from Reckitt & Cloman (Bristol, UK).

3.2. Animals

Adult male Brown Norway (BN) rats (3-4 months old) were obtained from Charles River Canada (Saint-Constant, QC) and housed at the McIntyre Animal Resources Centre of McGill University. Rats (3 per cage) were kept under controlled light (14-h light, 10-h dark) and temperature (22°C) and had access to regular rat chow and water *ad libitum*. All animal studies were conducted in accordance with the principles and procedures outlined in the Guide to the Care and use of Experimental Animals prepared by the Canadian Council on Animal Care (Animal Use Protocol no. 206).

Rats were separated into 11 groups (n=6/group): sham-operated; orchidectomized and implanted sc. with an empty 2.5-cm Silastic capsule (-T groups) and sacrificed at 0.5, 1, 2, 3 or 7 days after surgery; or orchidectomized and implanted sc. with a T-filled 2.5-cm Silastic capsule (+T groups) and sacrificed at 0.5, 1, 2, 3 or 7 days after surgery. Rats were anaesthetized by an intramuscular injection of ketamine, xylazine, and acepromazine (5:2.5:1) in normal saline (0.1ml/100g body weight) and received buprenorphine (0.001mg/100g body weight) after surgery. Bilateral orchidectomy was done as described elsewhere (17) and capsules were implanted sc. at the time of surgery. Implants were made according to the method of Stratton et al. (28) and had a T release rate of 30µg/cm/day, releasing T to an equivalent amount to serum T. To ensure a steady rate of T release, implants were bathed for 2 days prior to surgery in a solution of normal saline containing 1% BSA and 0.1% sodium azide. At the time of death, blood was collected as well as epididymides that were separated into IS, Ca, Co, and Cd regions, frozen in liquid nitrogen and kept at -80°C.

3.3. Serum testosterone analysis

Serum was isolated from blood samples by centrifugation. Supernatants were kept at -80°C until used. Concentrations of serum T were measured using a commercially available Testosterone ELISA kit (Fitzgerald Industries International Inc., Acton, MA) following the manufacturer's instructions. Sensitivity of the assay was 0.1ng/ml. Intra-assay variation was 4.5%, whereas inter-assay variation was 6.9%.

3.4. RNA extraction, oligo arrays and hybridization

RNA was isolated from the IS, Ca, Co and Cd of sham-operated, 0.5 day (-T), 1 day (-T), 0.5 day (+T), and 1 day (+T) groups using Qiagen Mini-prep (Qiagen Inc., Mississauga, ON) following manufacturer's instructions. DNase treatment was done using the RNase-free DNase set (Qiagen Inc.) following the manufacturer's instructions. Concentration and quality of RNA were verified by measuring OD at 260nm and 280nm (DU7 spectrophotometer, Beckman,

Mississauga, ON). Quality of RNA was also assessed by running the samples in non-denaturing 1% agarose gels. Each RNA sample was extracted from a single epididymal region from an individual rat; no samples were pooled.

RNA samples were transcribed into biotin-labeled cRNA following the True-Amp protocol from SABiosciences (SABiosciences, Frederick, MD). Briefly, 1.5 µg to 2 µg of RNA was reverse transcribed into cDNA followed by overnight transcription and amplification into biotin-labeled cRNA using the GeneAmp PCR System 2400 machine (PerkinElmer, Woodbridge, ON). Four micrograms of biotin-labeled cRNA were then hybridized to apoptosis-focused oligo GEArrays (ORN-012, SABiosciences) following manufacturer's instructions; these arrays contain 112 probes, description of which can be found in table 1. Five arrays for each of the four epididymal region for the two time points after orchidectomy and sham (n=5/region/time; 100 samples) were hybridized and referred to as replicates. Hybridized membranes were visualized by exposing them to an ECL film (GE Healthcare, Baie d'Urfe, QC) for 20 sec. Arrays on films were scanned (ScanJet ADF, Hewlett Packard, Kirkland, QC) on grayscale with a resolution of 600dpi and saved as tiff- files. Scanned images were imported into the GEASuite software (http://geasuite.superarray.com) from SABiosciences where the arrays were aligned and raw data obtained. Raw data were then imported into Excel for background correction, which was done by subtracting the average value of 5 probe sets (*Blk*, *Cideb*, *Lyst*, *Rem2*, and *Xiap*) that had the lowest expression on every array to the raw data obtained. The data were analyzed using GeneSpring GX 7.2 software (Agilent Technologies, Mississauga, ON), where a custom genome was created. Two normalization steps were applied. First, every value below 0.01 was transformed into 0.01. Then, a per chip normalization was applied by normalizing every gene against all the others. This was followed by a per gene normalization where every gene was normalized to the median value of its measurements. For a gene to be considered expressed, its expression had to be two-fold above background. Genes were considered differentially expressed if they were up- or down-regulated by at least 1.5 fold (i.e. 50% increase or 33% decrease) as compared to sham-operated in at least 3 arrays out

of 5. In order to identify putative AREs in the promoter region of affected genes, we used the "Find potential regulatory sequences" tool. We used the following sequence AGAACCnnnTGTTCT allowing for a maximum of 3 unknown bases.

Pathway analysis was done using PathwayStudio 7 (Ariadne Genomics, Rockville, MD) and ResNet-7 database to visualize relationships between transcripts differentially affected by orchidectomy with and without T implants, the androgen receptor (AR), T, and growth factors. Objects were proteins and small molecule*s*; pathways and relationships were limited to expression, regulation, and promoter binding.

3.5. Quantitative Real-Time RT-PCR

Real-Time RT-PCR was done on selected genes (Table 2) to quantify their expression levels using the QuantiTect RT-PCR SybrGreen kit (Qiagen Inc.) and the LightCycler system (Roche Applied Science, Laval, QC) as described previously (29). Each sample was assayed in duplicate. Changes in gene expression were normalized against *peptidylprolyl isomerase A* (*Ppia*, *cyclophilin A*) expression. *Ppia* is a housekeeping gene; its mRNA expression is not affected by androgen manipulation (30). Transcript-specific primers were designed using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi/), except for *Tnfrsf11b*; those were ordered from QIAGEN (catalog no. QT00177170, QuantiTect Primer Assays). All other primers were synthesized by AlphaDNA (www.alphadna.com; Montreal, QC).

3.6. Dot blot

First-strand cDNA synthesis was done using 1µg total RNA, random primers (Invitrogen), 10mM dNTP mix (Invitrogen), and SuperScript™ III RT (Invitrogen). The synthesized cDNA was then used as a template for PCR amplification using primers for *Tnfrsf11b* (forward 5'-TGAGACGTCATCGAAAGCAC-3'; reverse 5'-CTGGCAGCTTTGCACAATTA-3') and *18S rRNA* (forward 5'-AAACGGCTACCACATCCAAG-3'; reverse 5'-

AGTCGGCATCGTTTATGGTC-3') designed using Primer3 software and synthesized by AlphaDNA. The cycling conditions were as follow: 2 min at 94^0C, 40 cycles of 30 sec at 94^0C, 1 min at 56^0C, and 1 min at 72^0C, followed by 5 min at 72^0C, and 4^0C O/N. For dot blot analysis, PCR samples were prepared by adding 0.5M EDTA, 6N NaOH, and 2M NH_4OAc. Samples were loaded into a dot-blot manifold (Bio-Rad, Mississauga, ON) to be transferred onto a nitrocellulose membrane (Bio-Rad). Filter was removed and soaked for 15 sec in 6X SSC+0.1% SDS. The membrane was cross-linked under UV light for 4 min. Membranes were soaked for 2-4h at 42^0C in pre-hybridization solution [20X SSC, 50X Denhardt's, 20mg/ml tRNA (Roche Applied Science), 20% SDS, and ddH_2O]. The internal oligonucleotide probe for *Tnfrsf11b* (TGGGAATGAAGATCCTCCAG) and *18S rRNA* (CGCGGTTCTATTTTGTTGGT) were designed using Primer3 software and synthesized by AlphaDNA. Fifty ng of oligonucleotide probe was labeled using γP32 (PerkinElmer), kinase buffer (Roche Applied Science), and T4 kinase (Roche Applied Science). It was incubated for 1-2h at 37^0C and passed trough a G-25 sephadex column. An activity of 10^4-10^5 cpm/ng was considered good. Labeled oligonucleotide was added to the hybridization solution (20X SSC, 20% SDS, and ddH_2O) at a concentration of $6x10^6$cmp/ml.

3.7. Western blot analysis

Whole cell extracts (n=5/group) were prepared in RIPA buffer (150mM NaCl, 1% NP-40 substitute, 0.5% sodium deoxycholate/DOC, 0.1% SDS, 50mM TRIS pH 7.4). For each ml of RIPA buffer, the following proteinase inhibitors were added: 4µl bestatin (10mg/ml), 1µl PMSF (24mg/ml), 2µl leupeptin (5mg/ml), and 3µl aprotinin (2mg/ml). Protein concentrations were determined by the Bradford method using the Bio-Rad protein assay (Bio-Rad Laboratories, Mississauga, ON) following the manufacturer's protocol. For each sample, 20µg protein per lane was separated on a 12% acrylamide SDS-PAGE gel; a testis sample was used as a positive control. Prestained All Blue Precision Plus Protein Standards (Bio-Rad Laboratories) were used as molecular weight markers.

Separated proteins were transferred to a PVDF Hybond-P membrane (GE Healthcare). Blots were blocked in 5% non-fat dried milk in TBS-T (137 mM NaCl, 20 mM Tris, 0.5% Tween 20, pH 7.6) for 1h at room temperature and then incubated overnight at 4°C with a primary rabbit antibody against human TNRSF11B (1:500, AB2125P, Millipore, Billerica, MA). The membrane was then probed with a donkey anti-rabbit IgG horseradish peroxidase linked whole antibody (1:10 000, NA934V, GE Healthcare). Constant loading was assessed by probing the membrane with a primary goat antibody against ACTIN (1:10 000, sc-1616, Santa Cruz Biotechnology, Santa Cruz, CA) and detecting it with a donkey anti-goat IgG horseradish peroxidase conjugated antibody (1:10 000, sc-2056, Santa Cruz Biotechnology).There was no blocking peptide for the TNFRSF11B antibody available from Millipore. Signals were detected with the Enhanced Chemiluminescence Plus kit (GE Healthcare) and visualized on Hyperfilm enhanced chemiluminescence (GE Healthcare). Quantification of western blot data was done by densitometry analysis using a Chemilmager 4000 imaging system (Cell Biosciences, Santa Clara, CA) with AlphaEase (version 5.5 software, Cell Biosciences). Expression of TNFRSF11B was expressed relative to the corresponding expression of ACTIN for all groups.

3.8. Immunohistochemistry

Tissue preparation (n=5) for immunohistochemistry was done as described elsewhere (12). Sections (5 μm thick) were incubated overnight at 4°C with a primary rabbit antibody against human TNFRSF11B (1:250, AB2125P, Chemicon International) and stained using the Rabbit Vectastain Elite ABC Kit (Vector Laboratories, Burlington, ON). The DAB substrate kit for peroxidase (SK-4100, Vector Laboratories) was used to reveal staining. The negative control was obtained by incubating the slides with no primary antibody against TNFRSF11B followed by incubation with the secondary antibody. Slides were counterstained with a 0.005% methylene blue solution and examined under a light microscope (Laborlux D, Leica, Allendale, NJ). Micrographs were taken with a CoolSnap camera (Roper Scientific, Tucson, AZ).

3.9. Statistical analysis

Parametric data were analyzed by one-way ANOVA followed by Dunnet's *post hoc* test, whereas non-parametric data were analyzed by Kruskal-Wallis one-way ANOVA on ranks followed by Dunn's *post hoc* test. Serum T concentrations were log transformed before being analyzed. Statistical differences between time (0.5 day or 1 day) across treatment (-T and +T) were determined by unpaired t-tests. If normality could not be assumed, data were analyzed using the Mann-Whitney Rank Sum test. Significance was set at $p<0.05$.

4. Results

4.1. Orchidectomy, with or without testosterone replacement, changed serum testosterone concentration and sex accessory tissue weights

Previous studies on the effects of orchidectomy on the structure of the epididymis had been conducted in the Sprague-Dawley (SD) rat model system (10-12). However, the outbred SD rat strain was inappropriate to conduct genomic studies and hence we chose to work with Brown Norway (BN) rats.

In order to assess if the BN rat was a suitable model for the study of the effects of androgen withdrawal on the epididymis, we evaluated the consequences of orchidectomy with or without testosterone replacement on serum T levels (fig.1A) and on weights of ventral prostate (fig.1B), empty seminal vesicles (fig.1C) and epididymis (fig.1D). We found that by 0.5 day after orchidectomy, serum T levels had decreased below the detection limit of the assay, i.e., by more than 97%, whereas the presence of T implant maintained serum T concentrations in the normal physiological range (fig.1A). At 0.5 day, the seminal vesicles were the first tissues to show a significant change in tissue weight between the (-T) and (+T) groups ($p<0.05$) (fig. 1C), whereas the epididymis showed the first significant effect of treatment at 1 day ($p<0.05$) (fig. 1D). The prostate only showed a significant difference between the (-T) and (+T) groups at 7 days ($p<0.05$) (fig. 1B). For all tissues, androgen withdrawal significantly decreased their weights at 7 days ($p<0.05$). These results were similar to the ones found for SD rats (data not shown). In order to assess how androgen withdrawal or

replacement after orchidectomy affects gene transcription, we selected the 0.5 and 1.0 day time points, thus optimizing the identification of early response genes while minimizing the direct impact of apoptosis, as this process is minimal at these early times (12).

4.2. Testosterone differentially affected transcription of genes in the different regions of the epididymis

Out of the 96 apoptotic and survival probe sets expressed on the arrays, 43 probe sets were known to be pro-apoptotic, 17 anti-apoptotic, 12 were involved in repair, and 16 had apoptosis-related functions. Overall, combining all treatment groups and regions, 53 transcripts were increased or decreased by at least 1.5 fold in the epididymis.

When we compared the number of transcripts that were up- and down-regulated in the different regions of the epididymis (fig. 2), we found that T replacement greatly decreased the number of affected transcripts at 0.5 day after orchidectomy in the IS (up-regulated: 5 to 1; down-regulated: 7 to 4; fig. 2A) and Co (up-regulated: 5 to 1; down-regulated: 10 to 3; fig. 2C). In the Ca (fig. 2B), T replacement slightly increased the number of down-regulated transcripts (from 3 to 6), but had no effect in the Cd (fig. 2D). At 1 day after orchidectomy, T replacement caused the largest decrease in the number of affected transcripts in the Cd (up-regulated: 10 to 5; down-regulated: 7 to 1; fig. 2D), whereas the Co (fig. 2C) had only a decrease in down-regulated transcripts from 10 to 3 genes. Without T replacement, four transcripts (*Mcl1*, *Tnfrsf11b*, *Cd40lg*, and *Birc5*) were differentially affected in all regions of the epididymis (table 3). Six transcripts (*Bad*, *Bnip3*, *Bnip3l*, *Rad52*, *Birc3*, and *Traip*) were specifically affected in the distal regions of the epididymis, whereas 3 transcripts (*Casp4*, *Ltbr*, and *Tnfrsf26*) were specifically affected in the IS (table 3). *Bmf* was the only transcript that was affected in all regions, except in the Cd (table 3). With T replacement, 5 transcripts were specifically affected in the IS (*Bcl2l10*, *Bmf, Mcl1, Casp8, Tnfrsf11b)*, whereas 11 transcripts (*Bax, Bik, Casp11, Tnfrsf1a, Tnfrsf1b, CD70, Tnfsf10, Birc3, Dapk3, Traip,* and *Cntnap1*), 4 transcripts (*Bak1,*

Bcl2l2, Casp7, and *Dffa*), and 9 transcripts (*Bcl2l1, Tnfrsf4, Tnfrsf12a, Cd40lg, Tnfsf9, Tnfsf15, Rad50, Rad52,* and *Tnfaip2*) were affected in the Ca, Co, and Cd, respectively (table 4). Together, these data suggested that each epididymal region responded differently to orchidectomy with or without T replacement.

4.3. Testosterone and androgen receptor regulation of gene transcription

We used PathwayStudio software to query Pubmed to determine known regulatory relationships between androgens, either as T or through the androgen receptor (AR), and the affected transcripts. Only 7 transcripts (*Bax, Bcl2, Bmf, Birc5, Casp6, Tnfrsf1a,* and *Tnfrsf11b*) had been previously shown to be directly regulated by T; only 2 transcripts, *Bcl2* and *Cflar*, showed a direct regulation by AR (fig. 3A). We then identified potential proteins (kinases and transcription factors) that could act as mediators of AR and T action (fig. 4A and B, respectively). We found that 12 transcripts could be indirectly regulated by AR through the action of TERT, CDKN1A, MAPK1, MYC, CTNNB1, KRAS, and PTK2, whereas 19 transcripts could be indirectly regulated by T through 17 different potential mediators. Out of the 12 transcripts indirectly regulated by AR, only 8 could also be regulated by T. Most transcripts had only one potential mediator of AR or T action; *Mcl1* and *Birc3* were the two transcripts with the highest number of potential mediators, 7 and 5, respectively (fig. 4B).

A search for putative androgen response elements (AREs) up to 3kb upstream of differentially affected transcripts (table 5) revealed that out of the 53 transcripts that were affected by orchidectomy with or without T replacement, only 28 had published promoter sequences; all of those 28 genes showed putative AREs (fig. 3B). All apoptotic and cell survival gene families showed genes with potential AREs in their promoter sequences. *Cntnap1* had the lowest number of putative AREs (5 AREs), whereas *Bmf* had the highest number (22 AREs). Sixteen genes had more than 15 putative AREs whereas 12 genes had less than 15 putative AREs. All the putative AREs were scattered in the 3kb upstream promoter sequences. These results demonstrate that AR could directly regulate the transcription of a proportion of pro- and anti-apoptotic genes in the epididymis.

At 0.5 day and 1 day after orchidectomy, although serum T concentrations had decreased below detection limit, we could not exclude the participation of luminal factors, in particular growth factors, still present in the epididymis (31) to contribute to changes seen at the transcriptional level. We then determined which growth factors known to be present in the epididymis (EGFR, FGF2, IGF1, IGF1R, VEGFA, VEGFB, and TGFA) (32) could affect gene transcription and found that 23 out of the 53 transcripts affected in any condition could be regulated by growth factors (fig. 5). In fact, 7 genes were known to be regulated by EGFR, 10 by FGF2, 10 by IGF1/IGF1R, 8 by VEGFA, 5 by VEGFB, and 6 by TGFA (fig. 5).

4.4. Orchidectomy with or without testosterone replacement affected the transcription of *Bmf*, *Mcl-1*, *Rad52*, and *Tnfrsf11b*

We focused on four transcripts (*Bmf*, *Mcl1*, *Rad52*, and *Tnfrsf11b*), that showed region-specific changes in transcription after orchidectomy with or without T replacement (tables 2 and 3) and that belonged to three major apoptotic and cell survival gene families: Bcl2 family (*Bmf* and *Mcl1*), ATM and p53 family (*Rad52*), and TNFR (*Tnfrsf11b*). They also participated in promoting either cell death (*Bmf*), survival (*Mcl1* and *Tnfrsf11b*) or DNA repair (*Rad52*) (fig. 6).

Rad52. *Rad52* showed the highest basal level of expression in the Ca (0.68±0.07 fig. 7B) and the lowest in the Cd (0.008±0.001; fig. 7D). In all regions, at 1d after orchidectomy with T replacement, *Rad52* expression was undetectable (fig. 7). In addition, *Rad52* mRNA expression was significantly different from sham-operated levels in all regions and treatments ($p<0.05$) (fig. 7). Interestingly, at 1 day after orchidectomy without T replacement, the IS was the only region to show a significant increase in *Rad52* mRNA ($p<0.05$). In all regions and all time points, except at 0.5 day in the Cd (fig. 7D), T replacement significantly repressed *Rad52* mRNA expression ($p<0.05$). This indicated that T was repressing *Rad52* expression in the epididymis.

Mcl1. *Mcl1* showed the highest basal level of expression in the IS and Co (1.66±0.23 and 1.92±0.41, respectively; fig. 8A and 8C) and the lowest in the Ca and Cd (0.86±0.24 and 0.98±0.07, respectively; fig. 8B and 8D). At 0.5 day after orchidectomy, T replacement significantly repressed *Mcl1* mRNA expression in all regions except Cd ($p<0.05$) (fig. 8). At 1 day after orchidectomy, in the proximal regions, T replacement repressed *Mcl1* mRNA expression (fig. 8A-B), whereas in the distal regions, T replacement significantly increased *Mcl1* expression ($p<0.05$) (fig. 8C-D). This indicated that T was repressing *Mcl1* expression in the proximal regions of the epididymis, whereas T was activating *Mcl1* expression in the distal regions.

Bmf. In general, the proximal regions showed the highest basal levels of *Bmf* expression (2.22±0.47 and 1.06±0.13 for IS and Ca, respectively) and the distal regions the lowest (0.43±0.05 and 0.37±0.05 for Co and Cd, respectively) (fig. 9). In addition, the IS showed the highest induction of *Bmf* mRNA after orchidectomy with or without T replacement (fig. 9A). In all regions, except Ca, at 0.5 day after orchidectomy, T replacement repressed *Bmf* mRNA expression. On the other hand, at 1 day after orchidectomy, T replacement increased *Bmf* expression in the proximal regions, but had little effects on the distal regions (fig. 9). This indicated that *Bmf* was differentially regulated by T in the proximal and distal regions of the epididymis.

Tnfrsf11b. Compared to other reproductive tissues, the epididymis expressed *Tnfrsf11b* at an average level (fig. 10). The IS had the highest basal level of *Tnfrsf11b* mRNA expression (2.37±0.63, fig. 11A) and the Ca the lowest (0.55±0.14; fig. 11B). For all regions at 0.5 day after orchidectomy, T replacement significantly repressed *Tnfrsf11b* mRNA expression ($p<0.05$) as compared to orchidectomy without T replacement. Compared to control, T replacement also repressed *Tnfrsf11b* expression at 0.5 day after orchidectomy in the proximal regions, but increased *Tnfrsf11b* expression in the Cd. At 1 day, orchidectomy without T replacement significantly ($p<0.05$) repressed *Tnfrsf11b* expression in all regions, except Ca (fig. 11). This indicated that *Tnfrsf11b* was repressed by T in the epididymis. However, at the protein level, there were no

significant differences between the treatment groups, although T seemed to repress TNFRSF11B protein expression in all regions at 0.5 day after orchidectomy (fig. 11).

We also assessed whether *Tnfsf11* (Receptor Activator of NF-κB Ligand - RANKL) and *Tnfrsf11a* (RANK), two proteins involved in osteogenesis (33) and differentiation of the immune system (34), two processes affected by TNFRSF11B (33;35), were expressed in the epididymis. We found that they were both expressed in the epididymis. Interestingly, both *Tnfsf11* (fig. 12A) and *Tnfrsf11a* (fig. 12B) had the highest mRNA expression in the Co epididymidis.

Immunolocalization of TNFRSF11B in the epididymis revealed that in all regions, this protein was expressed specifically in principal cells (fig. 13). Interestingly, in the proximal regions, TNFRSF11B immumolocalized throughout the cytoplasm, whereas, in the distal regions, TNFRSF11B was apically localized (fig. 13F-I).

5. Discussion

In the control epididymis, only 5 probe sets (*Blk*, *Cideb*, *Lyst*, *Rem2*, and *Xiap*) present on the arrays were not expressed. One of them, XIAP (X-linked inhibitor of apoptosis) is the most characterized and potent inhibitor of apoptosis protein (IAP) (36). XIAP is not only up-regulated in cancers, but is also expressed in normal tissues, in particular the testis, where it is highly expressed (37). Given its ubiquitous expression, the lack of expression of *Xiap* in the epididymis would not have been predicted. This highlights the particular nature of the epididymis.

In the epididymis, expression of *Tp53*, a central regulator of many biological processes, including apoptosis (38), was not changed after orchidectomy with or without testosterone replacement. Although it has been shown that TP53 is not involved in the response of the IS to testicular factors removal by efferent duct ligation (14), one could have expected removal of both testicular factors and androgens by orchidectomy to trigger a decreased expression of *Tp53* to prevent apoptosis. Unfortunately, the arrays did not contain a probe for another central regulator of apoptosis, the tumor suppressor

retinoblastoma 1 (*Rb1*) (39); expression of this marker might have been changed in the epididymis after androgen orchidectomy with or without T replacement.

Two thirds of the probe sets present on the arrays showed a 1.5 fold increase or decrease in expression in at least one treatment and/or region. The finding that apoptotic and cell survival genes are expressed in a region-specific manner is similar to previous reports on the region-specific expression profiles of genes in the epididymis (17;19;40-42). For example, Ezer and Robaire (17) have identified *Tnfrsf1a* as a transcript specifically expressed in the Ca epididymidis, a finding reproduced in this study. However, they have also shown that transcripts involved in metabolism, calcium-binding proteins (CABPs), and heat shock proteins (Hsps) are affected across all regions after orchidectomy (17). In this study, we also identified 4 transcripts (*Birc5*, *Cd40lg*, *Mcl1*, and *Tnfrsf11b*) that were affected across all regions after androgen withdrawal; these four transcripts will be discussed in more detail in the next paragraphs.

Birc5. BIRC5 is an inhibitor of apoptosis protein (IAP), which is known as a protein highly expressed in cancers, but not in terminally-differentiated tissues (43). The epididymis is a terminally-differentiated tissue with very rare cases of cancers (1) making *Birc5* presence in the epididymis unexpected. BIRC5 can act as an anti-apoptotic protein or regulate cell division (44). Given that the epididymis has a very low mitotic index (45), one can assume that BIRC5 would be acting as an anti-apoptotic protein in the epididymis.

Cd40lg. CD40 ligand is a transmembrane TNF ligand expressed in non-inflammatory conditions by activated T lymphocytes, activated B lymphocytes, and platelets; during inflammation, monocytes, natural killer cells, mast cells, and basophils also express it (46). CD40 ligand could be expressed by halo cells, the immune cells of the epididymis that comprise helper T lymphocytes, cytotoxic T lymphocytes, and monocytes (47). The expression of *Cd40lg* after androgen withdrawal suggests the involvement of an immune response, which could be triggered by damaged cells in the lumen.

Mcl1. Mcl-1 is an anti-apoptotic Bcl2 family member that acts by directly binding pro-apoptotic Bcl2 members and thereby inhibiting cytochrome c release

from the mitochondria. Contrary to other Bcl2 member, Mcl-1 has a short half-life and can be up-regulated rapidly (48) making Mcl-1 a good target for transcriptional control. This suggests an involvement of the intrinsic mitochondrial pathway in the response of the epididymis to androgen withdrawal. We found that T regulated *Mcl1* expression in opposite ways in the proximal and distal epididymides: T repressed *Mcl1* expression in the proximal regions, whereas it increased *Mcl1* expression in the distal regions. These results highlight the differences in gene regulation between the different regions of the epididymis (17;19;40-42).

Tnfrsf11b. TNFRSF11B is a soluble TNFR that has been first discovered as an inhibitor of osteoclast formation (49). TNFRSF11B prevents the binding between TNFRSF11A (receptor activator of NF-kappaB; RANK) expressed by osteoclasts and its ligand TNFSF11 (RANKL) found in osteoblasts; binding of TNSF11 to TNFRSF11A on osteoclasts triggers the maturation of osteoclasts hence maintaining bone homeostasis (33;50). TNFRSF11A/TNFSF11 are also important for differentiation of the immune system (34), whereas TNFRSF11B increases humoral immune response (35). In fact, we have identified *Tnfrsf11a* and *Tnfsf11* as being expressed in the epididymis. Furthermore, during apoptosis, TNFRSF11B, which lacks a transmembrane domain and hence cannot signal, acts by binding to TNFSF10 (TNF-related apoptosis-inducing ligand, TRAIL), thereby preventing the activation of TNFRSF10A/B and the caspase cascade (49;51). TNFRSF11B is also a survival factor for human prostate cancer cells (52). In our arrays, *Tnfsf10* expression was not changed after orchidectomy with or without T replacement. At 0.5 day after orchidectomy, we found that *Tnfrsf11b* mRNA expression is repressed by T replacement. This result is consistent with the study by Hofbauer et al. (53) where they demonstrate that androgens decrease *Tnfrsf11b* mRNA expression. However, Hofbauer et al. (53) also show that androgens decrease TNFRS11B protein expression, which we have not found. This suggests that, although TNFRSF11B can be regulated at the transcriptional level by T, other mechanisms are probably in place to regulate its protein expression in the epididymis. Taken together, the multiple roles of

TNFRS11A/TNFRSF11B/TNFSF11, the expression of *Tnfrsf11a*, *Tnfrsf11b*, and *Tnfsf11* in the epididymis and the changes in expression of *Tnfrsf11b* after androgen withdrawal, one can assume a dual role of TNFRSF11B in immunity in the control epididymis and as an anti-apoptotic protein after androgen withdrawal. In the epididymis, TNFRSF11B was localized in the cytoplasm of principal cells, the androgen-responsive and major secretory cells of the tissue (54). However, halo cells are the primary immune cells (47). It is possible that principal cells secrete TNFRSF11B to regulate functions of immune cells and/or protect spermatozoa from degradation by immune cells (1). It is also tempting to speculate that TNFRSF11A and TNFSF11 would localize to halo cells.

We further characterized changes in expression for *Rad52* and *Bmf*, two transcripts that showed region-specific changes in expression.

Rad52. RAD52 is involved in DNA double strand break repair through homologous recombination. In fact, RAD52 is essential for the formation of the DNA repair complex and the recruitment of downstream effectors (55;56). Failure of the RAD52 complex to repair DNA damage will lead to apoptosis, whereas success will lead to cell survival (55). *Rad52* expression was repressed by T in the epididymis. This is the first time that androgens are shown to regulate the expression of a repair protein. We also found that the IS was the only region to show a significant increase in *Rad52* mRNA expression at 1 day after orchidectomy without T replacement. This suggests a higher sensitivity of the IS to the effects of androgen withdrawal and perhaps an attempt to counteract these effects. It is well established that the IS is particularly sensitive to androgen withdrawal by orchidectomy (12).

Bmf. BMF is a pro-apoptotic BH3-only Bcl2 family member that is linked to the actin cytoskeleton. When the cell detaches from the basal lamina, BMF is released from the cytoskeleton, allowing it to bind and inhibit the anti-apoptotic Bcl2 triggering a very specific form of cell death called anoikis (57-59). In addition, BMF has been localized to the subacrosomal space of postmeiotic spermatids from step 4 to 16 of spermiogenesis in the testis (60). We found that the IS showed the highest induction of *Bmf* mRNA in all treatments suggesting

that it is more sensitive to cell death by anoikis. In addition, at 0.5 day after orchidectomy without T replacement, we showed that there was an increase in *Bmf* expression. On the other hand, Show et al. (60) have demonstrated that decreased T in the testis causes a decrease in *Bmf* mRNA expression. At 1 day after orchidectomy, we found that T replacement increased *Bmf* expression in the proximal regions, but had little effect in the distal ones; this highlights the differences in gene regulation between the different regions of the epididymis (17;19;40-42).

This study demonstrates that androgens regulate the expression of both pro- and anti-apoptotic transcripts in a region-specific manner in the epididymis, some of which could potentially be transcriptionally-regulated by androgens. This suggests an ability of the epididymis to maintain a balance between pro- and anti-apoptotic responses and hence explain why although there are some apoptotic cells in the epididymis after androgen withdrawal by orchidectomy, their number is limited (12).

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Table 1: Description of genes represented on the apoptosis-focused arrays

Position	Gene symbol	Description	Gene family	RefSeq	UniGene	Entrez Gene ID
1	*Ppia*	Peptidylprolyl isomerase A (Cyclophilin A)	Other genes family	NM_017101	Rn.1463	25518
2	*Apaf1*	Apoptotic peptidase activating factor 1	CARD family	NM_023979	Rn.64522	78963
3	*Pycard*	Apoptosis-associated speck-like protein containing a CARD	CARD family	NM_172322	Rn.64522	282817
4	*Atm*	Ataxia telangiectasia mutated homolog (human)	p53 and ATM pathway	XM_236275	Rn.7817	300711
5	*Bad*	Bcl2-associated death promoter	Bcl2 family	NM_022698	Rn.98962	64639
6	*Baiap2*	Brain-specific angiogenesis inhibitor 1-associated protein 2	Other genes family	NM_057196	Rn.36696	117542
7	*Bak1*	Bcl2-antagonist/killer 1	Bcl2 family	NM_053812	Rn.95155	116502
8	*Bax*	Bcl2-associated X protein	Bcl2 family	NM_017059	Rn.14598	24887
9	*Bcl10*	B-cell CLL/lymphoma 10	Bcl2 family	NM_031328	Rn.10668	83477
10	*Bcl2*	B-cell leukemia/lymphoma 2	Bcl2 family	NM_016993	Rn.13007	24224
11	*Bcl2a1*	B-cell leukemia/lymphoma 2 related protein A1	Bcl2 family	NM_133416	Rn.9996	170929
12	*Bcl2l1*	Bcl2-like 1	Bcl2 family	NM_031535	Rn.19770	24888
13	*Bcl2l10*	Bcl2-like 10	Bcl2 family	NM_053733	Rn.10323	114552
14	*Bcl2l11*	Bcl2-like 11 (apoptosis facilitator)	Bcl2 family	NM_022612	Rn.67084	64547
15	*Bcl2l2*	Bcl2-like 2	Bcl2 family	NM_021850	Rn.82709	60434
16	*Becn1*	Beclin 1 (coiled-coil, myosin-like BCL2-interacting protein)	Bcl2 family	NM_053739	Rn.44267	114558
17	*Hrk*	BH3 interacting (with Bcl2 family) domain, apoptosis agonist	Bcl2 family	NM_057130	Rn.2776	117271
18	*Bik*	Bcl2-interacting killer-like	Bcl2 family	NM_053704	Rn.89639	114496
19	*Naip2*	Baculoviral IAP repeat-containing 1b	IAP family	XM_226742	Rn.38487	191568
20	*Birc3*	Inhibitor of apoptosis protein 1	IAP family	NM_023987	Rn.92423	78971
21	*Xiap*	X-linked inhibitor of apoptosis	IAP family	NM_022231	Rn.64578	63879
22	*Birc5*	Baculoviral IAP repeat-containing 5	IAP family	NM_022274	Rn.91239	64041
23	*Blk*	B lymphoid kinase (predicted)	IAP family	XM_344419	Rn.54471	364403
24	*Bmf*	Bcl-2 modifying factor	Bcl2 family	NM_139258	Rn.20030	246142
25	*Bnip1*	Bcl2/adenovirus E1B 19kDa-interacting protein 1	Bcl2 family	NM_080897	Rn.72585	140932
26	*Bnip3*	Bcl2/adenovirus E1B 19 kDa-interacting protein 3	Bcl2 family	NM_053420	Rn.16757	84480
27	*Bnip3l*	Bcl2/adenovirus E1B 19 kDa-interacting protein 3-like	Bcl2 family	NM_080888	Rn.2060	140923
28	*Bok*	Bcl-2-related ovarian killer protein	Bcl2 family	NM_017312	Rn.827	29884

Position	Gene symbol	Description	Gene family	RefSeq	UniGene	Entrez Gene ID
29	*Casp11*	Caspase 11	Caspase family	NM_053736	Rn.44461	114555
30	*Casp12*	Caspase 12	Caspase family	NM_130422	Rn.16195	156117
31	*Casp2*	Caspase 2	Caspase family	NM_022522	Rn.81078	64314
32	*Casp6*	Caspase 6	Caspase family	NM_031775	Rn.1438	83584
33	*Casp7*	Caspase 7	Caspase family	NM_022260	Rn.88160	64026
34	*Casp8*	Caspase 8	Caspase family	NM_022277	Rn.53995	64044
35	*Casp8ap2*	Caspase 8 associated protein 2	Death domain family	XM_232860	Rn.54474	313128
36	*Casp9*	Caspase 9	Caspase family	NM_031632	Rn.9052	58918
37	*Cflar*	CASP8 and FADD-like apoptosis regulator	Death domain family	NM_057138	Rn.32199	117279
38	*Chek1*	Checkpoint kinase 1 homolog (S. pombe)	p53 and ATM pathway	NM_080400	Rn.28010	140583
39	*Cidea*	Cell death-inducing DNA fragmentation factor, alpha subunit-like effector A (predicted)	CIDE domain family	XM_214551	Rn.33267	291541
40	*Cideb*	Cell death-inducing DNA fragmentation factor, alpha subunit-like effector B (predicted)	CIDE domain family	XM_344410	Rn.8171	364388
41	*Cntnap1*	Contactin associated protein 1	Other genes family	NM_032061	Rn.91559	84008
42	*Cradd*	CASP2 and RIPK1 domain containing adaptor with death domain (predicted)	Death domain family	XM_235061	Rn.88654	314756
43	*Dap3*	Death associated protein 3 (predicted)	Death domain family	XM_215627	Rn.85739	295238
44	*Dapk2*	Similar to Death-associated protein kinase 2 (DAP kinase 2) (DAP-like kinase) (Dlk) (ZIP-kinase)	Death domain family	NM_022546	Rn.1566	64391
45	*Dffa*	DNA fragmentation factor, alpha subunit	CIDE domain family	NM_053679	Rn.60353	114214
46	*Dffb*	DNA fragmentation factor, beta subunit	CIDE domain family	NM_053362	Rn.48799	84359
47	*E2f3*	Similar to E2f3 protein (LOC291105), mRNA	p53 and ATM pathway	XM_214476	Rn.67077	291105
48	*E2f5*	E2F transcription factor 5	p53 and ATM pathway	XM_574892	Rn.73967	116651
49	*E2f6*	E2F transcription factor 6	p53 and ATM pathway	XM_233986	Rn.127928	313978
50	*Fadd*	Fas (TNFRSF6)-associated via death domain	Death domain family	NM_152937	Rn.79506	266610
51	*Gadd45a*	Growth arrest and DNA-damage-inducible 45 alpha	p53 and ATM pathway	NM_024127	Rn.16183	25112
52	*Card9*	Caspase recruitment domain protein 9	CARD family	NM_022303	Rn.10250	64171
53	*Cd40lg*	Tumor necrosis factor (ligand) superfamily, member 5 (CD40 ligand)	TNF ligand family	NM_053353	Rn.64486	84349
54	*Ltb*	Lymphotoxin B	TNF ligand family	NM_212507	Rn.44218	361795
55	*Ltbr*	Lymphotoxin B receptor (predicted)	TNFR family	NM_001008315	Rn.128906	297604
56	*Lyst*	Lysosomal trafficking regulator	Other genes family	NM_053518	Rn.19329	85419

Position	Gene symbol	Description	Gene family	RefSeq	UniGene	Entrez Gene ID
57	*Mcl1*	Myeloid cell leukemia sequence 1	Bcl2 family	NM_021846	Rn.44274	60430
58	*Myd88*	Myeloid differentiation primary response gene 88	Death domain family	NM_198130	Rn.4067	301059
59	*Ngfrap1*	Nerve growth factor receptor associated protein 1	TNFR family	NM_053401	Rn.37341	117089
60	*Rad1*	Similar to Rad1p (LOC294800), mRNA	p53 and ATM pathway	XM_215497	Rn.3126	294800
61	*Rad23a*	Similar to UV excision repair protein RAD23 homolog A (MHR23A) (LOC361381), mRNA	p53 and ATM pathway	XM_341660	Rn.140834	361381
62	*Rad50*	RAD50 homolog (S. cerevisiae)	p53 and ATM pathway	NM_022246	Rn.51136	64012
63	*Rad52*	Similar to Rad52 protein	p53 and ATM pathway	NM_001106617	Rn.8154	297561
64	*Chek2*	Protein kinase Chk2	p53 and ATM pathway	NM_053677	Rn.18487	114212
65	*Rem2*	Rad and gem related GTP binding protein 2	Other genes family	NM_022685	Rn.48804	64626
66	*Rfng*	Radical fringe gene homolog (Drosophila)	Other genes family	NM_021849	Rn.44231	60433
67	*Ripk2*	Similar to receptor-interacting protein 2 (LOC362491), mRNA	Death domain family	XM_342810	Rn.139983	362491
68	*Rrad*	Ras-related associated with diabetes	Other genes family	NM_053338	Rn.11189	83521
69	*Tank*	TRAF family member-associated Nf-kappa B activator	TRAF family	NM_145788	Rn.89906	252961
70	*Tnfaip2*	Similar to [Mouse primary response gene B94 mRNA, 3end.], gene product	Other genes family	XM_216791	Rn.34387	299339
71	*Tnfrsf26*	Tumor necrosis factor receptor superfamily, member 26 (predicted)	TNFR family	XM_341968	Rn.138243	361685
72	*Tnfrsf10b*	Similar to TRAIL receptor2 KILLER/DR5 homologue (LOC364420), mRNA	TNFR family	XM_344431	Rn.105558	364420
73	*Tnfrsf11b*	Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	TNFR family	NM_012870	Rn.9792	25341
74	*Tnfrsf12a*	Tumor necrosis factor receptor superfamily, member 12a	TNFR family	NM_181086	Rn.105040	302965
75	*Tnfrsf1a*	Tumor necrosis factor receptor superfamily, member 1a	TNFR family	NM_013091	Rn.11119	25625
76	*Tnfrsf1b*	Tumor necrosis factor receptor superfamily, member 1b	TNFR family	NM_130426	Rn.83633	156767
77	*Tnfrsf4*	Tumor necrosis factor receptor superfamily, member 4	TNFR family	NM_013049	Rn.48883	25572
78	*Tnfrsf8*	Tumor necrosis factor receptor superfamily, member 8	TNFR family	NM_019135	Rn.11322	25069
79	*Tnfsf10*	Tumor necrosis factor (ligand) superfamily, member 10	TNF ligand family	NM_145681	Rn.83627	246775
80	*Tnfsf12*	Tumor necrosis factor ligand superfamily member 12	TNF ligand family	NM_001001513	Rn.3211	360548
81	*Tnfsf13*	Tumor necrosis factor ligand superfamily, member 13	TNF ligand family	NM_001009623	Rn.19955	287437
82	*Tnfsf15*	Tumor necrosis factor (ligand) superfamily, member 15	TNF ligand family	NM_145765	Rn.84873	252878
83	*CD70*	Similar to CD70 protein (CD27 ligand) (LOC301132), mRNA	TNF ligand family	XM_217320	Rn.103013	301132
84	*Tnfsf9*	Tumor necrosis factor (ligand) superfamily, member 9	TNF ligand family	NM_181384	Rn.46783	353218

Position	Gene symbol	Description	Gene family	RefSeq	UniGene	Entrez Gene ID
85	*Tnip2*	TNFAIP3 interacting protein 2 (predicted)	Other genes family	NM_001024771	Rn.17607	305451
86	*Tp53*	Tumor protein p53	p53 and ATM pathway	NM_030989	Rn.54443	24842
87	*Zranb1*	Zinc finger, RAN-binding domain containing 1 (predicted)	Other genes family	XM_215101	Rn.259	360216
88	*Tradd*	TNFRSF1A-associated via death domain	TRAF family	XM_341671	Rn.18545	246756
89	*Traf2*	Tnf receptor-associated factor 2 (predicted)	TRAF family	XM_231032	Rn.105232	311786
90	*Traf4*	Similar to TNF receptor associated factor 4 (LOC303285), mRNA	TRAF family	XM_220640	Rn.3219	303285
91	*Traip*	TRAF-interacting protein (predicted)	TRAF family	XM_345981	Rn.8891	367167
92	*Uba3*	Ubiquitin-activating enzyme E1C	Other genes family	NM_057205	Rn.2141	117553
93	*Uba1*	Similar to ubiquitin-protein ligase (EC 6.3.2.19) E1 - mouse	Other genes family	NM_001014080	Rn.11800	314432
94	*Ube2d2*	Ubiquitin-conjugating enzyme E2D 2	Other genes family	NM_031001	Rn.114675	79435
95	*Ube2d3*	Ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast)	Other genes family	NM_031237	Rn.2778	81920
96	*Ube2i*	Ubiquitin-conjugating enzyme E2I	Other genes family	NM_013050	Rn.2274	25573
97	*Ube2n*	Ubiquitin-conjugating enzyme E2N (homologous to yeast UBC13)	Other genes family	NM_053928	Rn.101834	116725
98	Blank	Blank	N/A	N/A	N/A	N/A
99	PUC18	PUC18 plasmid DNA	N/A	L08752	N/A	N/A
100	Luc1	Luciferase probe 1	N/A	N/A	N/A	N/A
101	Luc2	Luciferase probe 2	N/A	N/A	N/A	N/A
102	AS1R2	Artificial sequence 1 related 2 (80% identity) (48/60)	N/A	N/A	N/A	N/A
103	AS1R1	Artificial sequence 2 related 1 (90% identity) (56/60)	N/A	N/A	N/A	N/A
104	AS1	Artificial sequence 1	N/A	N/A	N/A	N/A
105	Rpl32	Ribosomal protein L32	N/A	NM_013226	Rn.110966	28298
106	Ldha	Lactate dehydrogenase A	N/A	NM_017025	Rn.107896	24533
107	Aldoa1	Aldolase A	N/A	NM_012495	Rn.1774	24189
108	Aldoa2	Aldolase A	N/A	NM_012495	Rn.1774	24189
109	Gapd1	Glyceraldehyde-3-phosphate dehydrogenase	N/A	NM_017008	Rn.91450	24383
110	Gapd2	Glyceraldehyde-3-phosphate dehydrogenase	N/A	NM_017008	Rn.91450	24383
111	BAS2C1	Biotinylated artificial sequence 2 complementary sequence	N/A	N/A	N/A	N/A
112	BAS2C2	Biotinylated artificial sequence 2 complementary sequence	N/A	N/A	N/A	N/A

Table 2: Real-Time RT-PCR primers

Gene name	Gene symbol	Accession no.	Forward primer sequence (5'→ 3')	Reverse primer sequence (5'→ 3')
Peptidylprolyl isomerase A (Cyclophilin A)	*Ppia*	NM_017101	GTGGTCTTTGGG AAGGTGAA	GTTGTCCACAGT CGGAGATG
RAD52 homolog (S. cerevisiae)	*Rad52*	XM_216230	CAAACCTCTGTC ACCCGAAC	TCCACGAACCTC TGCTACCT
Myeloid cell leukemia sequence 1	*Mcl1*	NM_021846	TCTTTTGGTGCCT TTGTGG	CCATCCCAGCCT CTTTGTT
Bcl2 modifying factor	*Bmf*	NM_139258	TTGTGGGGTGAC AGAGGAA	TATGAAGCCGAT GGAACTGG
Tumor necrosis factor receptor superfamily, member 11b	*Tnfrsf11b*	NM_012870	QuantiTect Primer Assays (Qiagen Inc.) QT00177170	
Tumor necrosis factor (ligand) superfamily, member 11 (RANKL)	*Tnfsf11*	NM_057149	QuantiTect Primer Assays (Qiagen Inc.) QT00195125	
Tumor necrosis factor receptor superfamily, member 11a (RANK)	*Tnfrsf11a*	XM_573424	QuantiTect Primer Assays (Qiagen Inc.) QT01689905	

Table 3: Transcripts up- or down-regulated by at least 1.5 fold at 0.5 and/or 1 day after orchidectomy without testosterone replacement

Common gene name	Gene symbol	RefSeq ccession no.	Control		0.5 day		1 day	
			Normalized data	SEM	Normalized data	SEM	Normalized data	SEM
Initial Segment								
Bcl2								
Bcl2-modifying factor	*Bmf*	NM_139258	0.40934043	0.054239	**0.789424**	0.167924	**1.031204**	0.051191
Myeloid cell leukemia sequence 1	*Mcl1*	NM_021846	0.37085665	0.060043	**0.570475**	0.11591	0.397362	0.046517
Caspase								
Caspase 4	*Casp4*	NM_053736	0.48801043	0.075825	**0.690645**	0.194611	0.669297	0.194153
Caspase 12	*Casp12*	NM_130422	0.15114109	0.042499	**0.226997**	0.086834	0.15187	0.074924
TNFR								
Tumor necrosis factor receptor superfamily, member 11b	*Tnfrsf11b*	NM_012870	1.78893458	0.332313	**1.142863**	0.395951	**0.828257**	0.206559
Lymphotoxin B receptor	*Ltbr*	NM_001008315	0.10107365	0.022974	**0.143557**	0.064115	0.140888	0.028291
Tumor necrosis factor receptor superfamily, member 26	*Tnfrsf26*	XM_341968	0.26014051	0.050132	**0.167951**	0.05687	0.476541	0.086893
TNF ligand								
Tumor necrosis factor (ligand) supefamily, member 5	*Cd40lg*	NM_053353	0.12102131	0.035183	**0.047314**	0.012696	**0.278893**	0.051197
p53 and ATM pathway								
Rad50 homolog (S. cerevisiae)	*Rad50*	NM_022246	0.32456447	0.020979	**0.181667**	0.066575	**0.518392**	0.034832
IAP								
Baculoviral IAP repeat-containing 5	*Birc5*	NM_022274	0.7717686	0.378798	0.406471	0.088167	**0.299685**	0.118111
Other related genes								
Similar to ubiquitin-protein ligase (EC 6.3.2.19) E1 - mouse	*Uba1*	XM_234520	3.70594412	0.790705	**2.073322**	0.58954	2.93136	0.354547
Ubiquitin-conjugating enzyme E2D 2	*Ube2d2*	NM_031001	1.78672549	0.82728	**0.588141**	0.164307	**0.62278**	0.157824
Ubiquitin-conjugating enzyme E2N (homologous to yeast UBC13)	*Ube2n*	NM_053928	2.54147794	0.394155	**1.261239**	0.365329	**1.141944**	0.297575
Caput								
Bcl2								
Myeloid cell leukemia sequence 1	*Mcl1*	NM_021846	0.321628	0.032479	**0.501661**	0.097503	0.434955	0.066995

Common gene name	Gene symbol	RefSeq ccession no.	Control		0.5 day		1 day	
			Normalized data	SEM	Normalized data	SEM	Normalized data	SEM
Caput								
Caspase								
Caspase 2	*Casp2*	NM_022522	0.909961	0.1789	**1.344735**	0.288303	1.289438	0.159017
Caspase 6	*Casp6*	NM_031775	0.866245	0.20476	**1.309098**	0.237492	**1.301303**	0.161052
Caspase 7	*Casp7*	NM_022260	0.137724	0.049584	0.135561	0.040675	**0.20962**	0.057518
TNFR								
Tumor necrosis factor receptor superfamily, member 1a	*Tnfrsf1a*	NM_013091	2.50914	0.42541	**4.074626**	0.979213	2.995805	0.257133
Tumor necrosis factor receptor superfamily, member 11b	*Tnfrsf11b*	NM_012870	0.769079	0.125149	**1.32231**	0.314527	0.738478	0.150291
TNF ligand								
Tumor necrosis factor (ligand) supefamily, member 5	*Cd40lg*	NM_053353	0.265458	0.047244	**0.155737**	0.033577	**0.133152**	0.055813
IAP								
Baculoviral IAP repeat-containing 5	*Birc5*	NM_022274	0.965077	0.404256	**0.322728**	0.076115	**0.440603**	0.120408
CIDE domain								
Cell death-inducing DNA fragmentation factor, alpha subunit-like effector A	*Cidea*	XM_214551	1.058603	0.112453	0.7288	0.105002	**0.691197**	0.112754
DNA fragmentation factor, alpha subunit	*Dffa*	NM_053679	0.160563	0.040715	0.103453	0.010754	**0.07765**	0.006382
Other related genes								
Contactin associated protein 1	*Cntnap1*	NM_032061	0.315036	0.100303	0.380396	0.050984	**0.570146**	0.095345
Ubiquitin-conjugating enzyme E2D 2	*Ube2d2*	NM_031001	2.282172	0.890252	**0.681419**	0.124342	**0.729433**	0.099234
Ubiquitin-conjugating enzyme E2N (homologous to yeast UBC13)	*Ube2n*	NM_053928	2.555367	0.458624	1.800853	0.397431	**1.298846**	0.160252
Corpus								
Bcl2								
Bcl2-associated death promoter	*Bad*	NM_022698	5.3308382	1.8032903	**3.2062952**	0.8756033	**3.47862088**	0.82938844
B-cell leukemia/lymphoma 2	*Bcl2*	NM_016993	0.9716471	0.1288422	**0.5991197**	0.0314996	0.63368987	0.07806264
Bcl2-modifying factor	*Bmf*	NM_139258	0.0978459	0.0133886	0.1267606	0.0215375	**0.21856063**	0.07740242
Bcl2/adenovirus E1B 19 kDa-interacting protein 3	*Bnip3*	NM_053420	5.0056228	1.4611783	**3.1709671**	0.7874351	**3.21834374**	0.70533985
Bcl2/adenovirus E1B 19 kDa-interacting protein 3-like	*Bnip3l*	NM_080888	5.3632147	1.7543086	**2.991277**	0.7986549	**3.29487174**	0.70413188

Common gene name	Gene symbol	RefSeq ccession no.	Control		0.5 day		1 day	
			Normalized data	SEM	Normalized data	SEM	Normalized data	SEM
Corpus								
Bcl2								
Bcl2-related ovarian killer protein	*Bok*	NM_017312	0.5003537	0.049399	**0.3230292**	0.0829255	**0.29939159**	0.09808904
TNFR								
Tumor necrosis factor receptor superfamily, member 11b	*Tnfrsf11b*	NM_012870	0.7703931	0.2212263	**1.2551548**	0.1890399	0.80033398	0.15642697
Tumor necrosis factor receptor superfamily, member 12a	*Tnfrsf12a*	NM_181086	0.2624038	0.0547693	**0.4036038**	0.0551533	**0.38967649**	0.10625918
TNF ligand								
Tumor necrosis factor (ligand) supefamily, member 5	*Cd40lg*	NM_053353	0.3284984	0.1143968	**0.2058245**	0.0868718	0.25778231	0.07277238
Tumor necrosis factor (ligand) supefamily, member 12	*Tnfsf12*	NM_001001513	3.6955812	0.6973497	**2.0042274**	0.259968	**2.17746348**	0.33715339
p53 and ATM pathway								
Similar to Rad52 protein	*Rad52*	XM_216230	0.4645132	0.0933588	0.5750735	0.0826803	**0.96485882**	0.24169737
IAP								
Baculoviral IAP repeat containing 1b	*Naip2*	XM_226742	0.6585121	0.1139367	**0.3987811**	0.0971684	**0.42945071**	0.11802074
Inhibitor of apoptosis protein 1	*Birc3*	NM_023987	0.5038832	0.127144	**0.8044672**	0.1380889	**0.58024889**	0.11953076
Baculoviral IAP repeat-containing 5	*Birc5*	NM_022274	0.6319154	0.227017	**0.2992543**	0.1227835	**0.41559259**	0.1917546
Death domain								
Similar to death-associated protein kinase 3	*Dlk*	NM_022546	0.774055	0.1033771	1.0438983	0.0921475	**1.33152326**	0.11336774
TRAF								
TNFRSF1A-associated via death domain	*Tradd*	XM_341671	3.9400301	0.6654751	2.7907911	0.5632055	**2.43985168**	0.38720894
TRAF-interacting protein	*Traip*	XM_345981	4.6974083	1.2622602	**3.0746429**	0.6787619	3.8357509	1.05507702
CARD								
Apoptotic peptidase activating factor 1	*Apaf1*	NM_023979	1.6521423	0.1147028	1.3388849	0.1689797	**1.09864884**	0.16728705
CIDE								
Cell death-inducing DNA fragmentation factor, alpha subunit-like effector A (predicted)	*Cidea*	XM_214551	1.3807691	0.2498749	**0.8771426**	0.1095429	1.26181598	0.21580838
Other related genes								
Ubiquitin-conjugating enzyme E2I	*Ube2i*	NM_013050	5.2085371	1.4617699	**3.3758484**	0.9269434	**3.08150866**	0.63197458

Common gene name	Gene symbol	RefSeq ccession no.	Control		0.5 day		1 day	
			Normalized data	SEM	Normalized data	SEM	Normalized data	SEM
Cauda								
Bcl2								
Bcl2-associated death promoter	*Bad*	NM_022698	5.21315366	1.20808695	3.9156339	1.03681733	**3.29775994**	1.11484272
BCL2/adenovirus E1B 19 kDa-interacting protein 3	*Bnip3*	NM_053420	4.35857576	0.9159602	3.7752926	0.92499396	**2.8460127**	0.80051414
Bcl2/adenovirus E1B 19 kDa-interacting protein 3-like	*Bnip3l*	NM_080888	4.9231564	1.10688026	3.96280222	1.07685546	**3.1145131**	0.90385031
Myeloid cell leukemia sequence 1	*Mcl1*	NM_021846	0.3195984	0.05000459	0.45736013	0.08336547	**0.63172688**	0.10782476
Caspase								
Caspase 6	*Casp6*	NM_031775	0.72878328	0.14782277	**1.04637876**	0.08009275	0.87752878	0.1056822
Caspase 12	*Casp12*	NM_130422	0.07709069	0.01927363	**0.04257377**	0.00770932	n/d	n/d
TNFR								
Tumor necrosis factor receptor superfamily, member 4	*Tnfrsf4*	NM_013049	0.25862187	0.06699828	0.33339111	0.03732649	**0.56268449**	0.11219838
Tumor necrosis factor receptor superfamily, member 11b	*Tnfrsf11b*	NM_012870	0.53216731	0.13778912	**1.41210513**	0.15755346	**1.01937554**	0.18914176
Tumor necrosis factor receptor superfamily, member 12a	*Tnfrsf12a*	NM_181086	0.30653683	0.0837452	0.36228225	0.03117065	**0.50315919**	0.12659097
Nerve growth factor receptor associated protein 1	*Ngfrap1*	NM_053401	4.6027577	0.95689212	3.70730262	0.85403306	**2.82476324**	0.56814126
TNF lignad								
Tumor necrosis factor (ligand) supefamily, member 5	*Cd40lg*	NM_053353	0.14546239	0.03108485	**0.25173048**	0.06661363	**0.32459246**	0.04783272
p53 and ATM pathway								
Rad50 homolog (S. cerevisiae)	*Rad50*	NM_022246	0.19885073	0.03568353	0.27336273	0.0453464	**0.34182224**	0.05862731
Similar to Rad52 protein	*Rad52*	XM_216230	0.38358991	0.14034804	0.51761002	0.09661152	**0.72248745**	0.18221105
IAP								
Baculoviral IAP repeat-containing 5	*Birc5*	NM_022274	0.70780642	0.19942366	0.53386061	0.18267595	**0.39679955**	0.14592724
Death domain								
Similar to receptor-interacting protein 2	*Ripk2*	XM_342810	0.47223832	0.06337911	0.73078114	0.05605016	0.84593964	0.03207672

Common gene name	Gene symbol	RefSeq ccession no.	Control		0.5 day		1 day	
			Normalized data	SEM	Normalized data	SEM	Normalized data	SEM
Cauda								
TRAF								
TRAF family member –associated Nf-kappa B activator	*Tank*	NM_145788	0.36958771	0.03997937	**0.55466787**	0.09456229	**0.65841861**	0.08775091
TRAF-interacting protein	*Traip*	XM_345981	4.69989904	0.96909914	4.71978772	1.8488873	**3.32413358**	1.06187451
Other related genes								
Radical fringe gene homolog (Drosophila)	*Rfng*	NM_021849	0.14926334	0.03061862	**0.22319633**	0.01991499	**0.38869603**	0.05208173
Ras-related associated with diabetes	*Rrad*	NM_053338	0.53649085	0.07495119	0.60912473	0.06880419	**0.80291418**	0.05889224
Similar to ubiquitin-protein ligase (EC 6.3.2.19) E1 - mouse	*Uba1*	XM_234520	3.40950014	0.49078175	2.94092978	0.48397088	**2.19792514**	0.38929124

Transcripts up- or down-regulated by at least 1.5 fold are identified in bold; n/d stands for non-detectable

Table 4: Transcripts up- or down-regulated by at least 1.5 fold at 0.5 and/or 1 day after orchidectomy with testosterone replacement

Common gene name	Gene symbol	RefSeq ccession no.	Control		0.5 day		1 day	
			Normalized data	SEM	Normalized data	SEM	Normalized data	SEM
Initial Segment								
Bcl2								
Bcl2-like 10	*Bcl2l10*	NM_053733	0.32874248	0.068308	0.382972	0.053767	**0.174795**	0.059813
Bcl2-modifying factor	*Bmf*	NM_139258	0.40934043	0.054239	**0.714838**	0.125488	**0.642543**	0.178543
Bcl2-related ovarian killer protein	*Bok*	NM_017312	0.426749	0.011513	0.314023	0.033077	**0.231021**	0.029642
Myeloid cell leukemia sequence 1	*Mcl1*	NM_021846	0.37085665	0.060043	0.470733	0.116133	**0.5543**	0.064327
Caspases								
Caspase 8	*Casp8*	NM_022277	0.86235821	0.100975	0.613806	0.094203	**0.521426**	0.098404
TNFR								
Tumor necrosis factor receptor superfamily, member 11b	*Tnfrsf11b*	NM_012870	1.78893458	0.332313	1.254544	0.436622	**1.153427**	0.206147
p53 and ATM pathway								
E2F transcription factor 6	*E2f6*	XM_233986	0.13430787	0.014984	0.182829	0.054708	**0.046384**	0.004163
IAP								
Baculoviral IAP repeat-containing 5	*Birc5*	NM_022274	0.7717686	0.378798	0.453868	0.087976	**0.249139**	0.093363
TRAF								
TNF-receptor-associated factor 2	*Traf2*	XM_231032	0.27788451	0.029784	**0.126436**	0.041348	0.347265	0.143119
CIDE domain								
Cell death-inducing DNA fragmentation factor, alpha subunit-like effector A	*Cidea*	XM_214551	0.82618949	0.119574	**0.500751**	0.10317	0.6724	0.103812
Other related genes								
Ubiquitin-conjugating enzyme E2D 2	*Ube2d2*	NM_031001	1.78672549	0.82728	**0.571827**	0.144049	**0.63545**	0.050453
Ubiquitin-conjugating enzyme E2N (homologous to yeast UBC13)	*Ube2n*	NM_053928	2.54147794	0.394155	**1.303364**	0.230394	**1.562261**	0.184123
Caput								
Bcl2								
Bcl2-associated X protein	*Bax*	NM_017059	3.261147	0.910384	**5.60284132**	1.936713184	3.7373389	0.823627
Bcl2-interacting killer-like	*Bik*	NM_053704	0.674765	0.156344	0.703170416	0.09399545	**0.9020971**	0.0895715
Caspase								
Caspase 11	*Casp11*	NM_053736	0.471132	0.141405	0.324365968	0.078483832	**0.2012063**	0.0374507
Caspase 12	*Casp12*	NM_130422	0.228392	0.101024	**0.12429134**	0.022921861	0.0453879	0.0191903

Common gene name	Gene symbol	RefSeq ccession no.	Control		0.5 day		1 day	
			Normalized data	SEM	Normalized data	SEM	Normalized data	SEM
Caput								
TNFR								
Tumor necrosis factor receptor superfamily, member 1a	*Tnfrsf1a*	NM_013091	2.50914	0.42541	**3.77729182**	0.413842043	3.3499968	0.5107648
Tumor necrosis factor receptor superfamily, member 1b	*Tnfrsf1b*	NM_013091	0.726376	0.168016	**1.08112889**	0.14622327	**1.0285431**	0.0814386
TNF ligand								
Similar to CD70 protein (CD27 ligand) (LOC301132), mRNA	*CD70*	XM_217320	0.992105	0.231605	**1.45572486**	0.447748332	1.056433	0.0271561
Tumor necrosis factor (ligand) supefamily, member 10	*Tnfsf10*	NM_145681	2.215064	0.403791	**3.52851214**	0.609029946	**3.4290664**	0.9023652
IAP								
Inhibitor of apoptosis protein 1	*Birc3*	NM_023987	0.680104	0.109772	**0.450842432**	0.065024514	0.7722568	0.1617907
Baculoviral IAP repeat-containing 5	*Birc5*	NM_022274	0.965077	0.404256	**0.222522902**	0.045682092	**0.3426076**	0.1051455
Death effector domain								
CASP8 and FADD-like apoptosis regulator	*Cflar*	NM_057138	0.614871	0.144279	0.51081597	0.127109058	**0.386857**	0.070083
Similar to death-associated protein kinase 3	*Dlk*	NM_022546	1.124439	0.082425	1.2254541	0.214785644	**1.8013025**	0.1619393
TRAF								
TRAF-interacting protein	*Traip*	XM_345981	3.981135	1.167827	**6.16929032**	2.008548637	4.7354594	1.5602951
CIDE domain								
Cell death-inducing DNA fragmentation factor, alpha subunit-like effector A	*Cidea*	XM_214551	1.058603	0.112453	**0.51320383**	0.113172024	0.7163516	0.1330793
Other related genes								
Contactin associated protein 1	*Cntnap1*	NM_032061	0.315036	0.100303	0.247506749	0.06009225	**0.5247461**	0.0619264
Radical fringe gene homolog (Drosophila)	*Rfng*	NM_021849	0.13559	0.01668	0.22531383	0.083382485	**0.2542247**	0.0438113
Ubiquitin-conjugating enzyme E2D 2	*Ube2d2*	NM_031001	2.282172	0.890252	**0.87422604**	0.133592443	**0.57911**	0.0574263
Ubiquitin-conjugating enzyme E2N (homologous to yeast UBC13)	*Ube2n*	NM_053928	2.555367	0.458624	**1.36877858**	0.127590711	**1.6573192**	0.1224044
Corpus								
Bcl2								
BCL2-antagonist/killer 1	*Bak1*	NM_053812	1.4280128	0.2161611	1.80348332	0.25137763	**2.287699**	0.5510879
Bcl2-like 2	*Bcl2l2*	NM_021850	0.1096359	0.0234872	0.29967214	0.12357943	**0.2882156**	0.1266244
Bcl2-related ovarian killer protein	*Bok*	NM_017312	0.5003537	0.049399	0.40033829	0.050869	**0.304047**	0.0792966

Common gene name	Gene symbol	RefSeq ccession no.	Control		0.5 day		1 day	
			Normalized data	SEM	Normalized data	SEM	Normalized data	SEM
Corpus								
Caspase								
Caspase 7	*Casp7*	NM_022260	0.1502441	0.0303747	0.19072327	0.04898823	**0.2931508**	0.0530957
Caspase 12	*Casp12*	NM_130422	0.0924266	0.0424318	0.10695932	0.04284573	**0.1801705**	0.1005149
TNF ligand								
Tumor necrosis factor (ligand) supefamily, member 5	*Cd40lg*	NM_053353	0.3284984	0.1143968	**0.15973511**	0.03887752	0.27547	0.0545888
Tumor necrosis factor (ligand) superfamily, member 15	*Tnfsf15*	NM_145765	0.3092401	0.0662743	0.36258112	0.04533234	**0.1917702**	0.0559313
p53 and ATM pathway								
Similar to E2f3 protein (LOC291105), mRNA	*E2f3*	XM_214476	0.1375977	0.0289928	**0.1048704**	0.03948196	0.1811454	0.0809456
E2F transcription factor 6	*E2f6*	XM_233986	0.0978354	0.0203553	**0.16431959**	0.04088362	0.1685533	0.0367578
Death effector domain								
CASP8 and FADD-like apoptosis regulator	*Cflar*	NM_057138	0.6857112	0.067224	0.64714758	0.10932038	**0.4283372**	0.063359
CIDE domain								
Cell death-inducing DNA fragmentation factor, alpha subunit-like effector A	*Cidea*	XM_214551	1.3807691	0.2498749	**0.7238651**	0.11842147	0.9442768	0.2052631
DNA fragmentation factor, alpha subunit	*Dffa*	NM_053679	0.0885367	0.0177638	0.09880605	0.03442712	**0.1737388**	0.0557716
Cauda								
Bcl2								
Bcl2-like 1	*Bcl2l1*	NM_031535	0.1897063	0.06413434	0.21776464	0.02620002	**0.292752369**	0.10149012
TNFR								
Tumor necrosis factor receptor superfamily, member 4	*Tnfrsf4*	NM_013049	0.25862187	0.06699828	**0.46926169**	0.0642675	0.304896006	0.05207210
Tumor necrosis factor receptor superfamily, member 12a	*Tnfrsf12a*	NM_181086	0.30653683	0.0837452	**0.45390488**	0.14050504	0.20970613	0.06192642
TNF ligand								
Tumor necrosis factor (ligand) superfamily, member 5	*Cd40lg*	NM_053353	0.14546239	0.03108485	0.20639962	0.03596576	**0.243658534**	0.04745598
Tumor necrosis factor (ligand) superfamily, member 9	*Tnfsf9*	NM_181384	0.40851006	0.0535702	**0.60935561**	0.04594509	0.459303158	0.04571178
Tumor necrosis factor (ligand) superfamily, member 15	*Tnfsf15*	NM_145765	0.29663853	0.07108684	**0.78500072**	0.43939574	0.286680282	0.07449125

Common gene name	Gene symbol	RefSeq ccession no.	Control		0.5 day		1 day	
			Normalized data	SEM	Normalized data	SEM	Normalized data	SEM
Cauda								
p53 and ATM pathway								
Rad50 homolog (S. cerevisiae)	*Rad50*	NM_022246	0.19885073	0.03568353	**0.34221285**	0.0325397	**0.306989336**	0.03633621
Similar to Rad52 protein	*Rad52*	XM_216230	0.38358991	0.14034804	0.53235596	0.08149214	**0.5507258**	0.07253780
IAP								
Baculoviral IAP repeat-containing 5	*Birc5*	NM_022274	0.70780642	0.19942366	**0.41173006**	0.10105858	**0.314875226**	0.10642196
TRAF								
TNF-receptor-associated factor 2	*Traf2*	XM_231032	0.26672335	0.05671671	**0.45896248**	0.07433052	0.328456592	0.06471028
Other related genes								
Radical fringe gene homolog (Drosophila)	*Rfng*	NM_021849	0.14926334	0.03061862	**0.2380238**	0.05917722	0.179882786	0.03740126
Similar to [Mouse primary response gene B94 mRNA, 3end.], gene product	*Tnfaip2*	XM_216791	0.68077352	0.06653726	**1.04698491**	0.19870733	0.725659288	0.05318787

Transcripts up- or down-regulated by at least 1.5 fold are identified in bold.

Table 5: Identification of putative androgen response elements (AREs) up to 3kb upstream of available promoter sequences for the genes affected by orchidectomy with or without replacement in the epididymis.

Sequence: AGAACCnnnTGTTCT

Common gene name	Gene symbol	Genbank accession no.	Position (upstream)	Sequence
Bcl2-associated death promoter	*Bad*	XM_236275	235	GGAAGGAGCTGGTCT
			816	GGCTCCCGCTGCTCC
			887	CGATGTCAATGTCCT
			1876	ACAAGCCTCGGCTCA
			2062	AGCCTCCATCTTTCT
			2441	AGACCCCAGGGTCAC
			2590	AGACAGCACTGCACA
			2619	AGGACCCAGGGCTGT
			2777	AGCCCCAAGGGTACT
			2873	GACACACACTGGTCC
Brain-specific angiogenesis inhibitor 1-associated protein 2	*Baiap2*	NM_022698	159	ACAGCGCCCTGTCCA
			193	AGGACCCCTTGTTGC
			237	AGAAGCCAGGCCTGT
			1059	AGGTTCCTGTGTTCT
			1389	TAAACTCAGAGATCT
			1593	GGTCCCAACTGTCCT
			1658	CCAACCCAATATACA
			2019	ACTACCCACATTACC
			2101	AGACAGCAGTGTCCC
			2235	CAGGCCCACTTTTCC
			2348	TGGATTCCCTGTTTT
			2460	AGAACCCACGGCTTC
			2739	AGCACCCAGTTTGTT
			2812	GGAACTCACTCTGTA
Bcl2-associated X protein	*Bax*	NM_053812	962	AGTGCCAAATGTAGT
			1150	AGTATCTAGTGTAAT
			1329	CCACCCGACTCTTCT
			1436	AGTAGCCATGGCTGT
			1695	AAACTCTACTCTTCC
			1761	AACACTCAGGGTGCT
			1850	AGATCTCTATGTTCC
			1999	AAGGCCCAGGGTTCG
			2076	AAAACCCAACGCTTA
			2275	GGCAGTCACTGTCCC
			2364	AATCCCAACTTTTCT
			2408	GGGACCTACTTACCT
			2446	AGACACTCCTCTTCC
			2449	TGTAGACACTCCTCT
			2708	ACAACCTGCTCTCTT
			2711	CCAACAACCTGCTCT
			2750	AGCAGTCAGTGCTCT
			2752	AGAGCAGTCAGTGCT
			2996	AGTACGCAATATTGG
B-cell leukemia/lymphoma 2	*Bcl2*	NM_031328	296	AGATCATGCGGTCCT
			456	TGAGGACACAATTCT
			800	AAAACGCATTGGCCC
			892	ACCACACACAGTGCG
			1446	GCCACCCACGGTCCC
			1711	TGTACACACTTTACA
			1793	ACACTACACTGTTTA
			1883	ATAACATAATGTTTT
			1942	ACCACTCACTACTGT
			2061	ACTCACACCTGTTCT
			2064	AGCACTCACACCTGT

Common gene name	Gene symbol	Genbank accession no.	Position (upstream)	Sequence
B-cell leukemia/lymphoma 2	*Bcl2*	NM_031328	2404	AGGAAAAACTGTTCC
			2447	AGCACTCCCTGAGGT
			2654	CGAATGAACAGTTTT
			2695	AGCAGTCAGTGCTCT
			2697	AGAGCAGTCAGTGCT
			2880	AGGCCCACCTGGACT
Inhibitor of apoptosis protein 1	*Birc3*	XM_226742	242	AGAATCTAGTGTTTA
			398	ATAAAGCACAATTTT
			461	TAAACCAAAAGTTTT
			935	AGAGAAGACAGTTAT
			1247	ACAGCCAGCTCTTCT
			1443	AGTAACCCGTGCTCC
			1793	AGAAACTTAAGTCCT
			1892	GGGAACCTCTAGTCT
			2518	AGAAGAACTTGGTCT
			2727	TGGTCTCAATGTTTT
			2808	AGAAGCCCGTGTCTA
			2901	AAAACTGTTTGTTGT
B lymphoid kinase (predicted)	*Blk*	NM_022274	104	TGCAGCCACTATTTA
			118	TAAAACAGCGGTTCT
			388	AGAGCGCGGAGTTCT
			705	AGAGCCCCTAGTCCC
			865	AGAGCCCAGTTTACT
			904	GGAACCCCAAGTATT
			1203	AGATCTTTCTGGTCC
			1212	AGACCTGTCAGATCT
			1643	CTCACCCAGTTTTTT
			1724	GGAACTCACTGTAAA
			1832	AAGCCACACTCTTAT
			1834	GGAAGCCACACTCTT
			2157	AGGACCCAGAGGTAG
			2198	AGATACCAGTGAACA
			2403	ATCACCTATTCTTCG
			2406	CCAATCACCTATTCT
			2411	ATACCCCAATCACCT
			2717	GAAGCCCCCTCTTCA
Bcl-2 modifying factor	*Bmf*	NC_001025751	203	AGCTCCAATTGCGCT
			228	GGAACCGAATCCTCA
			260	GGGACTCTCTGTCAT
			268	AGAACCCAGGGACTC
			375	ATCTCCCAGTCTCCT
			416	AGCAACTCCTTTTTT
			554	GGATCCAAATGTCAT
			668	ACAGCCCTCTGATGC
			895	ATTCCCCATTCTTCA
			898	AAAATTCCCCATTCT
			951	AGCTCCCTGCCTTCT
			1230	AGGACCCACGTGGCA
			1469	AGAGACCACTGAGCC
			1485	ACAGCACACTGTAGG
			1572	AGAAACCTAAGTAAT
			2047	ACAGAGCACAGCTCT
			2054	AGCAACCACAGAGCA
			2293	CTAAGCTACTGGTCC
			2485	AGTGCTTTCTGTTGT
			2661	AAACCAATCTGTTCT
			2715	AGGAAGAACTGTTTG
			2963	AGAAACCAATCTGTT

Common gene name	Gene symbol	Genbank accession no.	Position (upstream)	Sequence
BCL2/adenovirus E1B 19 kDa-interacting protein 3	*Bnip3*	NM_080897	208	CGCGCCCCTTGTTCC
			282	AAAAACAACCGCCCT
			615	ACCACGCATGCTTCT
			975	ACACGCCCCTTCTCT
			1011	CTGACCCACTGCTGC
			1331	AAAACGAAAGGTTCA
			1638	AGACCATTCTGTTTC
			1837	AGATCTCTGAGTTCG
			2147	AGACCCAGCTGGCCT
			2486	AGACTCCTGTTTTAT
			2806	CCAGCCCAGCGTGCT
BCL2/adenovirus E1B 19 kDa-interacting protein 3-like	*Bnip3l*	NM_053420	176	AGACCCTATAGTCCG
			1085	AGAAGGCAACTTTGT
			1242	AGAGCTATCTGATAT
			1301	AGGACCTGATTTTCA
			1428	AGAATCCACATGTAC
			1863	AGTAACCAATGTGCT
			1612	AGACCAGACTGTACT
			1937	AGAAGACAGTGTTGA
			2114	AAAACCCACAGAAGA
			2303	AGCCCCTTCTTTTCA
			2359	CGAACCCAGGGCCTT
			2461	ATAAGACTCTGTTAC
			2628	GGAATCCAGTGCCCT
			2655	ATAGCCATCTGTGAT
Bcl-2-related ovarian killer protein	*Bok*	NM_080888	283	AGAACCCAAAATGAA
			353	AGAACCTATTGCCGC
			531	ACAAACACTGGTTCT
			1011	AGAGCTGGTTGTTCT
			1110	AGAATCCAGGATCTT
			1258	AGAACCCAGACTCTA
			1573	TGGACCAGCTGGTCA
			1700	CTACCACACTGTTCA
			1768	AGAACGGAGTCTAGT
			1812	AAATCCCATTGCTTG
			1960	AGATCCAATGCTTCT
			2046	AACACTGACTGCTCT
			2197	GGAACCGCCTCTTCT
			2333	AGACCCCTCACTTTA
			2405	AGAATAAACTGTCTG
			2448	GGAAGCCTTTTTCCT
			2691	ACAGCCTGGTGATCT
			2749	AAAAAGACCTGTTCT
			2793	AGAAACCAGGAGTGT
Caspase 6	*Casp6*	NM_022522	100	GGAAGCCACAGTGGC
			496	GGTCATCATTGTTCT
			506	ATCACCTACAGGTCA
			738	AGCCTGCCCTGTCCT
			979	CCAACCCTCTTTACT
			1144	ACAACTCACCTATTT
			1112	GAAAAGCACGGTTCT
			1734	ACAACCCTTTCATCT
			1738	AGAAACAACCCTTTC
			2367	AGAGCCTGCTGCTCT
			2857	GGTAACAACTCTTCT
Caspase 7	*Casp7*	NM_031775	44	AGACGCCCCTTTGCA
			954	AGGATCCACATATCC
			996	GGAATTCCCAGTTCA
			1204	AGAAACAAGTGGGCC
			1411	AGCACCTTCTGTGTG
			1705	AGACTCCACCTTCTT
			1730	GGAACGCAGAGTATT
			2116	AGACCACAAACTGCT
			2151	TGATGACCCTGTACT
			2953	TCCATCCACTGCTCT

Common gene name	Gene symbol	Genbank accession no.	Position (upstream)	Sequence
Caspase 8	*Casp8*	NM_022260	177	GGAACTTCCTGTTTT
			323	AAGAACTGCTTTTCT
			341	TGTATGCACTTTTCC
			402	CCAACAATCCGTTCT
			486	AGTGTCCAGTGGTAT
			646	AGAACCCTCAGGACC
			892	GGAAGGCCCTGCTCA
			1033	CGAACCCAGGGCCTT
			1111	AGAATTCTTTCTTTT
			1548	AAAACACTATGTAAT
			1630	AGCAACCATAGTTTT
			1647	ACAATCTAATGTCTT
			1678	CAAATAGACTGGTCT
			1778	GGAGCCCATAGCTTT
			1966	TGTACCCAAAGTGTT
			2289	AGAGGAAACAGTTTT
			2303	GGAAGCAAGTCTTCA
			2329	TGAGCTAGCTGTTCT
			2720	TATACCCCCTTTCCT
			2891	AGGACGAAAGGGTCT
			2971	ATAACCATGAGTTCC
CASP8 and FADD-like apoptosis regulator	*Cflar*	NM_031632	373	ATCACCGAGTTCTCT
			692	AGAATCCACAAAGCC
			718	CGAGCTCAAGGTCCT
			935	AGAATAGACAGTGCT
			1195	AGATCAGAGTGGTTT
			1235	ATAACTAACAGTTGG
			1245	TGAAACCACTATAAC
			1480	TTAAAGCACTGTTCT
			1827	AGAAGGCACAGTAGG
			2021	AGAACTGACCGCGCT
			2101	AAAACTGACTATTTT
			2163	CGAACCCAGGGCCTT
			2214	AAAACTGACTATTTT
			2498	GCAACCCAGTGATTT
			2532	AAACCACAGGCTTCT
			2912	AGGATCCACAGTCTC
Contactin associated protein 1	*Cntnap1*	XM_344410	2030	ACTCCCACCTGTTCT
			2193	AGAACTCAGACATAT
			2339	ATAAATCTCAGCTCT
			2511	AGCAGCCACTGACTC
			2684	AGAACTCCCAGTTCT
DNA fragmentation factor, alpha subunit	*Dffa*	NM_022546	125	AGAACCCCCTGTGGT
			151	AGAAACTACAACTCC
			424	TAAACCCTCTCTTCA
			942	TTTACCCACTGAGCT
			443	TGTACCCTATTTTCT
			952	AGCAAGCACTTTTAC
			1320	TGAACTCACAGAGAT
			1335	AGACCAGGCTGGCCT
			1349	GGAACTCACTCTGCA
			1451	GCAACGAACTGTACT
			1520	AAAAAACACCGTTAA
			1721	GAAAATCAACGTTCT
			1953	AGTACTACCTGCTCT
			2021	ACGCCCTCCTGTTAT
			2067	TTAATCCAATTTGCT
			2433	AGAAAGGAGTTTTTT
			2471	GGAAAAGATTGATCT

Common gene name	Gene symbol	Genbank accession no.	Position (upstream)	Sequence
DNA fragmentation factor, alpha subunit	*Dffa*	NM_022546	2506	ATAAAATACTGTTGA
			2568	AGGACAAACTTTTTA
			2694	AAAACAGACTTGTAT
			2798	ACAACCATCTCTAAT
			2856	AGCACTGACTGCTCT
			2857	GGTAACAACTCTTCT
			2924	AGCAATCACAATTCT
Growth arrest and 45 DNA-damage-inducible alpha	*Gadd45a*	NM_152937	156	CGGACCCTTTGTCCT
			230	ATGACCCAATGACCT
			409	AAAGCCCTCTGCACC
			760	ACACACAAATGTGCT
			1016	AGAAGGCAGTGTCAT
			1332	ACTGCCCAGTGACCT
			1648	GACAGCCAGTGTGCT
			1864	CAATCCCAATGTTGG
			1903	CTAGGCAACTGCTCT
			2036	ATTACACAATGTCCA
			2388	GGAACACAGTTTATT
			2405	GAATCCCAGAGTTCT
			2545	GACACCCACTGTACT
			2704	AGATCACGAGGTTTT
			2754	ATGTTCCACTAGTCT
			2980	AGAGCCTACTTCATT
Ras-related associated with diabetes	*Rrad*	XM_342810	75	TGACTCCAGGGTCCT
			267	ATTCCCCAGTGGTCT
			1075	AGACCTAATTTTTCT
			1369	TGCACCCCCTGAACC
			1991	CGCGCCCAATCTTCA
			2194	ACAGCCCACTGAGGT
			2291	CTTAGCCACTTTCCT
			2325	AGAACCCTGCATCAT
			2480	AGAAGTCTCTGATTC
			2612	ACACCTCAAGGTTCT
			2739	AAGATACACTGTTGA
TRAF family member-associated Nf-kappa B activator	*Tank*	NM_053338	177	AGTGAAGACTGTTTT
			331	AGAAGTCCCTTCTCA
			356	AGGAGTAACTGTCCA
			642	AAAACCAAATTACCT
			988	AGTCCCAAAAGTTGT
			1410	AGAAGCCACACATTA
			1532	AGATCCAATGTTACT
			1609	TGACACAAATGTTCA
			1730	TGCACTTACAGTTCC
			1799	AGCACTGACTATGCT
			2173	AGTAAGGTCTCTTCT
			2527	ATTACCCTTTTGTCT
			2254	AGTACCTACACTTAT
			2977	AGAAGGGGCTGTCCC
Tumor necrosis factor (ligand) superfamily, member 5 (CD40 ligand)	*Cd40lg*	NM_022303	2	AGCACTAATTGTGTT
			28	AGAAGACACCATTTC
			265	AGAAGAAACTCGTTT
			613	AGAGCCCTATGTTTT
			820	CGAAGCCACACATCA
			1470	AGAACCAATGCTTCT
			2185	AGAAACCATTCTAAG
			2392	AGACAAGACTGACCT
			2464	ATAACTCTCAGGTCT
			2621	GGTACCCAGTTTAGT
Tumor necrosis factor (ligand) superfamily, member 10	*Tnfsf10*	NM_019135	165	ATAACCTCCTCCCCT
			300	TGCTCCAGCAGTTCT
			341	AGATCCTGCAGCTTT
			358	ATTGCCCTGTGCTCT
			489	CTATCCCTCTGTCCA

Common gene name	Gene symbol	Genbank accession no.	Position (upstream)	Sequence
Tumor necrosis factor (ligand) superfamily, member 10	*Tnfsf10*	NM_019135	1013	AGCACACACTTTAAT
			1777	ACAGGCTACTCTTCA
			1986	ATACCCCACAGATAT
			2196	AAAAAGGACTTATCT
			2520	AGACTCCCCTGTACC
			2757	AGTGCCAAGTGTTAG
Tumor necrosis factor, (ligand) superfamily member 15	*Tnfsf15*	NM_001009623	675	ATATCCTTCTGTTTC
			711	ACAACCAGATATTCT
			758	AGAAGCCCATGTCCT
			800	ATAAACCACTGGCAT
			866	AAAAGCGAGTGTTTA
			1048	CGAAGCCAGTCTGGT
			1429	CCACCCCTCTTTTAT
			1969	GGAATTCACTTTAAT
			1986	AAAAGTCACCCTTCC
			2313	AGCAACTACAGCACT
			2332	AAAATAAAATATTCT
			2773	AGCAAGGACTGATAT
			2834	ATCCCCAGATGTTCT
			2837	AGAATCCCCAGATGT
Tumor necrosis factor receptor superfamily, member 1b	*Tnfsrf1b*	NM_013091	115	CCCACCCCCTGGTCT
			1040	AGTGGCTACAGGTCT
			1244	AGAGACCAGAGCTCT
			1380	TGGACTCACTGGACA
			1888	CGCAATCACTGTGCA
			1903	AGTAACTACTGTTTT
			2051	AGAAGCTCCTTGGCT
			2296	TGAACTCTCTGAGCT
			2340	AGCCCTGGCTGTCCT
			2750	TGAAACCTGTGTTGG
			2997	TGGAGTCACCGTGCT
Tumor necrosis factor receptor superfamily, member 4	*Tnfsrf4*	NM_130426	23	AAACCCCAGACTCCT
			80	TCCGCCTACTCTTCT
			175	AGGCCCCAGTGGCCC
			375	AGCACTCATGGTAAT
			566	AGCTTGTACTGTTCT
			657	AGAACCCAAATTAGG
			1102	TGGGACTTCTGTTCT
			1147	AGCCCACTCTGACCT
			1303	AGAGACCACGTGTCT
			1322	CAAGCCACCTGTCCT
			1384	GGACAGCAGAGTTCT
			1509	GGGACCTGCTGTCCT
			1594	GCCAGCTACTGATCT
			1773	AGTAGCAGCTGGACT
			1941	AGGTCACACTGCTTT
			2011	AGAACTCAGAATGAT
			2118	GGAACCTACTTCTAT
			2265	TTGGGCCAGTGTTCT
			2731	AAAAAAACCTGTTTT
			2789	AGGCCAGCCTGGTCT
Tumor necrosis factor, receptor superfamily member 11b	*Tnfsrf11b*	XM_344431	57	AGGGCCCAGGGTTCC
			159	AGAAATCAGCCATCT
			438	AAACCCCAGACTTCT
			648	AGAATTTATTCTTCT
			1700	AGACATAAATGTTTT
			1846	AGAACTAATTTATGT
			1940	ACACCCCACTCTCTC
			2101	AGATCTCTGTGAACT
			2211	AGATTCAAATATTCT
			2332	TGAGTGCAGTGTTCA

Common gene name	Gene symbol	Genbank accession no.	Position (upstream)	Sequence
Tumor necrosis factor receptor superfamily, member 12a	*Tnfsrf12a*	NM_012870	33	GGAGCAGACCGTTCT
			404	ATAACTCAGTGCTCG
			1302	ACAACCATCTGTAAT
			1361	AGAAATGGCTGCTCT
			1454	AGCAGACACTCGTAT
			1491	GGACCCCAGGGATCG
			1662	AGAATTTACTCAACT
			2030	ACACAACACTCTTCT
			2346	GGAGACCATTCTCCT
			2433	GTTACCCTCTGTTCT
			2541	AGACCCCAATGCCTT
			2697	AAAGCAGATTTTTCT
			2861	AAAAACGACAGCTCT
			2904	AAACCCCAAGCTTCC
Ubiquitin-conjugating enzyme E2I	*Ube2i*	NM_031237	223	AGACAGCACTGGTGC
			537	AGTAGTGATTGTCCT
			568	GGTGCGGACTTTTCT
			714	AGAGGCTGCTGGCCT
			940	AAATCCCAGTGCTCT
			1005	AACACCTATTGCCCT
			1142	AGGAACCTCTGGGAT
			1423	AGAACACACAGTAAT
			1529	ATGAACAGCTGTGCT
			1598	AGCCACCTCTGTCTT
			1601	ACAAGCCACCTCTGT
			1715	AGGAAAAAGTGGTCT
			1791	AGGCCAGCCTGGTCT
			1867	AGTCCCAGCCTTTCT
			2161	TAAACCAGGTATTCT
			2441	TGTACACTTTGTGCT
			2495	TGGACCCTGTGTTTT
			2652	CAAACTGCCTGTCCT
			2670	AGCAGCCCCTGTGGC
			2882	AGAAGCTAGAGTGCT
Ubiquitin-conjugating enzyme E2N (homologous to yeast UBC13)	*Ube2n*	NM_013050	1	GGTCTCCAGTTTTCT
			592	AGAAGCCCCTTTTTA
			686	AGGCCCCCCCCTTTT
			955	CGAACCCAGGGCTTT
			1047	AAAACACTCTTTGCG
			1049	ACAAAACACTCTTTG
			1052	AGAACAAAACACTCT
			1077	AGACCCCAGCATGCT
			1207	CGAACCCAGGGCCTT
			1272	AGATCACCCTCCCCT
			1680	ATAACCTACAACTTT
			1771	ACGATTCATTGCTCT
			1914	AGAAGGCACCAGCCT
			2081	GGAACTGTGTGTGCT
			2223	AGATGCCCCTGTCTC
			2349	ACATGGCTCTTTTCT
			2368	TAATCTGACTGTTTT
			2502	GGGAGCCATTGTTAG
			2676	TGGGAACAGTGTTCT
			2906	ACAAGACCCGGGTCT
			2959	AGAACCGTCTGTAAC

Letters highlighted in grey are conserved from the consensus sequence (AGAACCnnnTGTTCT) used to complete the analysis. Only sequences with at least 6 conserved nucleic acids have been kept.

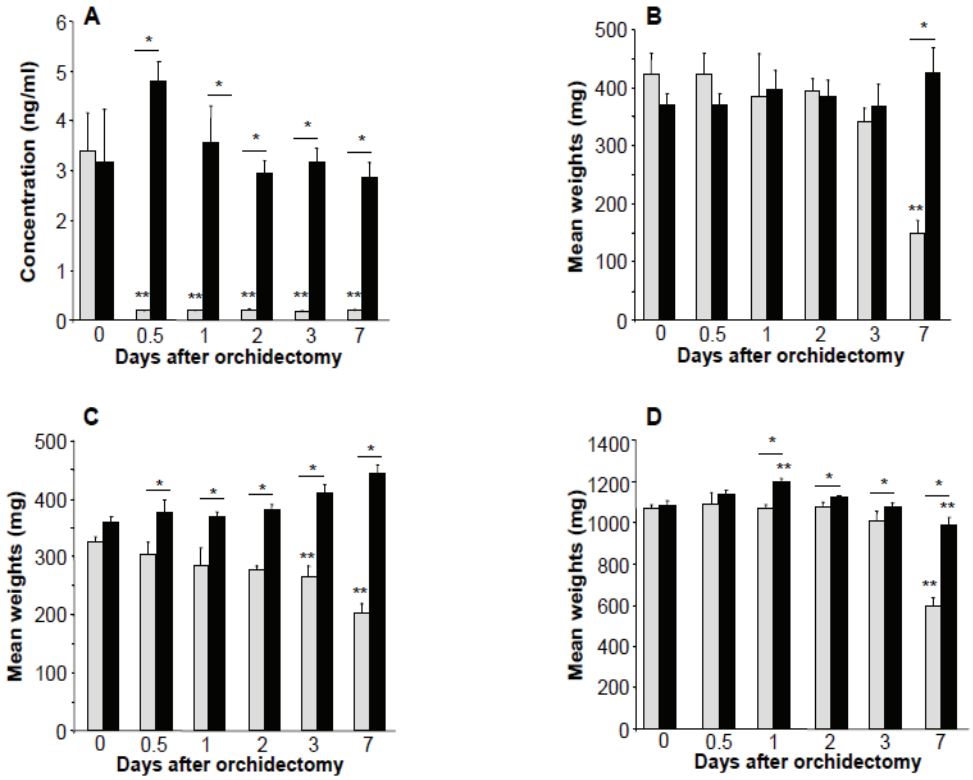


Figure 1: Effects of orchidectomy with and without testosterone replacement on serum testosterone concentration and weights of prostate, empty seminal vesicles, and epididymis. Rats were orchidectomized with empty (grey bars) or testosterone-filled implants (black bars) and sacrificed 0.5, 1, 2, 3, or 7 days after surgery. Serum testosterone concentration (A) was measured by ELISA, whereas weights of prostate (B), empty seminal vesicles (C), and epididymis (D) were recorded. Day 0 corresponds to sham-operated animals. Data are presented as mean ± SEM (n=5-6/group). Significant effects ($P<0.05$) of treatment on serum testosterone concentration and tissue weights are depicted by (*), whereas significant effects ($P<0.05$) of time on serum concentration and tissue weights are depicted by (**).

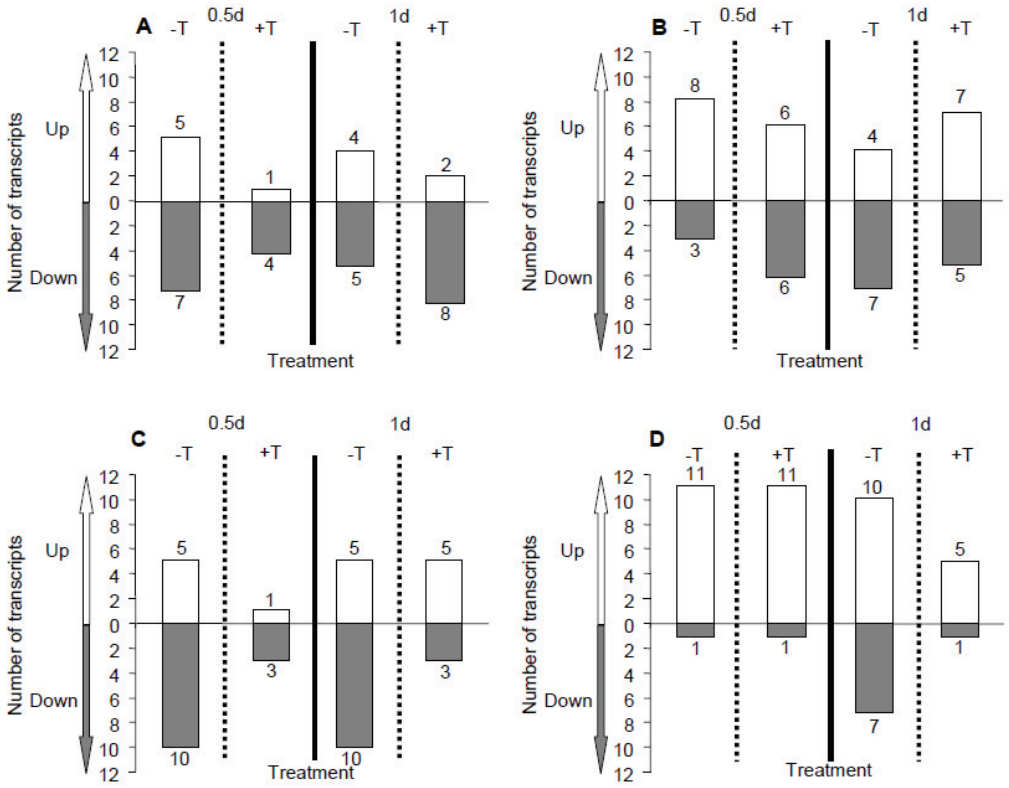


Figure 2: Number of transcripts changing in the different regions of the epididymis after orchidectomy with or without testosterone replacement. The number of transcripts changing by at least 1.5 fold in either direction (50% increase or decrease) (vertical axis) was determined for the IS (A), Ca (B), Co (C), and Cd (D). Fold change was determined at 0.5 and 1d after orchidectomy without (-T) or with testosterone replacement (+T) (horizontal axis) relative to sham-operated. The white bars indicate the number of transcripts increasing in expression (above x-axis), whereas the grey bars indicate transcripts decreasing in expression (below the x-axis). Each number was obtained independently at each treatment time relative to sham-operated.

Figure 3: Direct relationships between the androgen receptor, testosterone, and the affected transcripts. Using PathwayStudio, we identified genes that were known to be directly regulated by testosterone (T) and/or the androgen receptor (AR) (A). After promoter sequence analysis to uncover putative AREs, we identified new genes that could be transcriptionally regulated by AR. Genes that could not be linked either directly or indirectly to AR and/or T were represented and grouped according to their family (B). Interactions are expression, regulation or promoter binding; genes with putative AREs are connected to AR by two-head arrows. Genes known to be regulated by either AR or testosterone are in pink, genes with putative AREs are in blue, and genes not known to be regulated by T and/or AR are in yellow. Arrows in T indicate negative regulation, arrows with (+) sign indicate positive regulation, and simple arrows indicate regulation.

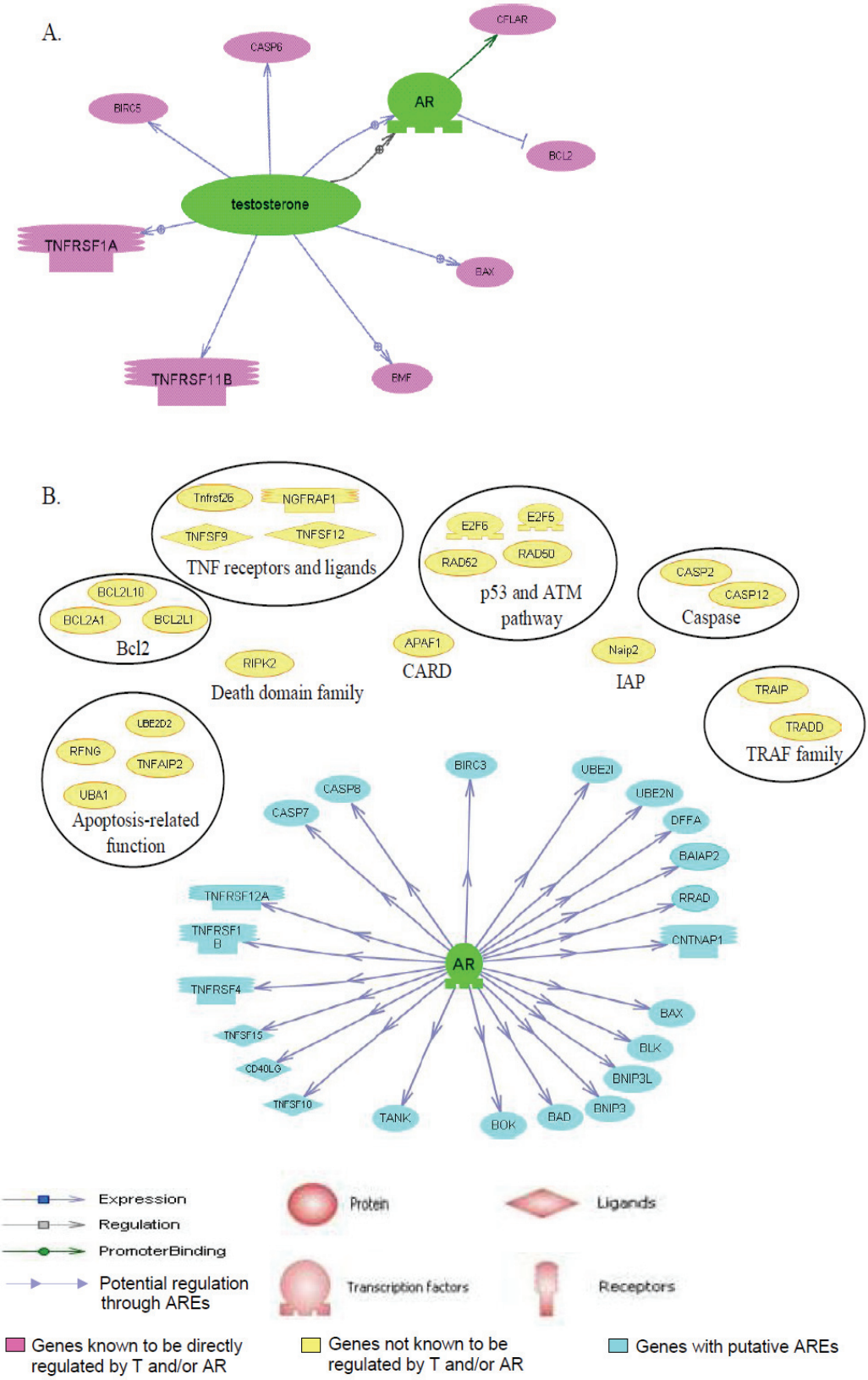

A.
CFLAR
CASP6
AR
BIRC5
BCL2
testosterone
TNFRSF1A
BAX
TNFRSF11B
BMF
B.
Tnfrsf26
NGFRAP1
TNFSF9
TNFSF12
TNF receptors and ligands
E2F6
E2F5
RAD52
RAD50
p53 and ATM pathway
CASP2
CASP12
Caspase
BCL2L10
BCL2A1
BCL2L1
Bcl2
APAF1
CARD
Naip2
IAP
RIPK2
Death domain family
TRAIP
TRADD
TRAF family
UBE2D2
RFNG
TNFAIP2
UBA1
Apoptosis-related function
BIRC3
UBE2I
CASP8
UBE2N
CASP7
DFFA
BAIAP2
TNFRSF12A
RRAD
TNFRSF1B
CNTNAP1
AR
TNFRSF4
BAX
TNFSF15
BLK
CD40LG
BNIP3L
TNFSF10
TANK
BOK
BAD
BNIP3
Expression
Regulation
PromoterBinding
Potential regulation through AREs
Protein
Ligands
Transcription factors
Receptors
Genes known to be directly regulated by T and/or AR
Genes not known to be regulated by T and/or AR
Genes with putative AREs

Figure 4: Potential pathways through which the androgen receptor and/or testosterone could regulate gene expression. Using PathwayStudio, we determined possible pathways through which the androgen receptor (AR) (A) and/or testosterone (T) (B) could regulate the expression of affected transcripts. The potential mediators of AR action were not present on the array, but are present in the epididymis. Interactions are expression, regulation or promoter binding. Potential mediators are in grey, genes with putative AREs and potentially indirectly regulated by AR and/or T are in blue, and genes potentially indirectly regulated by AR and/or T are in purple. Arrows in T indicate negative regulation, arrows with (+) sign indicate positive regulation, and simple arrows indicate regulation.

TERT: telomerase reverse transcriptase; KAT5: K(lysine) acetyltransferase 5; PPARGC1A: peroxisome proliferator-activated receptor gamma, coactivator 1 alpha; KIT: v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog; CDKN1A: cyclin-dependent kinase inhibitor 1A (p21, Cip); RAF1: v-raf-1 murine leukemia viral oncogene homolog 1; MAPK1: mitogen-activated protein kinase 1; MYC: v-myc myelocytomatosis viral oncogene homolog (avian); AKT1: v-akt murine thyoma viral oncogene homolog 1; CREB1: cAMP responsive element binding protein 1; HIF1A: hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor); MAPK14: mitogen-activated protein kinase 14; CTNNB1: catenin (cadherin-associated protein), beta 1, 88kDa; PRKCD: protein kinase C; RPS6KB1: ribosomal protein S6 kinase, 70kDa, polypeptide 1; ABL1: c-abl oncogene 1, receptor tyrosine kinase; GATA4: GATA binding protein 4; KRAS: v-Ki-ras 2 Kirsten rat sarcoma viral oncogene homolog; CEPBA: CCAAT/enhancer binding protein (C/EBP), alpha; RB1: retinoblastoma 1.

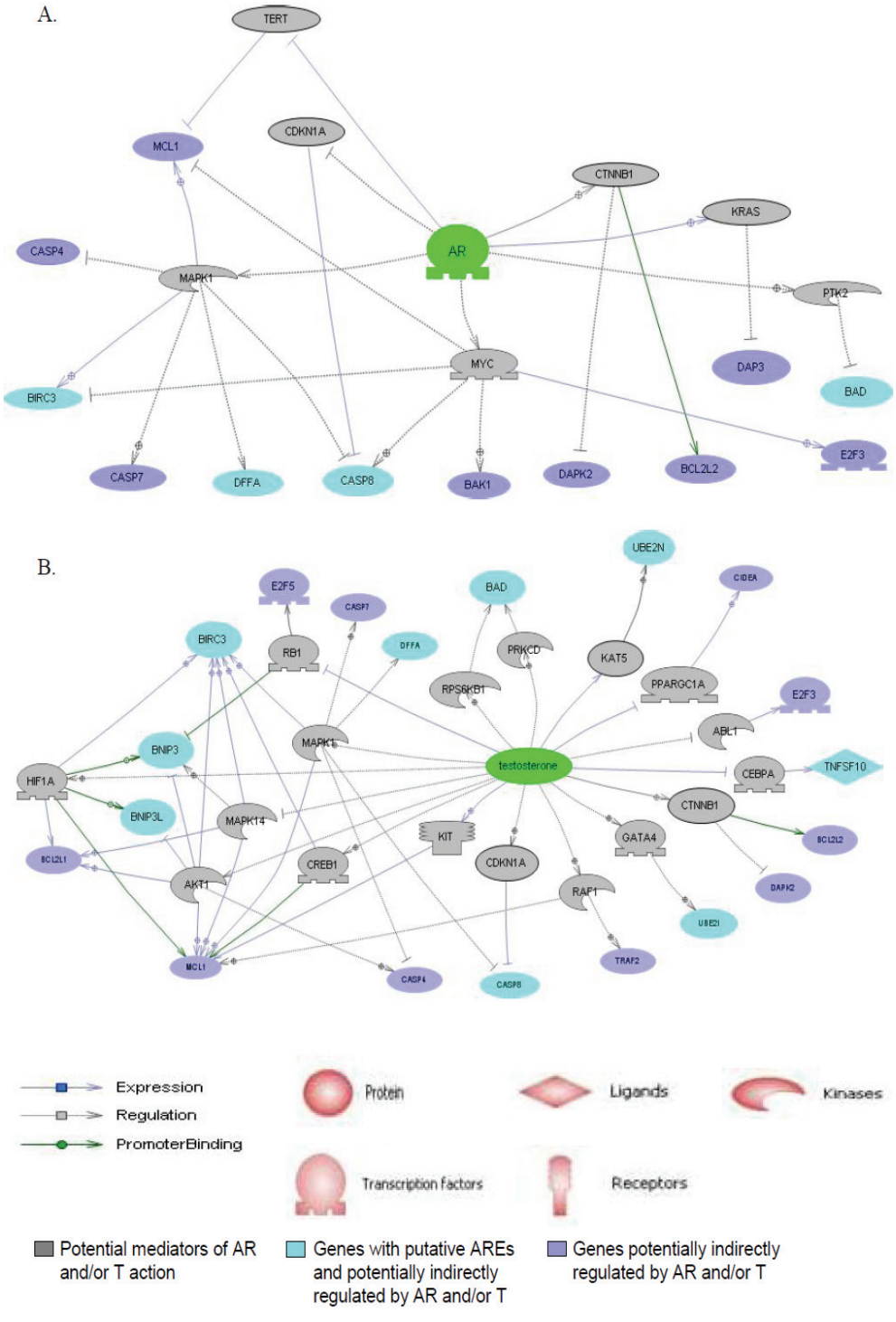
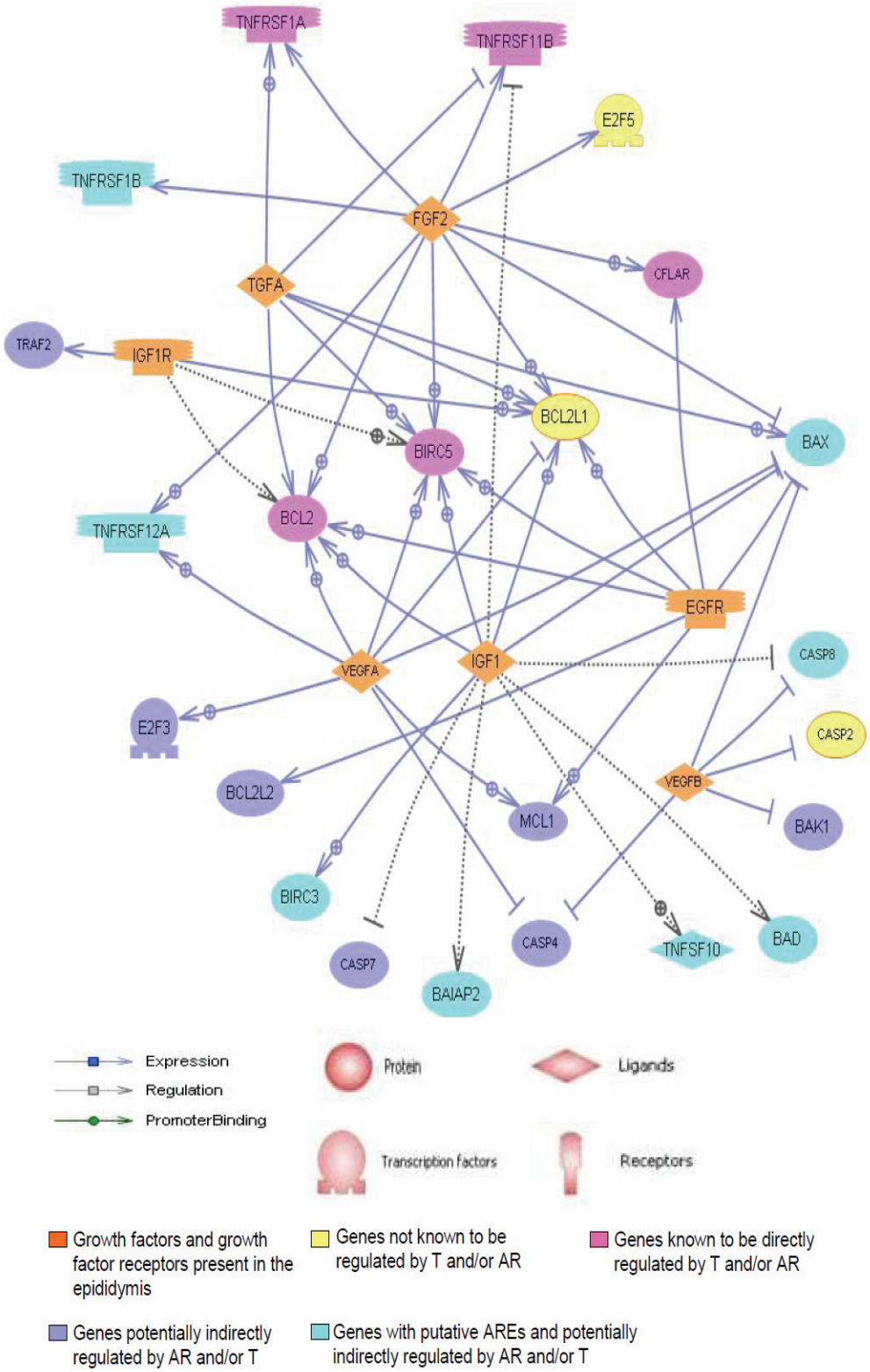

A.
TERT
MCL1
CDKN1A
CTNNB1
KRAS
AR
CASP4
MAPK1
PTK2
MYC
DAP3
BIRC3
BAD
E2F3
CASP7
DFFA
CASP8
BAK1
DAPK2
BCL2L2
B.
UBE2N
E2F5
CASP7
BAD
CIDEA
BIRC3
RB1
DFFA
PRKCD
KAT5
RPS6KB1
PPARGC1A
E2F3
ABL1
BNIP3
MAPK1
testosterone
HIF1A
CEBPA
TNFSF10
BNIP3L
MAPK14
CTNNB1
KIT
GATA4
BCL2L1
CREB1
CDKN1A
BCL2L2
AKT1
RAF1
DAPK2
UBE2I
MCL1
TRAF2
CASP4
CASP8
Expression
Regulation
PromoterBinding
Protein
Ligands
Kinases
Transcription factors
Receptors
Potential mediators of AR and/or T action
Genes with putative AREs and potentially indirectly regulated by AR and/or T
Genes potentially indirectly regulated by AR and/or T

Figure 5: Potential interactions between growth factors and affected genes. Using PathwayStudio, we determined the possible regulation by growth factors and growth factor receptors (EGFR, FGF2, IGF1, IGF1R, VEGFA, VEGFB, TGFA) (orange) known to be present in the epididymis to transcription of affected genes. Genes not known to be regulated by testosterone (T) and/or the androgen receptor (AR) are in yellow, genes known to be directly regulated by T and/or AR are in pink, genes potentially indirectly regulated by T and/or AR are in purple, and genes with putative AREs and potentially indirectly regulated by AR and/or T are in blue.

EGFR: epidermal growth factor receptor; FGF2: basic fibroblast growth factor; IGF1: insulin-like growth factor 1; IGF1R: insulin-like growth factor 1 receptor; VEGFA: vascular endothelial growth factor A; VEGFB: vascular endothelial growth factor B; TGFA: transforming growth factor alpha.


TNFRSF1A
TNFRSF11B
E2F5
TNFRSF1B
FGF2
CFLAR
TGFA
TRAF2
IGF1R
BCL2L1
BAX
BIRC5
BCL2
TNFRSF12A
EGFR
CASP8
VEGFA
IGF1
E2F3
CASP2
VEGFB
BCL2L2
MCL1
BAK1
BIRC3
CASP4
TNFSF10
BAD
CASP7
BAIAP2
Expression
Regulation
PromoterBinding
Protein
Ligands
Transcription factors
Receptors
Growth factors and growth factor receptors present in the epididymis
Genes not known to be regulated by T and/or AR
Genes known to be directly regulated by T and/or AR
Genes potentially indirectly regulated by AR and/or T
Genes with putative AREs and potentially indirectly regulated by AR and/or T

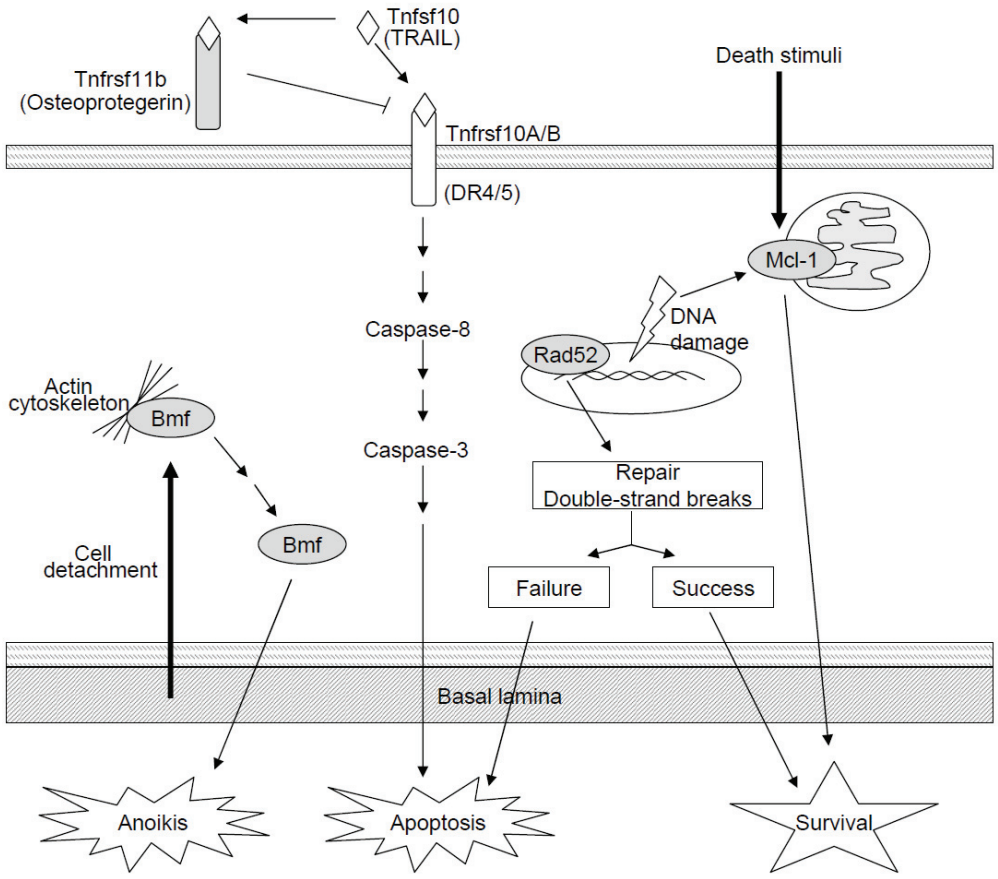


Figure 6: Roles of BMF, Mcl-1, TNFRSF11B, and Rad52 in the apoptotic, survival and repair responses. BMF is a pro-apoptotic Bcl-2 family member that is linked to the actin cytoskeleton. When the cell detaches from the basal lamina, BMF is released from the cytoskeleton causing anoikis (a form of cell death associated with detachment from the basal lamina) (55). Mcl-1 is an anti-apoptotic Bcl-2 family member that sits on the mitochondrial membrane. Upon death stimuli and/or DNA damage, Mcl-1 inhibits pro-apoptotic proteins leading to cell survival (44). TNFRSF11B is a soluble TNFR. It binds to TNFSF10, thereby preventing the activation of TNFRSF10A/B and the caspase cascade (47). Rad52 is involved in the repair of double-strand breaks after DNA damage. Failure or success in repairing breaks dictates cell fate (51).

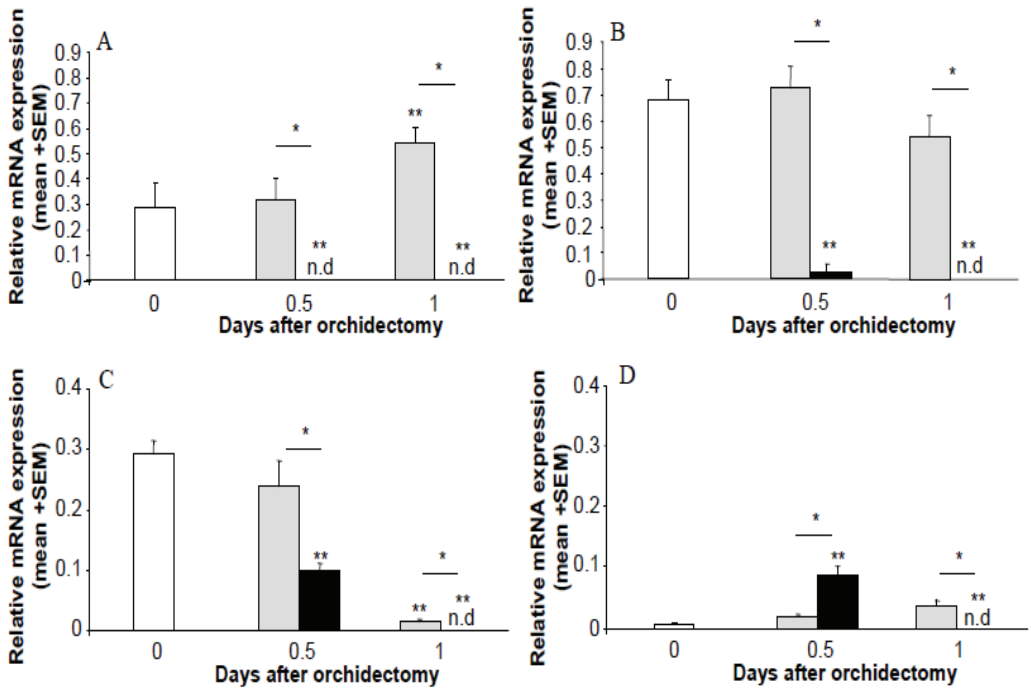


Figure 7: Effects of orchidectomy with or without testosterone replacement on *Rad52* expression. Rats were orchidectomized with empty (grey bars) or testosterone-filled (black bars) implants and sacrificed 0.5 day and 1 day after surgery. Changes in expression for *Rad52* were assayed by Real-Time RT-PCR for IS (A), Ca (B), Co (C), and Cd (D). Day 0 corresponds to sham-operated animals (white bars). *Rad52* expression was normalized to *Ppia* (*cyclophilin A*) expression. Data are presented as mean ± SEM (n=4-5/group). Significant effects ($p<0.05$) of treatment on transcript expression are depicted by (*) and significant changes as compared to sham-operated are depicted by (**).

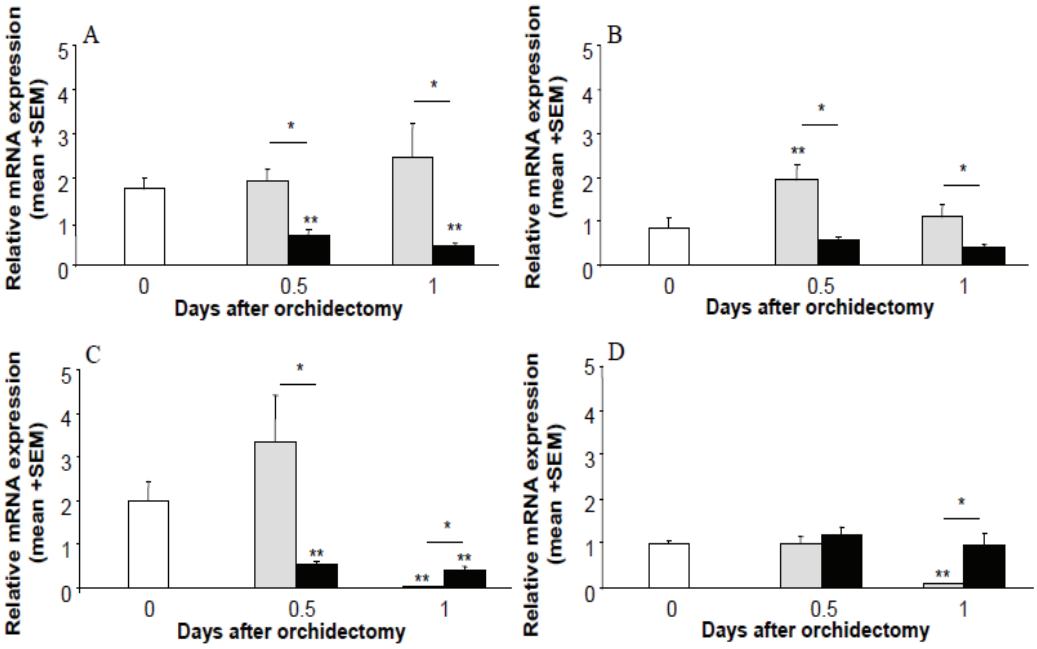


Figure 8: Effects of orchidectomy with or without testosterone replacement on *Mcl-1* expression. Rats were orchidectomized with empty (grey bars) or testosterone-filled (black bars) implants and sacrificed 0.5 day and 1 day after surgery. Changes in expression for *Mcl-1* were assayed by Real-Time RT-PCR for IS (A), Ca (B), Co (C), and Cd (D). Day 0 corresponds to sham-operated animals (white bars). *Mcl-1* expression was normalized to *Ppia* (*cyclophilin A*) expression. Data are presented as mean ± SEM (n=4-5/group). Significant effects ($p<0.05$) of treatment on transcript expression are depicted by (*) and significant changes as compared to sham-operated are depicted by (**).

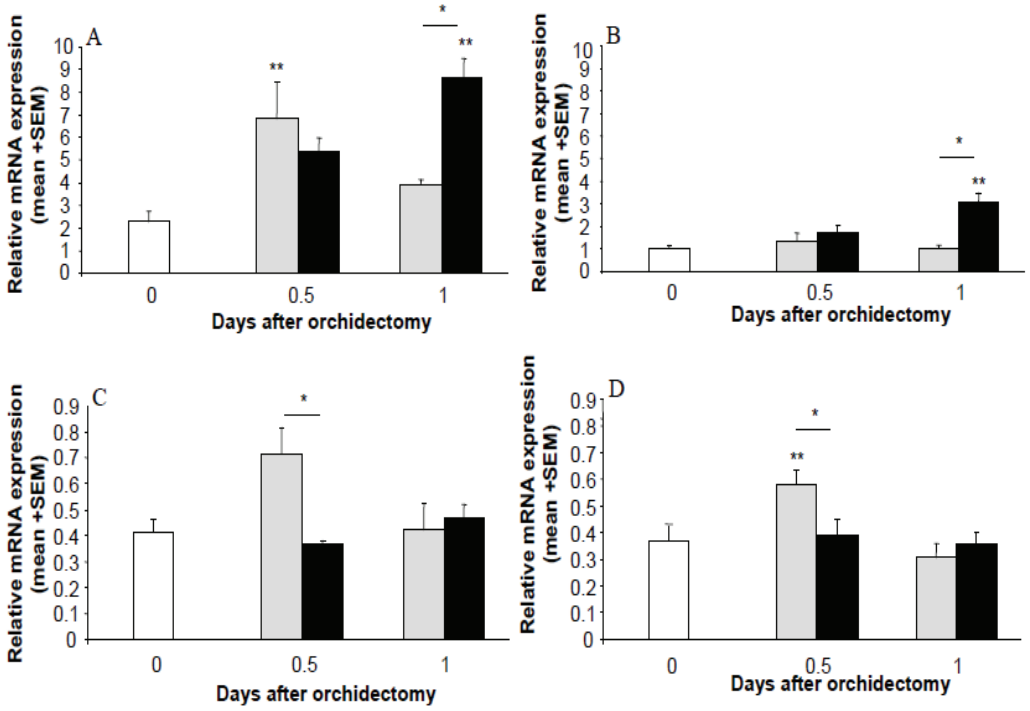


Figure 9: Effects of orchidectomy with or without testosterone replacement on *Bmf* expression. Rats were orchidectomized with empty (grey bars) or testosterone-filled (black bars) implants and sacrificed 0.5 day and 1 day after surgery. Changes in expression for *Bmf* were assayed by Real-Time RT-PCR for IS (A), Ca (B), Co (C), and Cd (D). Day 0 corresponds to sham-operated animals (white bars). *Bmf* expression was normalized to *PPia* (*cyclophilin A*) expression. Data are presented as mean ± SEM (n=4-5/group). Significant effects ($p<0.05$) of treatment on transcript expression are depicted by (*) and significant changes as compared to sham-operated are depicted by (**).

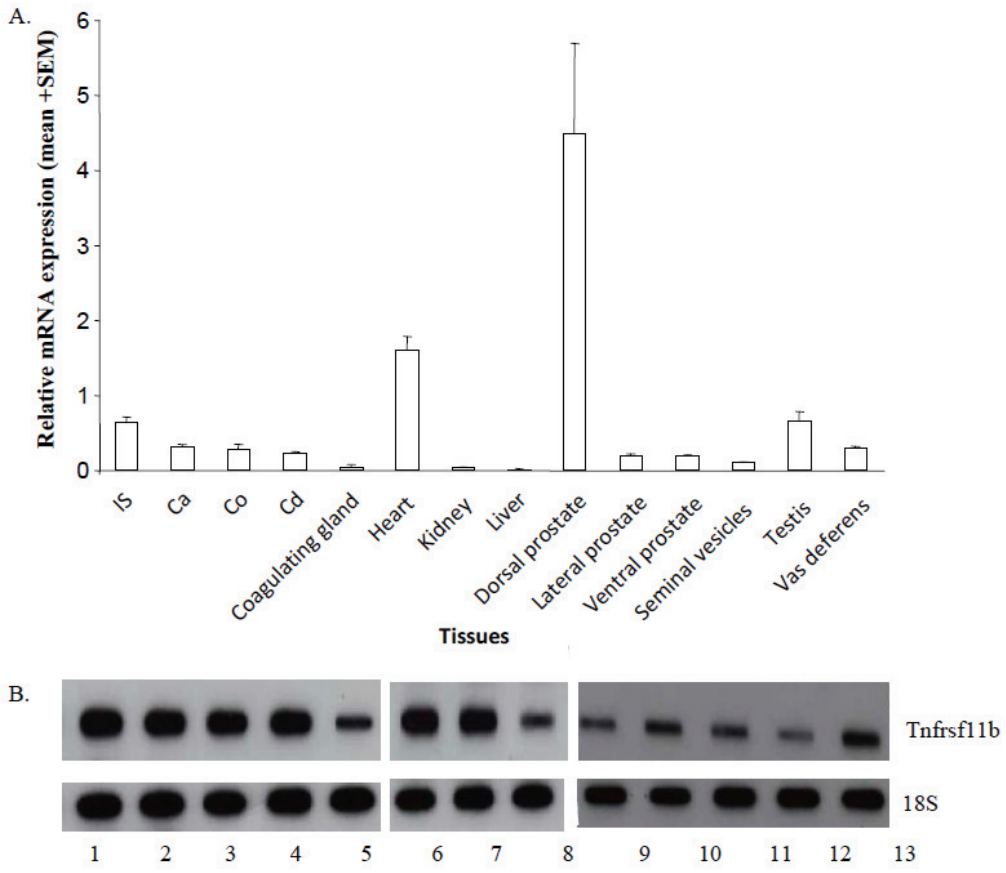
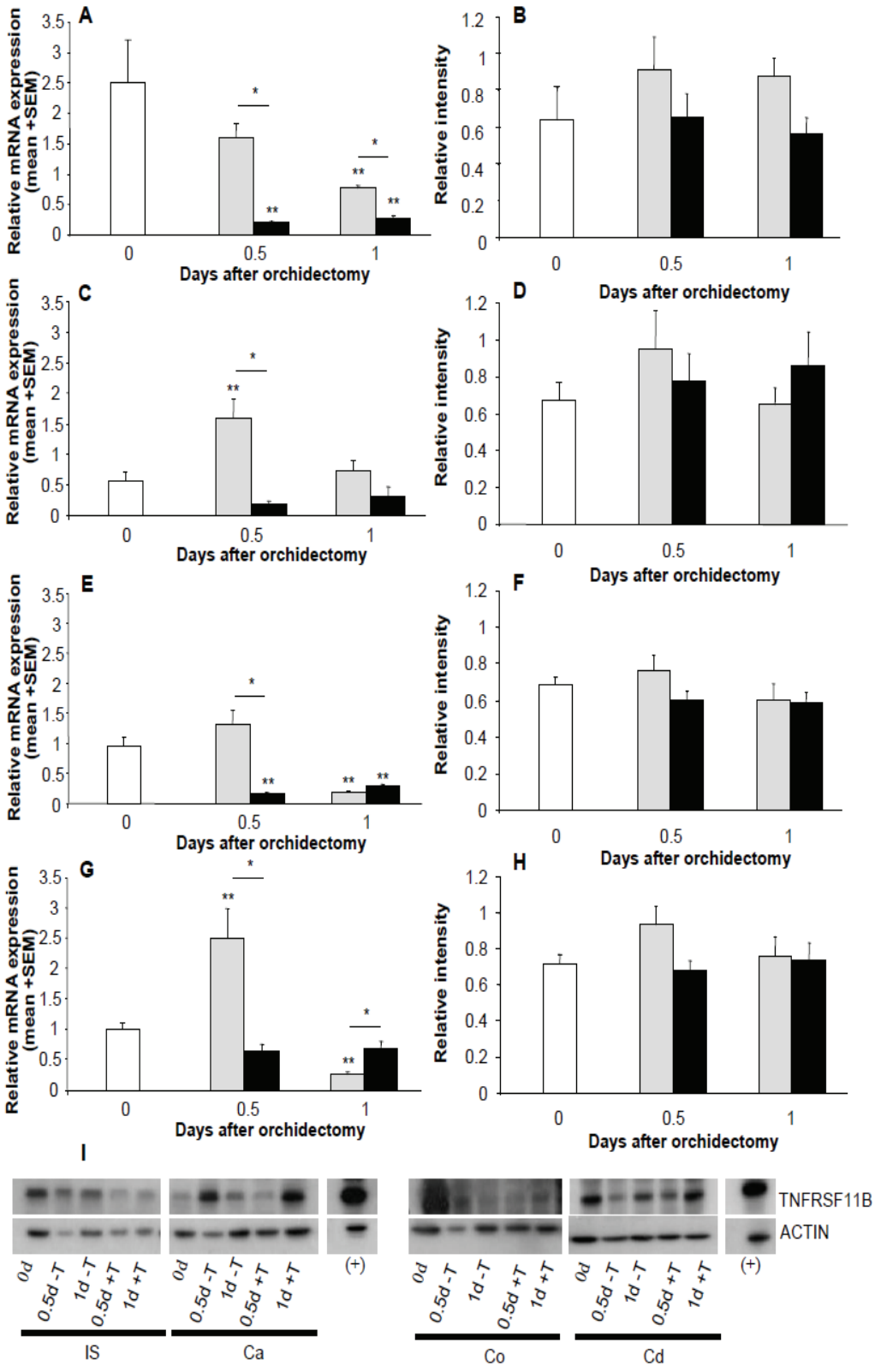


Figure 10: Identification of *Tnfrsf11b* in different rat tissues. Presence of *Tnfrsf11b* in IS, Ca, Co, Cd, coagulating gland, heart, kidney, liver, dorsal prostate, lateral prostate, ventral prostate, seminal vesicles, testis, and vas deferens was assessed quantitatively by Real-Time RT-PCR (A) and qualitatively by dot blot (B). To confirm equal loading of RNA in the dot blot experiment, membranes were probed with an 18S probe. *Tnfrsf11b* expression for the Real-Time RT_PCR experiment was normalized to *Ppia* (*cyclophilin A*) expression. Data are presented as mean ± SEM (n=3/group). 1: whole epididymis; 2: IS; 3: Ca; 4: Co; 5: Cd; 6: heart; 7: kidney; 8: ventral prostate; 9: testis; 10: coagulating gland; 11: dorsal prostate; 12: lateral prostate; 13: seminal vesicles; 14: vas deferens.

Figure 11: Effects of orchidectomy with or without testosterone replacement on TNFRSF11B transcript and protein expression. Rats were orchidectomized with empty (grey bars) or testosterone-filled (black bars) implants and sacrificed 0.5 day and 1 day after surgery. Changes in expression for *Tnfrsf11b* were assayed by Real-Time RT-PCR for IS (A), Ca (C), Co (E), and Cd (G). Day 0 corresponds to control (white bars). *Tnfrsf11b* expression was normalized to *Ppia* (*cyclophilin A*) expression. Amounts of TNFRSF11B were quantified and normalized relative to ACTIN in the IS (B), Ca (D), Co (F), and Cd (H) epididymides. Data are presented as mean ± SEM (n=4-5/group). Significant effects ($p<0.05$) of treatment on expression are depicted by (*) and significant changes as compared to sham-operated are depicted by (**). (I) are representative western blots with images for IS, Ca, Co, and Cd. The (+) sign indicates the positive control.


A
B
C
D
E
F
G
H
I
Relative mRNA expression (mean +SEM)
Relative intensity
Days after orchidectomy
0
0.5
1
TNFRSF11B
ACTIN
(+)
0d
0.5d -T
1d -T
0.5d +T
1d +T
IS
Ca
Co
Cd

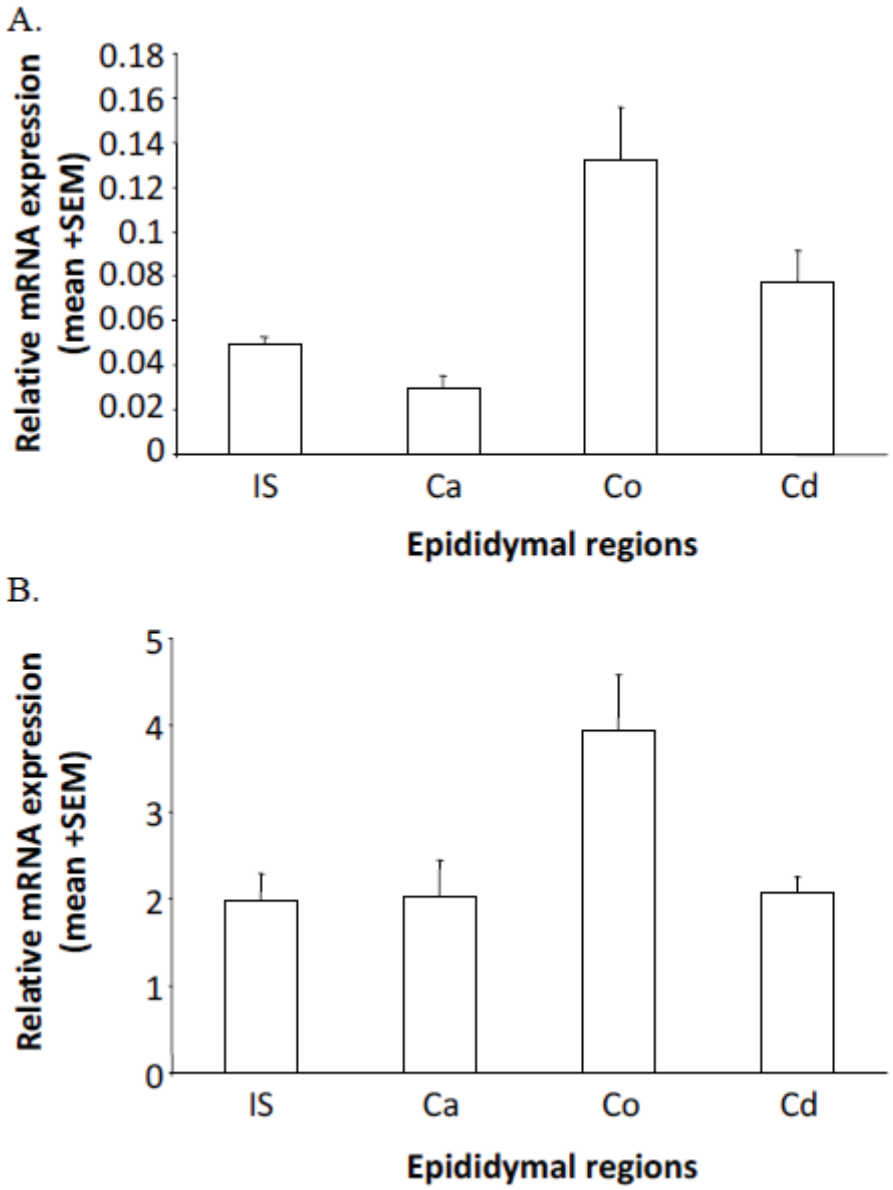
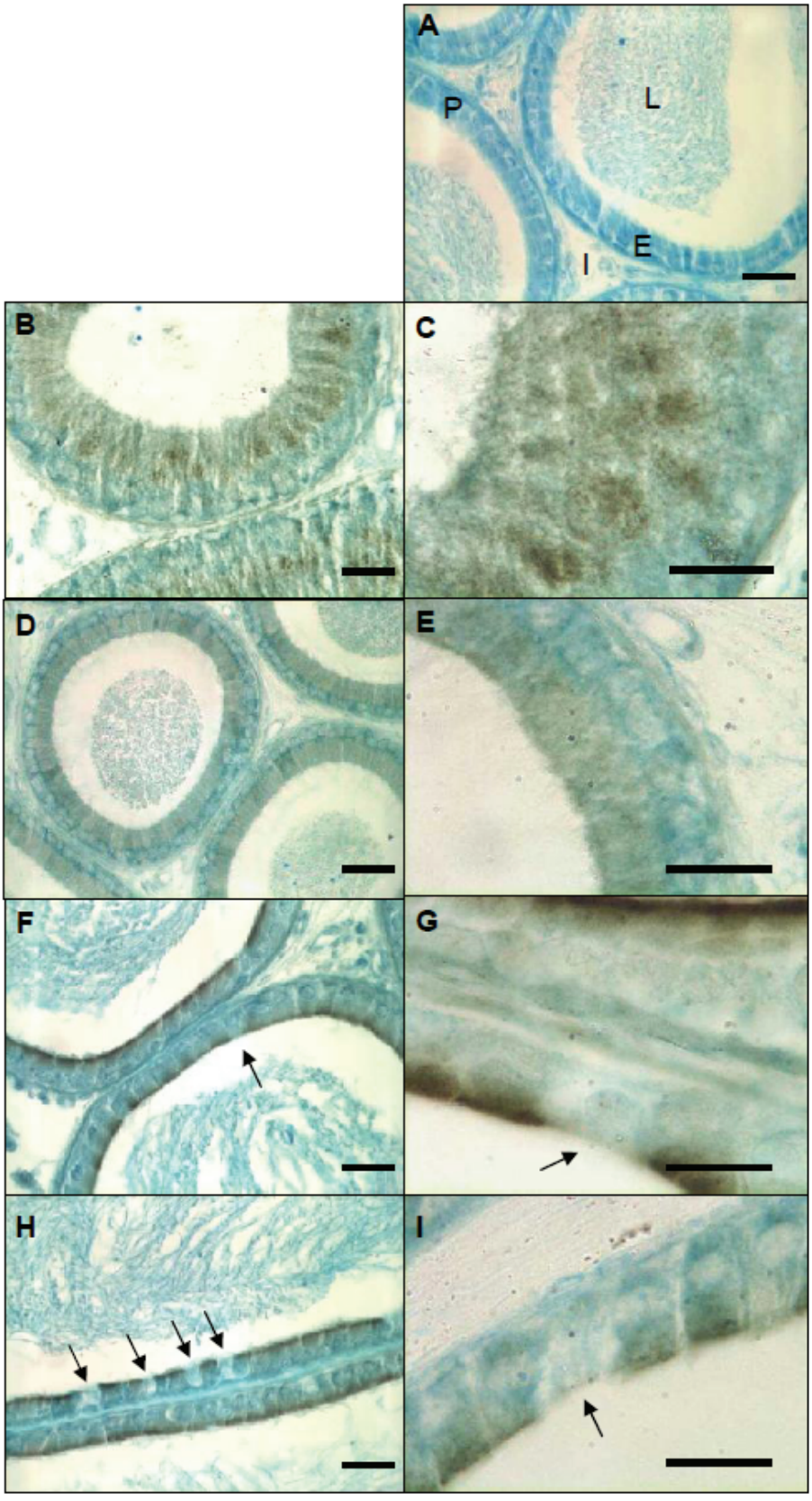


Figure 12: Identification of *Tnfsf11* and *Tnfrsf11a* in the different regions of the epididymis. Expression of *Tnfsf11* (A) and *Tnfrsf11a* (B) in the different regions of the epididymis was determined by Real-Time RT-PCR using Quantitect primers. *Tnfsf11* and *Tnfrsf11a* expressions were normalized to *Ppia* (*cyclophilin A*) expression. Data are presented as mean ± SEM (n=4-5/group).

Figure 13: Immunolocalization of TNFRSF11B in the different regions of the epididymis. Epididymides of control animals were fixed by Bouin's fixation, stained with an anti-TNFRSF11B antibody, and counterstained with methylene blue. Principal cells stained only in the cytoplasm in IS (B-C), Ca (D-E), Co (F-G), and Cd (H-I). (A) shows a slide incubated with only secondary antibody. E: epithelium; L: lumen; I: interstitium; P: principal cells; arrows indicate clear cells. The bar represents 2μm.


A
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CONNECTING TEXT

In the previous chapter, orchidectomy with or without testosterone replacement in the epididymis changed the expression of many apoptotic and cell survival transcripts, many of which could be regulated by IGF1, an important regulator of cell survival. In addition, Seenundun and Robaire (Endocrinology 2007 148(1):173-188) had previously shown that IGF1 was central to the response of the PC-1 mouse epididymal cell line to androgen withdrawal. The previous chapter also identified BIRC5, a downstream effector of the IGF1 signaling pathway, as being changed after orchidectomy with or without testosterone replacement in the epididymis. Therefore, the next chapter will determine the potential involvement of the IGF1 and BIRC5 survival signaling pathway in the response of the epididymis to androgen withdrawal.

CHAPTER 3

Androgen Withdrawal Regulates IGF1 and BIRC5 Expression in the Rat Epididymis

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1. Abstract

The epididymis is responsible for the proper maturation and storage of spermatozoa. Despite its dependence on androgens to maintain its functions, androgen withdrawal by orchidectomy causes little apoptosis. To investigate the potential involvement of the IGF1 and BIRC5 survival signaling pathway in the response of the epididymis to androgen withdrawal, we assessed changes in gene expressions for *Igf1*, *Igf1r*, *Birc5*, *Ide*, *Igfbp3*, *Bax*, *Bid*, and *Diablo* within a week after androgen withdrawal and/or replacement in the epididymis using qRT-PCR. Changes in protein expression for IGF1, IGF1R, and BIRC5 were also assessed by ELISA and western blots. We determined that orchidectomy with or without testosterone (T) replacement increased *Igf1* expression in all regions, but decreased *Igf1r* in the IS and Cd. We found that orchidectomy increased *Birc5* expression, whereas T replacement maintained it at control levels. Orchidectomy increased *Igfbp3* and *Bid* expressions, but decreased *Bax* expression, whereas *Ide* mRNA was transiently increased. In most regions, T replacement repressed *Igfbp3* and *Bax* expressions. *Diablo* showed region-specific changes in expression after orchidectomy with or without T replacement. Together, these results indicate that members of the IGF1 signaling pathway participate in the response of the epididymis to androgen withdrawal.

2. Introduction

The epididymis is a long coiled tubule that not only transports spermatozoa from the efferent ducts of the testis to the vas deferens but also provides optimal microenvironments for their proper maturation and storage (1). It is morphologically and functionally divided into four regions: initial segment (IS), caput (Ca), corpus (Co), and cauda (Cd). At the structural level, it comprises two compartments, a lumen where spermatozoa bathe and an epithelium composed of four major cell types (principal, basal, halo, and clear cells) (2;3). Epididymal functions are regulated by androgens, in particular testosterone (T) and its more active 5α –reduced metabolite dihydrotestosterone (DHT) (4), as well as by testicular factors that may include basic fibroblast growth factor (FGF2) (5) and androgen binding protein (ABP) (6).

Androgen withdrawal by orchidectomy causes a decrease in epididymal weight due to the loss of spermatozoa and luminal fluid, as well as a decrease in cell height (7;8); however, it triggers very little apoptosis (9). Despite the identification of apoptotic and cell survival genes affected in a region-specific manner in the epididymis after orchidectomy (chapter 2), little is known on the survival pathways activated after androgen withdrawal. A potential survival pathway that could be activated after androgen withdrawal is the IGF1 signaling pathway. We have shown that IGF1/IGF1R could regulate the expression of apoptotic and cell survival genes after orchidectomy with or without testosterone replacement (chapter 2). In addition, in the PC-1 mouse epididymal cell line, genes affected by androgen withdrawal have been directly linked to IGF1 by pathway analysis suggesting that it is central to the response of the PC-1 cells to androgen withdrawal (10). Hamzeh and Robaire (11) have also shown that in the regressed epididymis, IGF1 is a potential regulator of the expression of many affected genes.

IGF1/IGF1R promote cell survival through the activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway and the inhibition of pro-apoptotic proteins such as BAX and BID (12;13). IGF1 bioavailability is regulated by IGF binding proteins (IGFBPs). The most abundant one, IGFBP3

forms a ternary complex with IGF1 and the acid-labile subunit (ALS) that prolongs IGF1 half-life and prevents it from reaching its receptor (13). Once internalized, IGF1 is degraded by the insulin-degrading enzyme (IDE) leading to signal termination (14). Activation of IGF1R and the PI3K signaling cascade leads to increased androgen receptor (AR) expression (15). In addition, interaction between IDE and AR enhances AR DNA binding (16;17). On the other hand, AR regulates IGF1, IGF1R (18), and IGFBP3 (19) expression. IGF1 can promote survival in an AR-independent manner by increasing BIRC5 expression (20). BIRC5, an inhibitor of apoptosis protein (IAP), is known to be highly expressed in cancers, but not in terminally-differentiated tissues (21). However, we have identified *Birc5* as being expressed in the epididymis, a terminally-differentiated tissue (chapter 2). BIRC5 activity is inhibited by the binding of second mitochondria-derived activator of caspase/direct IAP binding protein with low pI (Smac/Diablo); its release from mitochondria is regulated by BID (22;23).

The objective of this study was to determine the involvement of members of the IGF1 signaling pathway in the response of the epididymis to androgen withdrawal. We assessed changes in expression for *Igf1*, *Igf1r*, *Birc5*, *Ide*, *Igfbp3*, *Bax*, *Bid*, and *Diablo* during a week after orchidectomy with or without T replacement using qRT-PCR.

3. Materials and Methods

3.1. Chemicals

T (4-Androsten-17β-ol-3-one) was purchased from Steraloids Inc. (Newport, RI). Bovine serum albumin (Fraction V) (BSA) was bought from Sigma-Aldrich Canada Ltd. (Oakville, ON). Normal saline (0.9% w/v NaCl in water) was bought from Roche Applied Science (Laval, QC), whereas sodium azide was bought from Thermo Fisher Scientific (Waltham, MA). Medical adhesive (Silicone type A, cat. no. 891) and tubing (cat. no. 602-305) to make the polydimethylsiloxane (Silastic) implants were purchased from Dow Corning Silicones (Midland, MI). NP-40 substitute and sodium deoxycholate/DOC were bought from Sigma-Aldrich Canada Ltd., whereas NaCl, SDS, and TRIS were

bought from Invitrogen Canada Inc. (Burlington, ON). Bestatin, PMSF, leupeptin, and aprotinin were bought from Roche Applied Science (Laval, QC). Ketamine was bought from Bioniche (Belleville, ON), acepromazine from Wyeth-Ayerst (St-Laurent, QC), xylazine from Novopharm (Montreal, QC), and buprenorphine from Reckitt & Cloman (Bristol, UK). All cell culture reagents were purchased from Wisent Inc. (St-Brunon, QC).

3.2. Animals

Adult male Brown Norway (BN) rats (3-4 months old) were obtained from Charles River Canada (St-Constant, QC) and housed at the McIntyre Animal Resources Centre of McGill University. Rats (3 per cage) were kept under controlled light (14-h light, 10-h dark) and temperature (22°C) and had access to regular rat chow and water *ad libitum*. All animal studies were conducted in accordance with the principles and procedures outlined in the Guide to the Care and use of Experimental Animals prepared by the Canadian Council on Animal Care (Animal Use Protocol no. 206).

Rats were separated into 11 groups (n=5/group): sham-operated; orchidectomized and implanted sc. with an empty 2.5-cm Silastic capsule (-T groups) and sacrificed at 0.5, 1, 3 or 7 days after surgery; or orchidectomized and implanted sc. with a T-filled 2.5-cm Silastic capsule (+T groups) and sacrificed at 0.5, 1, 3 or 7 days after surgery. Rats were anaesthetized by an intramuscular injection of ketamine, xylazine, and atravet (5:2.5:1) in normal saline (0.1ml/100g body weight) and received buprenorphine (0.001mg/100g body weight) after surgery. Bilateral orchidectomy was done as described elsewhere (24) and capsules were implanted sc. at the time of surgery. Implants were made according to the method of Stratton et al. (25) and had a T release rate of 30µg/cm/day, releasing T to an equivalent amount to serum T. To ensure a steady rate of T release, implants were bathed for 2 days prior to surgery in a solution of normal saline containing 1% BSA and 0.1% sodium azide. At the time of death, epididymides were collected, separated into IS, Ca, Co, and Cd regions, frozen in liquid nitrogen and kept at -80°C.

3.3. RNA extraction

RNA was isolated from the IS, Ca, Co and Cd of all treatment groups using Qiagen Mini-prep (Qiagen Inc., Mississauga, ON) following manufacturer's instructions. DNase treatment was done using the RNase-free DNase set (Qiagen Inc.) following the manufacturer's instructions. Concentration and quality of RNA were verified with a nanodrop 2000 spectrophotometer (Thermo Scientific Scientific). Each RNA sample was extracted from a single epididymal region from an individual rat; no samples were pooled.

3.4. Quantitative Real-Time RT-PCR

Real-Time RT-PCR was done to quantify expression of *Igf1*, *Igf1r*, *Birc5*, *Igfbp3*, *Bax*, *Bid*, and *Diablo* (Table 1) using the QuantiTect RT-PCR SybrGreen kit (Qiagen Inc.) and the LightCycler system (Roche Applied Science) as described elsewhere (10). Each sample was assayed in duplicate. Gene expressions were normalized against *peptidylprolyl isomerase A* (*Ppia*, *cyclophilin A*) expression. *Ppia* is a housekeeping gene, its mRNA expression is not affected by androgen manipulation (26). Gene-specific primers were designed using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi/), except when primers were ordered from Qiagen Inc. The primers were synthesized by AlphaDNA (www.alphadna.com, Montreal, QC).

3.5. Western blot analysis

Whole cell extracts (n=5/group) were prepared in RIPA buffer (150mM NaCl, 1% NP-40 substitute, 0.5% sodium deoxycholate/DOC, 0.1% SDS, 50mM TRIS pH 7.4). For each ml of RIPA buffer, the following proteinase inhibitors were added: 4µl bestatin (10mg/ml), 1µl PMSF (24mg/ml), 2µl leupeptin (5mg/ml), and 3µl aprotinin (2mg/ml). Protein concentrations were determined by the Bradford method using the Bio-Rad protein assay (Bio-Rad Laboratories, Mississauga, ON) following the manufacturer's protocol. For each sample, 20µg protein per lane was separated on an 8% or 15% acrylamide SDS-PAGE gel to determine changes in protein expression for IGF1R and BIRC5, respectively.

Prestained All Blue Precision Plus Protein Standards (Bio-Rad Laboratories) were used as molecular weight markers. Separated proteins were transferred to a PVDF Hybond-P membrane (GE Healthcare, Baie d'Urfe, QC). Blots were blocked in 5% non-fat dried milk in TBS-T (137 mM NaCl, 20 mM Tris, 0.5% Tween 20, pH 7.6) for 1h at room temperature and then incubated overnight at 4°C with a primary rabbit antibody against human BIRC5 (1:500, #2808, Cell Signaling Technology, Danvers, MA) or IGF1R (1: 1000, #3018, Cell Signaling Technology). Membranes were then probed with a donkey anti-rabbit IgG horseradish peroxidase linked whole antibody (1:2 000, NA934V, GE Healthcare). Constant loading was assessed by probing the membranes with a primary goat antibody against ACTIN (1:10 000, sc-1616, Santa Cruz Biotechnology, Santa Cruz, CA) and detecting it with a donkey anti-goat IgG horseradish peroxidase conjugated antibody (1:5 000, sc-2056, Santa Cruz Biotechnology). Signals were detected with the Enhanced Chemiluminescence Plus kit (GE Healthcare) and visualized on Hyperfilm enhanced chemiluminescence (GE Healthcare). Quantification of western blot data was done by densitometry analysis using a Chemilmager 4000 imaging system (Cell Biosciences, Santa Clara, CA) with AlphaEase (version 5.5 software, Cell Biosciences). The expression of IGF1R and BIRC5 was expressed relative to the corresponding expression of ACTIN for all groups.

3.6. IGF1 ELISA

Quantification of IGF1 expression in the epididymis (n=5/group) was done using the mouse IGF1 quantikine immunoassay (MG100, R&D Systems Inc., Minneapolis, MN) following the manufacturer's instructions. The minimum detectable dose was 3.5pg/ml. Intra-assay CV was 4.3% and inter-assay CV was 6%.

3.7. Statistical analysis

Significant differences between treatment groups within each epididymal region were assessed by two-way ANOVA followed by the Holm-Sidak *post-hoc*

test. When the normality test failed, data were transformed by the square root or log methods and analyzed as previously described. When the two-way ANOVA could not identify in which treatment group there was a significant difference, two one-way ANOVAs were done followed by the Dunnett's *post hoc* test. In those cases, comparisons between the (-T) and (+T) groups were done by multiple t-test with Bonferroni correction. Significance was set at $p<0.05$.

4. Results

4.1. Effects of orchidectomy with or without testosterone replacement on *Igf1* and *Igf1r* expression

In order to determine if the IGF1 signaling pathway could play a role in the response of the epididymis to orchidectomy with or without T replacement, we assessed changes in *Igf1* (fig. 1) and *Igf1r* (fig. 2) mRNA expression.

In all regions, androgen withdrawal triggered an increase in *Igf1* expression at 3 and 7 days (fig. 1A-D); in the distal regions, we observed a significant ($p<0.05$) decrease at 1 day after orchidectomy (fig. 1C-D). T replacement also caused a significant ($p<0.05$) increase in *Igf1* expression at 3 and 7 days in all regions. T replacement significantly ($p<0.05$) increased *Igf1* expression as compared to (-T) group in the IS at 3 and 7 days (fig. 1A), as well as at 1 day in the distal regions (fig. 1C-D). After androgen withdrawal, *Igf1r* expression was decreased in all regions, except at 7 days in the IS and at 1 day in the Ca where it was increased. T replacement was not able to prevent the decrease in *Igf1r* expression in the IS (fig. 2A) and Cd (fig. 2D). In the Ca, T replacement significantly ($p<0.05$) increased *Igf1r* mRNA at all time points except at 7 days (fig. 2B). The Cd was the only region to show significant ($p<0.05$) decrease in *Igf1r* mRNA at all time points and treatments (fig. 2D). T replacement significantly ($p<0.05$) increased *Ig1r* expression in the Ca and Co, but decreased its expression in the IS and Cd as compared to (-T) group. Together, the data suggested that IGF1/IGF1R could play a role in the response of the epididymis to androgen withdrawal.

4.2. Effects of orchidectomy with or without testosterone replacement on upstream regulators of IGF1 signaling

We assessed the effects of orchidectomy with or without testosterone replacement on two genes known to regulate IGF1 signaling, *Ide* (fig. 3) and *Igfbp3* (fig. 4). After androgen withdrawal, *Ide* mRNA expression was significantly ($p<0.05$) increased in all regions as early as 0.5 day after orchidectomy. In the distal regions, this increase was followed by a significant ($p<0.05$) decrease from 1 day on (fig. 3C-D). T replacement could not prevent the significant increase ($p<0.05$) at 0.5 and 1 day in the proximal regions (fig. 3A-B) or the significant ($p<0.05$) decrease at 3 and 7 days in the distal regions (fig. 3C-D). However, in the IS and Co, T replacement significantly ($p<0.05$) decreased *Ide* expression at 0.5 day. For *Igfbp3*, androgen withdrawal caused a significant ($p<0.05$) increase in expression all time points in the IS (fig. 4A) and Cd (fig. 3D). In the Ca (fig. 3B) and Co (fig. 3C), *Igfbp3* was only increased at 7 days after orchidectomy. T replacement prevented the increase in *Igfbp3* mRNA expression in all regions. In fact, in all regions, T replacement significantly ($p<0.05$) repressed *Igfbp3* expression as compared to (-T) group from 1 day onward. This suggested that IDE and IGFBP3 could be involved in regulating IGF1 signaling after androgen withdrawal.

4.3. Effects of orchidectomy with or without testosterone replacement on *Birc5* and *Diablo* expression

We determined the effects of orchidectomy with or without testosterone replacement on *Birc5* (fig. 5) and *Diablo* (fig. 7) mRNA expression in the different regions of the epididymis (fig. 5); DIABLO is a known regulator of BIRC5 activity. After androgen withdrawal, *Birc5* mRNA was significantly ($p<0.05$) increased from 1 day on in the proximal regions (fig. 5A-B). In the distal regions, *Birc5* mRNA was increased at 3 day after orchidectomy (fig. 5C-D). T replacement prevented the increase in *Birc5* expression in all regions, except Co at 3 days (fig. 5C). This suggested that *Birc5* could be transcriptionally-regulated by androgens. In fact, we found 5 putative androgen-response elements (AREs) in

the upstream promoter region of *Birc5* (fig. 6). In the proximal regions, androgen withdrawal caused a significant ($p<0.05$) increase in *Diablo* expression at 0.5 day followed by a decrease in expression at 3 days and a significant ($p<0.05$) increase at 7 days (fig. 7A-B). In the distal regions, androgen withdrawal caused a decrease in *Diablo* expression (fig. 7C-D). T replacement could not prevent the increased expression in the proximal regions and the decreased expression in the distal regions.

4.4. Effects of orchidectomy with or without testosterone replacement on downstream signaling molecules

We determined the effects of orchidectomy with or without testosterone replacement on *Bax* (fig. 8) and *Bid* (fig. 9) expression, two pro-apoptotic Bcl2 genes targeted by the IGF1 signaling cascade. After androgen withdrawal, in the IS, *Bax* was significantly ($p<0.05$) decreased from 1 day onward (fig. 8A). In the Co, *Bax* mRNA was first significantly ($p<0.05$) increased at 1 day and then decreased at 3 and 7 days (fig. 8C). T replacement could not prevent the decrease in *Bax* expression in the IS (fig. 8A). In the Ca, T replacement could not prevent the decrease in *Bax* expression at 0.5 day, but significantly ($p<0.05$) increased its expression at 1 and 3 days (fig. 8B). In all regions, except Co at 0.5 and 1 day, T replacement significantly repressed *Bax* expression as compared to the (-T) group. For *Bid*, androgen withdrawal significantly ($p<0.05$) increased its expression in all regions at 3 and 7 days (fig. 9). In the Cd, increased expression was preceded by a significant ($p<0.05$) decrease at 0.5 and 1 day (fig. 9D). T replacement could not prevent the increase in *Bid* expression in all regions, except Cd at 1 and 3 days. In all regions, T replacement significantly ($p<0.05$) repressed *Bid* mRNA expression as compared to the (-T) group at 3 and 7 days (fig. 9).

4.5. Effects of orchidectomy with or without testosterone replacement on IGF1, IGF1R, and BIRC5 expression

As IGF1 (fig. 10), IGF1R (fig. 11), and BIRC5 (fig. 12) are the main effectors in the IGF1 signaling pathway, we determined changes in protein expression after androgen withdrawal and/or replacement in the epididymis.

At the protein level, IGF1 expression increased significantly ($p<0.05$) at 3 days after orchidectomy without T replacement in the proximal regions and this increase was prevented by T replacement (fig. 10A-B). However, there were no significant changes in IGF1R expression after androgen withdrawal and/or replacement (fig. 11). For BIRC5 orchidectomy significantly ($p<0.05$) decreased its expression in the proximal regions (fig. 12A-B). In the IS, this decrease was observed as early as 1 day and reached a low at 7 days where BIRC5 could not be detected (fig. 12A). In the Ca, the decrease was observed from 3 days onward (fig. 12B). T replacement prevented the decreased expression in the Ca, but not in the IS, except at 7 days (fig. 12A-B). This suggested that BIRC5 could be post-transcriptionally regulated.

5. Discussion

The epididymis expresses many growth factors, in particular IGF1, which has been postulated to play important roles in the tissue (27). In fact, IGF1 null mice have an under-developed distal epididymis, suggesting that IGF1 is important for the development of this tissue (28). However, little is known on the exact role of the IGF1 signaling pathway in the epididymis and on its involvement in the response of the epididymis to androgen withdrawal.

When we assessed the effects of orchidectomy with or without T replacement on the expression of *Igf1*, *Igf1r*, *Igfbp3*, *Ide*, *Birc5*, *Bax*, *Bid*, and *Diablo*, we observed a bi-phasic response: an early response at 0.5 day and 1 day and a late response at 3 and 7 days; this response could be associated with the removal of luminal content. In fact, it takes 1 day for the IS to be emptied of luminal content after orchidectomy, whereas by 7 days all regions except Cd are emptied (29).

After orchidectomy with or without T replacement, *Igf1* mRNA did not change during the early response, but increased in the late response. On the other hand, *Igf1r* mRNA decreased in the early and late responses. The opposite direction in transcriptional regulation of *Igf1* and *Igf1r* suggests compensatory mechanisms to maintain the balance between the ligand and the receptor. The only exception to this pattern is the Ca, where *Igf1* increased over time as in the other regions, but *Igf1r* also increased. This suggests that the Ca is differentially regulated by IGF1/IGF1R compared to the other regions. In the early response, *Ide* mRNA first increased and then decreased in the late response. The lack of correlation between *Ide* and *Igf1* mRNA in the early response suggests that IDE may play another role than regulating IGF1 expression. In fact, interaction between IDE and AR leads to increased AR DNA binding (16;17). It is tempting to speculate that during the early response after androgen withdrawal, IDE expression increases to enhance AR DNA binding in the absence of androgens. After androgen withdrawal, *Igfbp3* mRNA increased over time paralleling the increase in *Igf1* mRNA, whereas with T replacement, *Igfbp3* remained around control levels even when *Igf1* increased. This suggests that when high levels of IGF1 are required such as after androgen withdrawal, IGFBP3 may stabilize IGF1 levels enabling IGF1 to act more efficiently (13) (fig. 13).

Orchidectomy triggered over time an increase in *Birc5* mRNA in all regions that was accompanied by an early increase in *Diablo* mRNA in the proximal regions and a decreased expression in the distal regions. With T replacement, *Birc5* mRNA remained around control levels in all regions except Co, whereas *Diablo* showed again an increased expression in the proximal regions and a decreased expression in the distal regions. As DIABLO negatively regulates BIRC5 activity (22), we can assume that *Diablo* transcription is negatively regulated to allow BIRC5 activity (fig. 14).

After an apoptotic stimulus, BID is cleaved to tBID and migrates to the mitochondrial membrane. Once there, tBID activates BAX leading to cytochrome c release and apoptosis (30). After orchidectomy with or without T replacement, as *Bid* expression increased, *Bax* expression decreased, which indicated a negative

correlation between *Bid* and *Bax* levels. This suggests a compensatory mechanism where two genes involved in the same pathway are inversely regulated, therefore preventing inappropriate activation (fig. 15).

After orchidectomy with or without testosterone replacement, *Igf1* and *Igf1r* mRNA expressions were changed, but not their protein expression. This finding suggests two possibilities: (i) although *Igf1* and *Igf1r* respond at the transcriptional level to orchidectomy with or without T replacement, they are not involved in the response of the epididymis to androgen withdrawal; and (ii) there are post-transcriptional regulatory mechanisms that regulate translation. The second option seems more probable because of the stability of IGF1 and IGF1R proteins. IGF1 can exist in three ways: in a ternary complex with IGFBP3 and ALS, in a binary complex with IGFBP3, or alone. This association within a complex affects IGF1 half-life such that IGF1 half-life is 10-16hrs in the ternary complex, 30-60min in the binary complex, and 10min alone (31); these half-lives are only relevant *in vivo* because *in vitro*, IGF1 is only degraded by 5% in 8hrs (31). The ternary complex is restricted to the circulation, whereas the binary complex can freely enter tissues, hence IGF1 half-life in the epididymis could vary from 10min to 60min (32). This rapid turnover of IGF1 in a control situation could explain why even under stress condition, we could not detect an increase in IGF1 production. On the other hand, IGF1R has a half-life of 22hrs, making it a very stable protein and hence less sensitive to changes in protein expression (33).

After orchidectomy, as *Birc5* mRNA increased, BIRC5 protein expression decreased in the proximal regions. With T replacement, both *Birc5* mRNA and BIRC5 remained around control levels in the Ca, while BIRC5 was still decreased in the IS. The IS and Ca are the two most sensitive regions of the epididymis to orchidectomy (9); the decreased expression of BIRC5 parallels the increased sensitivity of these two regions to orchidectomy. The IS also requires testicular factors to maintain its functions (9;34), which could explain why BIRC5 expression was not back to control levels after T replacement. Furthermore, BIRC5 expression is regulated by the ubiquitin-proteasome pathway causing a rapid turnover of BIRC5 with a half-life of 30min (35). We can speculate that in

the most sensitive regions, the ubiquitin-proteasome pathway would be more active leading to decreased expression of BIRC5 protein.

We have shown that members of the IGF1 and BIRC5 signaling pathway are modulated differently after orchidectomy with or without testosterone replacement with respect to time, region of the epididymis, and individual components of the pathway; this could lead to a fine-tuned response of the epididymis. This study gives insights into the potential involvement of the IGF1 and BIRC5 signaling pathway in the survival response of the epididymis to androgen withdrawal.

6. Acknowledgements

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Table 1: Real-Time RT-PCR primers

Gene name	Gene symbol	Genbank accession no.	Forward primer sequence (5'→ 3')	Reverse primer sequence (5'→ 3')
Peptidylprolyl isomerase A (Cyclophilin A)	*Ppia*	NM_017101	GTGGTCTTTGGGAAGGTGAA	GTTGTCCACAGTCGGAGATG
Baculoviral IAP repeat-containing 5	*Birc5*	NM_022274	ACCACCGGATCTACACCTTC	TCCCAGCCTTCCAGTTCCTT
Insulin-like growth factor 1	*Igf1*	NM_178866	TGTGGATGAGTGTTGCTTCC	CGTGGCATTTTCTGTTCCTC
Insulin-like growth factor 1 receptor	*Igf1r*	NM_052807	GAATGGAGGAGGTGACAGGA	GTGGAGGTGAAACGGAGAAC
Insulin-like growth factor binding protein 3	*Igfbp3*	NM_012588	QuantiTect Primer Assays (Qiagen Inc.) QT00186669	
Insulin-degrading enzyme	*Ide*	NM_013159	TCAAAGGGCTGGGTAAACAC	CCTTGCACTCTTGGAAAACC
Direct IAP-binding protein with low pI	*Diablo*	NM_001008292	QuantiTect Primer Assays (Qiagen Inc.) QT00372995	
BH3 interacting domain death agonist	*Bid*	NM_022684	QuantiTect Primer Assays (Qiagen Inc.) QT00189028	
BCL2-associated X protein	*Bax*	NM_017059	QuantiTect Primer Assay (Qiagen Inc.) QT01081752	

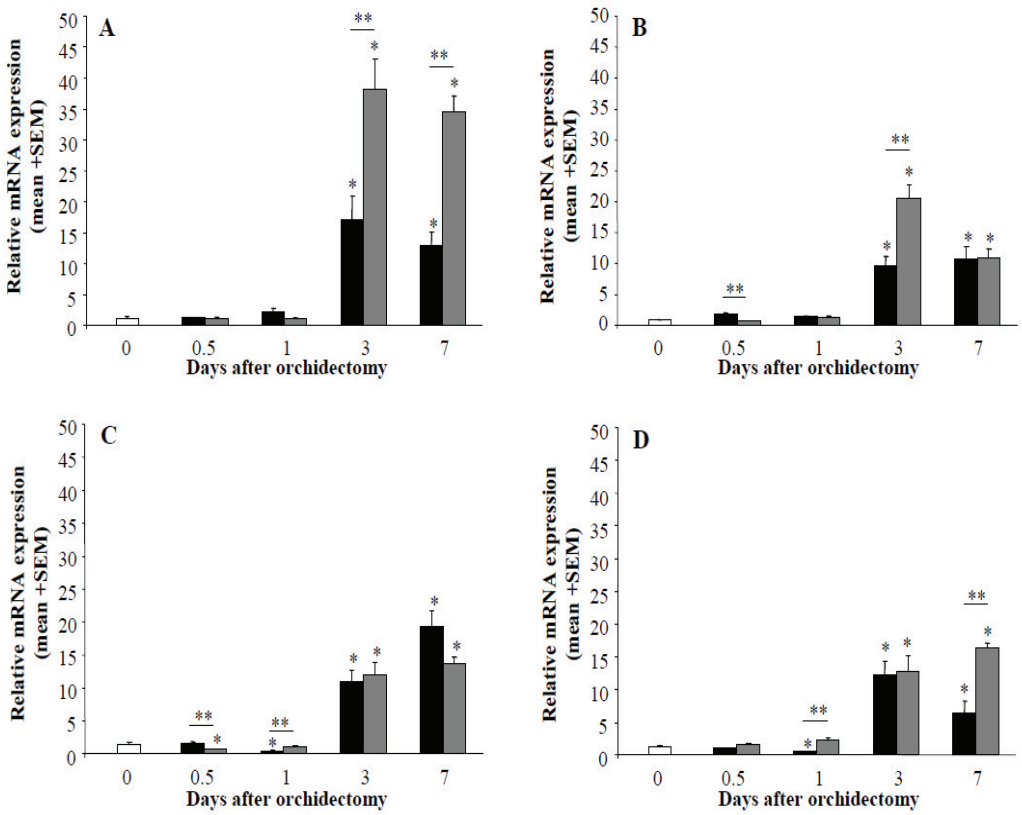


Figure 1: Effects of orchidectomy with or without testosterone replacement on *Igf1* mRNA expression in the epididymis. Rats were orchidectomized with empty (black bars) or testosterone-filled (grey bars) implants and sacrificed 0.5, 1, 3, and 7 days after surgery. Changes in *Igf1* mRNA were assayed by qRT-PCR in the IS (A), Ca (B), Co (C), and Cd (D). *Igf1* expression was normalized to *Ppia* (*cyclophilin A*) expression. Day 0 corresponds to sham-operated animals (white bars). Data are presented as mean ±SEM (n=5/group). Significant effects ($p<0.05$) of treatment on expression are depicted by (**) and significant changes as compared to sham-operated are depicted by (*).

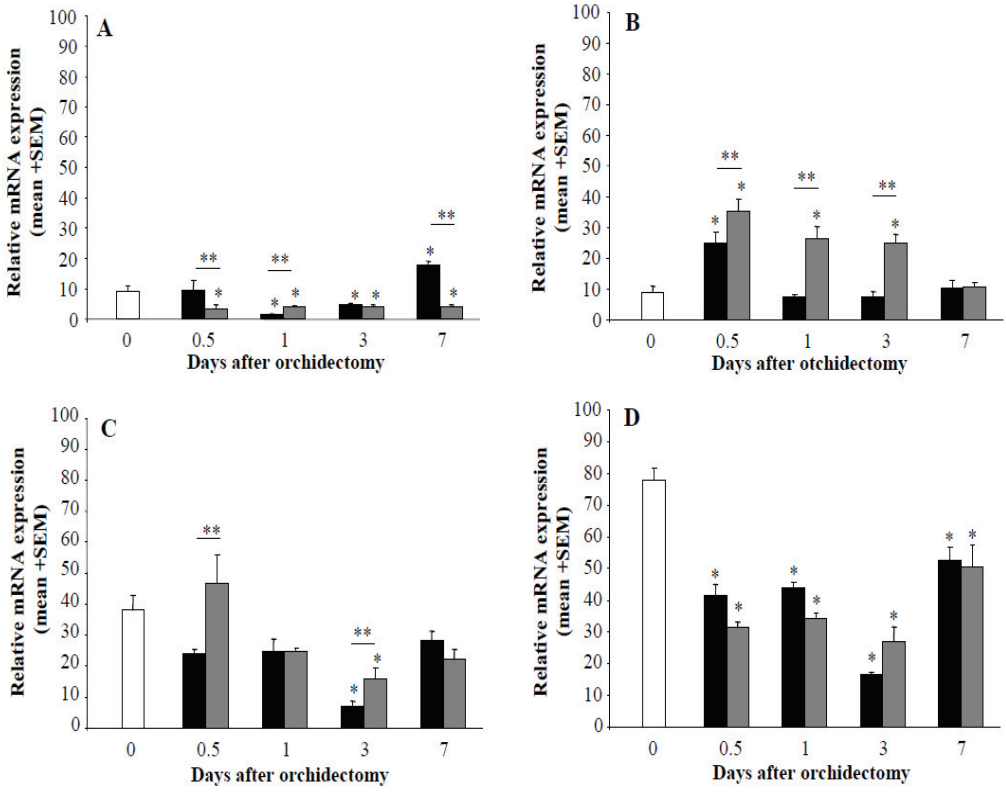


Figure 2: Effects of orchidectomy with or without testosterone replacement on *Igf1r* mRNA expression in the epididymis. Rats were orchidectomized with empty (black bars) or testosterone-filled (grey bars) implants and sacrificed 0.5, 1, 3, and 7 days after surgery. Changes in *Igf1r* mRNA were assayed by qRT-PCR in the IS (A), Ca (B), Co (C), and Cd (D). *Igf1r* expression was normalized to *Ppia* (*cyclophilin A*) expression. Day 0 corresponds to sham-operated animals (white bars). Data are presented as mean ±SEM (n=5/group). Significant effects ($p<0.05$) of treatment on expression are depicted by (**) and significant changes as compared to sham-operated are depicted by (*).

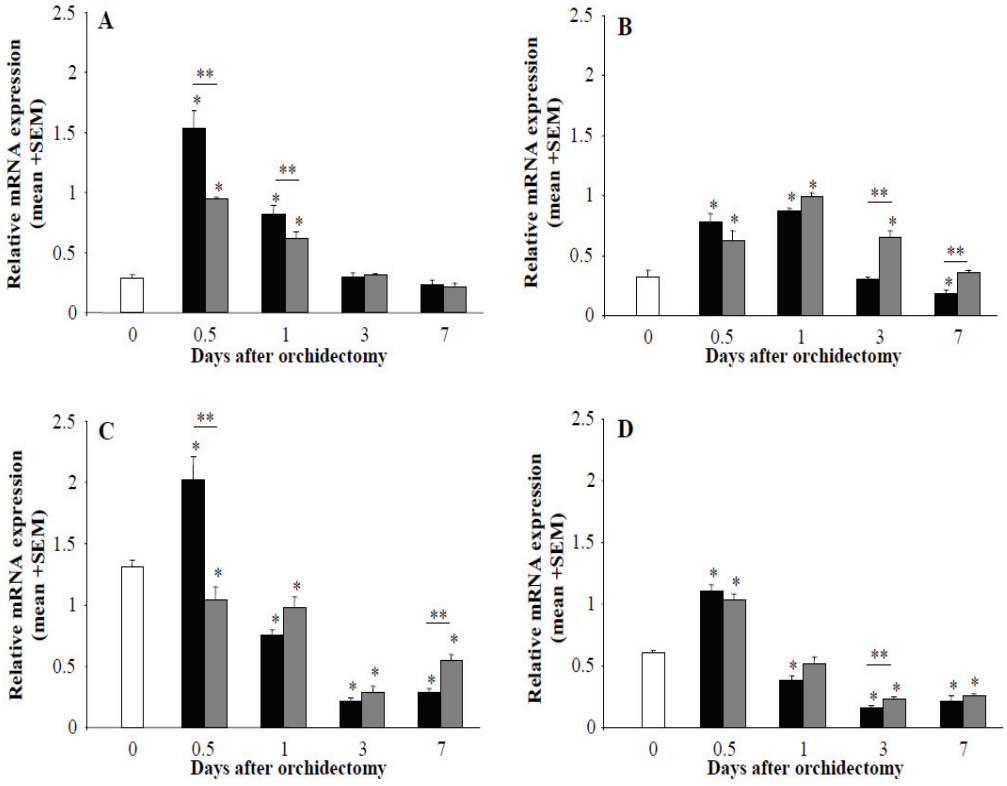


Figure 3: Effects of orchidectomy with or without testosterone replacement on *Ide* mRNA expression in the epididymis. Rats were orchidectomized with empty (black bars) or testosterone-filled (grey bars) implants and sacrificed 0.5, 1, 3, and 7 days after surgery. Changes in *Ide* mRNA were assayed by qRT-PCR for IS (A), Ca (B), Co (C), and Cd (D). *Ide* expression was normalized to *Ppia* (*cyclophilin A*) expression. Day 0 corresponds to sham-operated animals (white bars). Data are presented as mean ±SEM (n=5/group). Significant effects ($p<0.05$) of treatment on expression are depicted by (**) and significant changes as compared to sham-operated are depicted by (*).

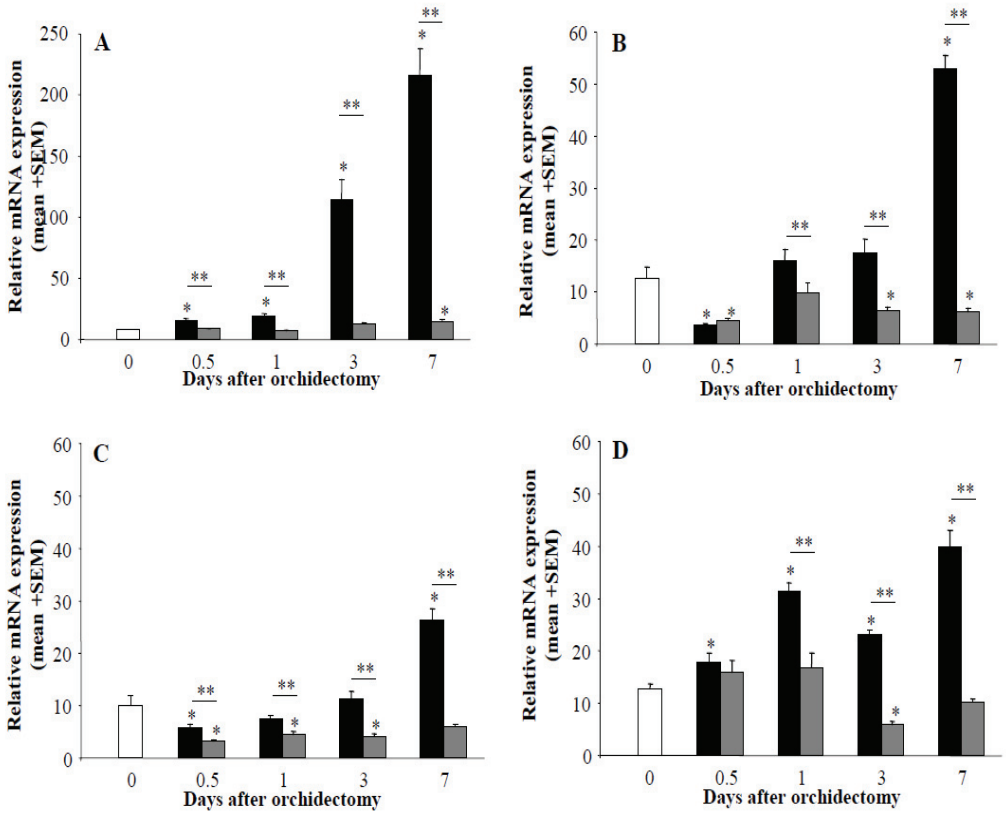


Figure 4: Effects of orchidectomy with or without testosterone replacement on *Igfbp3* mRNA expression in the epididymis. Rats were orchidectomized with empty (black bars) or testosterone-filled (grey bars) implants and sacrificed 0.5, 1, 3, and 7 days after surgery. Changes in *Igfbp3* mRNA were assayed by qRT-PCR for IS (A), Ca (B), Co (C), and Cd (D). *Igfbp3* expression was normalized to *Ppia* (*cyclophilin A*) expression. Day 0 corresponds to sham-operated animals (white bars). Data are presented as mean ±SEM (n=5/group). Significant effects ($p<0.05$) of treatment on expression are depicted by (**) and significant changes as compared to sham-operated are depicted by (*).

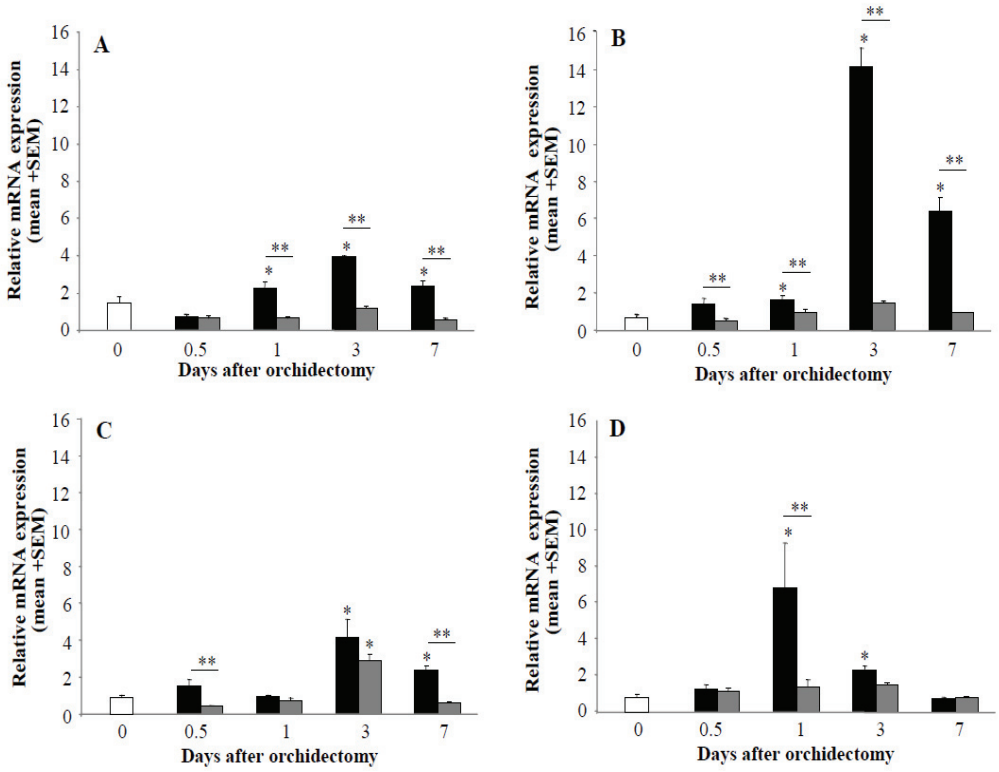


Figure 5: Effects of orchidectomy with or without testosterone replacement on *Birc5* mRNA expression in the epididymis. Rats were orchidectomized with empty (black bars) or testosterone-filled (grey bars) implants and sacrificed 0.5, 1, 3, and 7 days after surgery. Changes in *Birc5* mRNA were assayed by qRT-PCR in the IS (A), Ca (B), Co (C), and Cd (D). *Birc5* expression was normalized to *Ppia* (*cyclophilin A*) expression. Day 0 corresponds to sham-operated animals (white bars). Data are presented as mean ±SEM (n=5/group). Significant effects ($p<0.05$) of treatment on expressions are depicted by (**) and significant changes as compared to sham-operated are depicted by (*).

Figure 6: Potential androgen-response elements in the upstream promoter region of rat *Birc5*. *In silico* analysis was done to identify putative androgen-response elements in the 3kb upstream promoter region of rat *Birc5*.

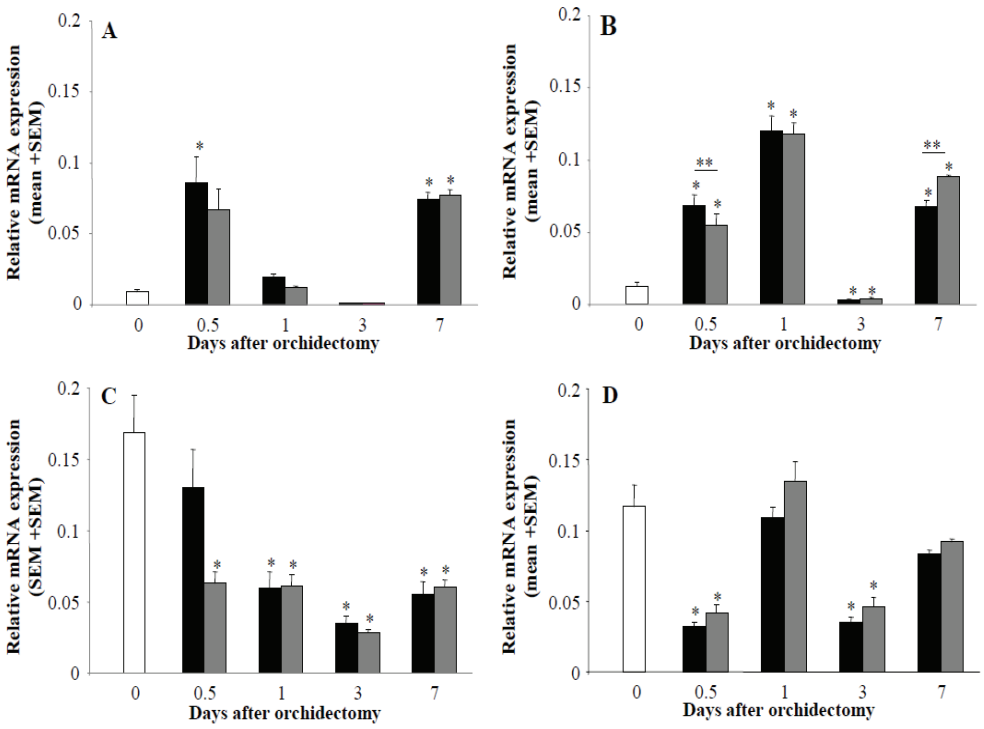


Figure 7: Effects of orchidectomy with or without testosterone replacement on *Diablo* mRNA expression in the epididymis. Rats were orchidectomized with empty (black bars) or testosterone-filled (grey bars) implants and sacrificed 0.5, 1, 3, and 7 days after surgery. Changes in mRNA were assayed by qRT-PCR for *Diablo* in the IS (A), Ca (B), Co (C), and Cd (D). *Diablo* expression was normalized to *Ppia* (*cyclophilin A*) expression. Day 0 corresponds to sham-operated animals (white bars). Data are presented as mean ±SEM (n=5/group). Significant effects ($p<0.05$) of treatment on expression are depicted by (**) and significant changes as compared to sham-operated are depicted by (*).

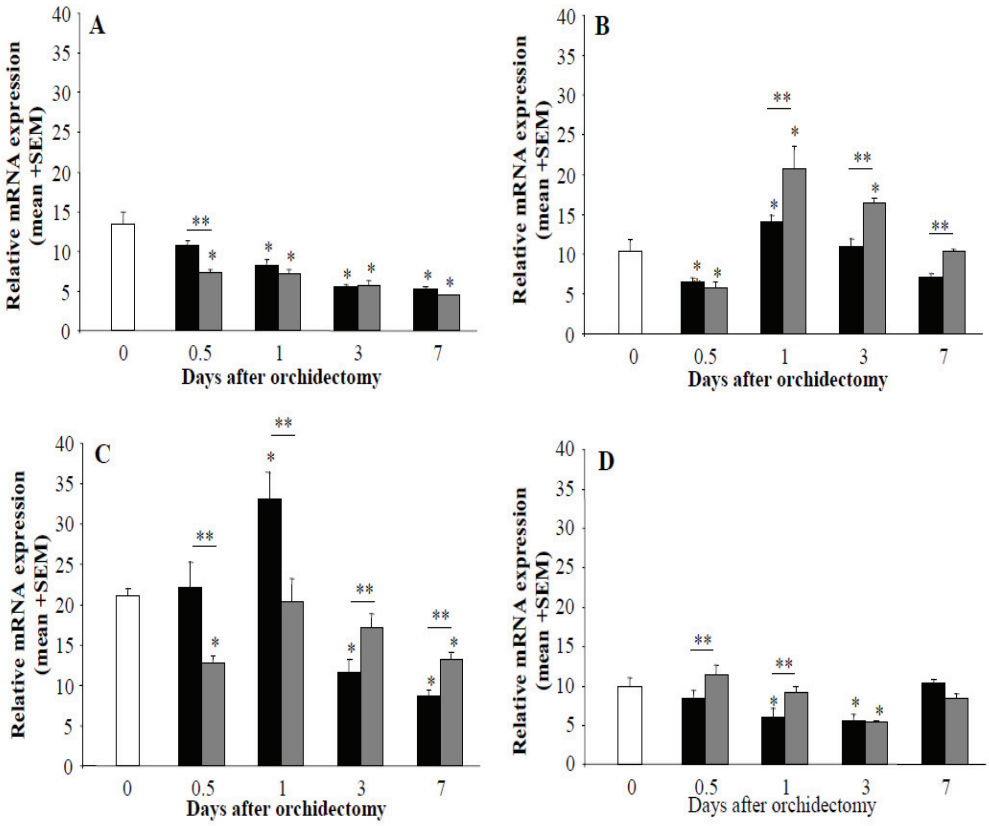


Figure 8: Effects of orchidectomy with or without testosterone replacement on *Bax* mRNA expression in the epididymis. Rats were orchidectomized with empty (black bars) or testosterone-filled (grey bars) implants and sacrificed 0.5, 1, 3, and 7 days after surgery. Changes in mRNA were assayed by qRT-PCR for *Bax* in the IS (A), Ca (B), Co (C), and Cd (D). *Bax* expression was normalized to *Ppia* (*cyclophilin A*) expression. Day 0 corresponds to sham-operated animals (white bars). Data are presented as mean ±SEM (n=5/group). Significant effects ($p<0.05$) of treatment on expression are depicted by (**) and significant changes as compared to sham-operated are depicted by (*).

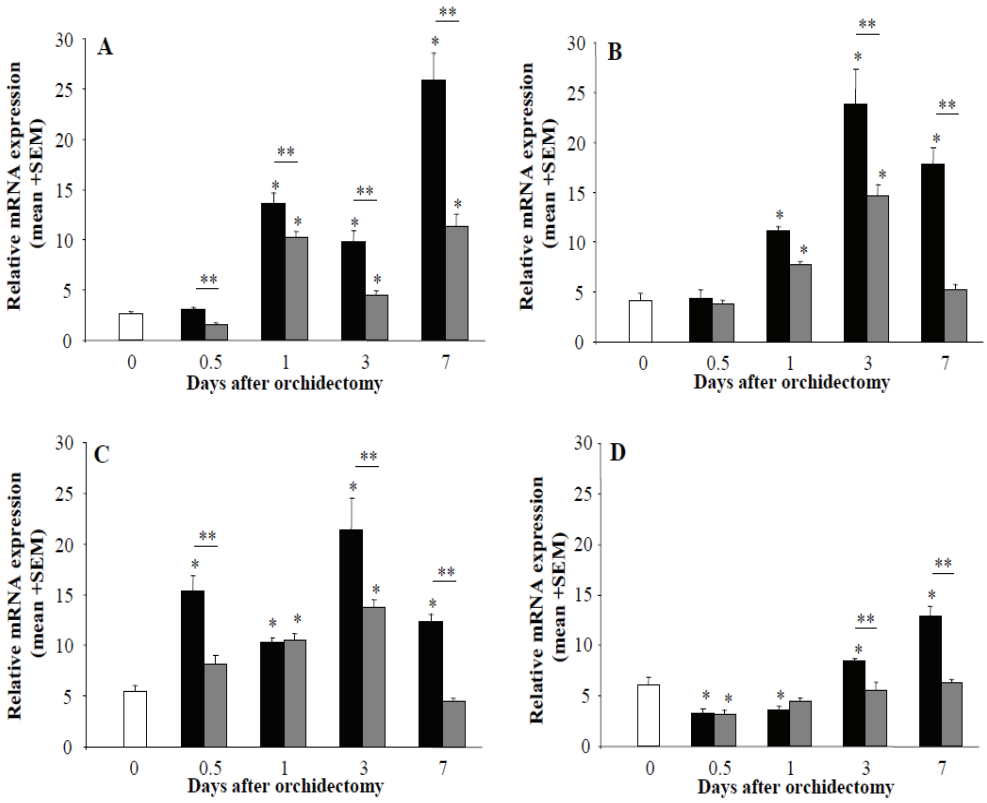


Figure 9: Effects of orchidectomy with or without testosterone replacement on *Bid* mRNA expression in the epididymis. Rats were orchidectomized with empty (black bars) or testosterone-filled (grey bars) implants and sacrificed 0.5, 1, 3, and 7 days after surgery. Changes in mRNA were assayed by qRT-PCR for *Bid* in the IS (A), Ca (B), Co (C), and Cd (D). *Bid* expression was normalized to *Ppia* (*cyclophilin A*) expression. Day 0 corresponds to sham-operated animals (white bars). Data are presented as mean ±SEM (n=5/group). Significant effects ($p<0.05$) of treatment on expression are depicted by (**) and significant changes as compared to sham-operated are depicted by (*).

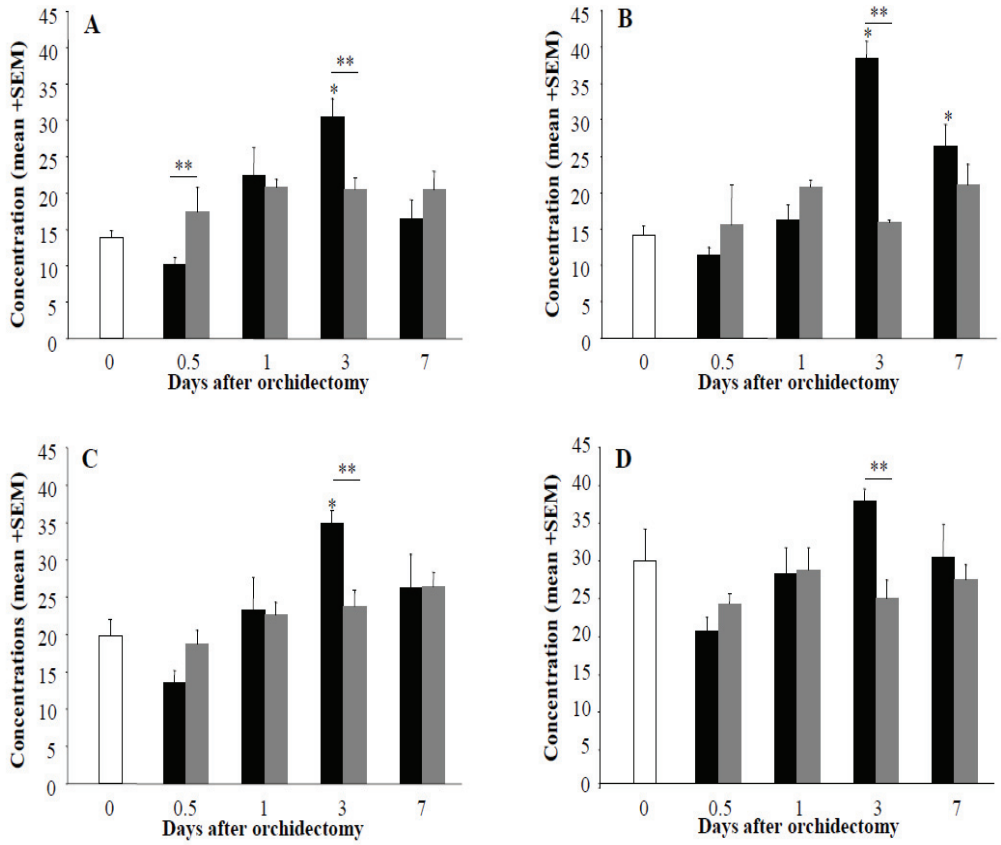


Figure 10: Effects of orchidectomy with or without testosterone replacement on IGF1 protein expression in the epididymis. Rats were orchidectomized with empty (black bars) or testosterone-filled (grey bars) implants and sacrificed 0.5, 1, 3, and 7 days after surgery. Concentrations of IGF1 in the IS (A), Ca (B), Co (C), and Cd (D) were assayed using the quantikine mouse IGF1 ELISA assay where every sample was assayed in duplicate. Day 0 corresponds to sham-operated animals (white bars). Data are presented as mean ±SEM (n=5/group). Significant effects ($p<0.05$) of treatment on expression are depicted by (**) and significant changes as compared to sham-operated are depicted by (*).

Figure 11: Effects of orchidectomy with or without testosterone replacement on IGF1R protein expression in the epididymis. Rats were orchidectomized with empty (black bars) or testosterone-filled (grey bars) implants and sacrificed 0.5, 1, 3, and 7d after surgery. IGF1 expression was determined by western blots in the IS (A), Ca (B), Co (C), and Cd (D). (E) Representative films for the different epididymal regions. Expression of IGF1R was quantified and normalized relative to ACTIN expression. Day 0 corresponds to sham-operated animals (white bars). Data are presented as mean ±SEM (n=5/group). Significant effects ($p<0.05$) of treatment on expression are depicted by (**) and significant changes as compared to sham-operated are depicted by (*).

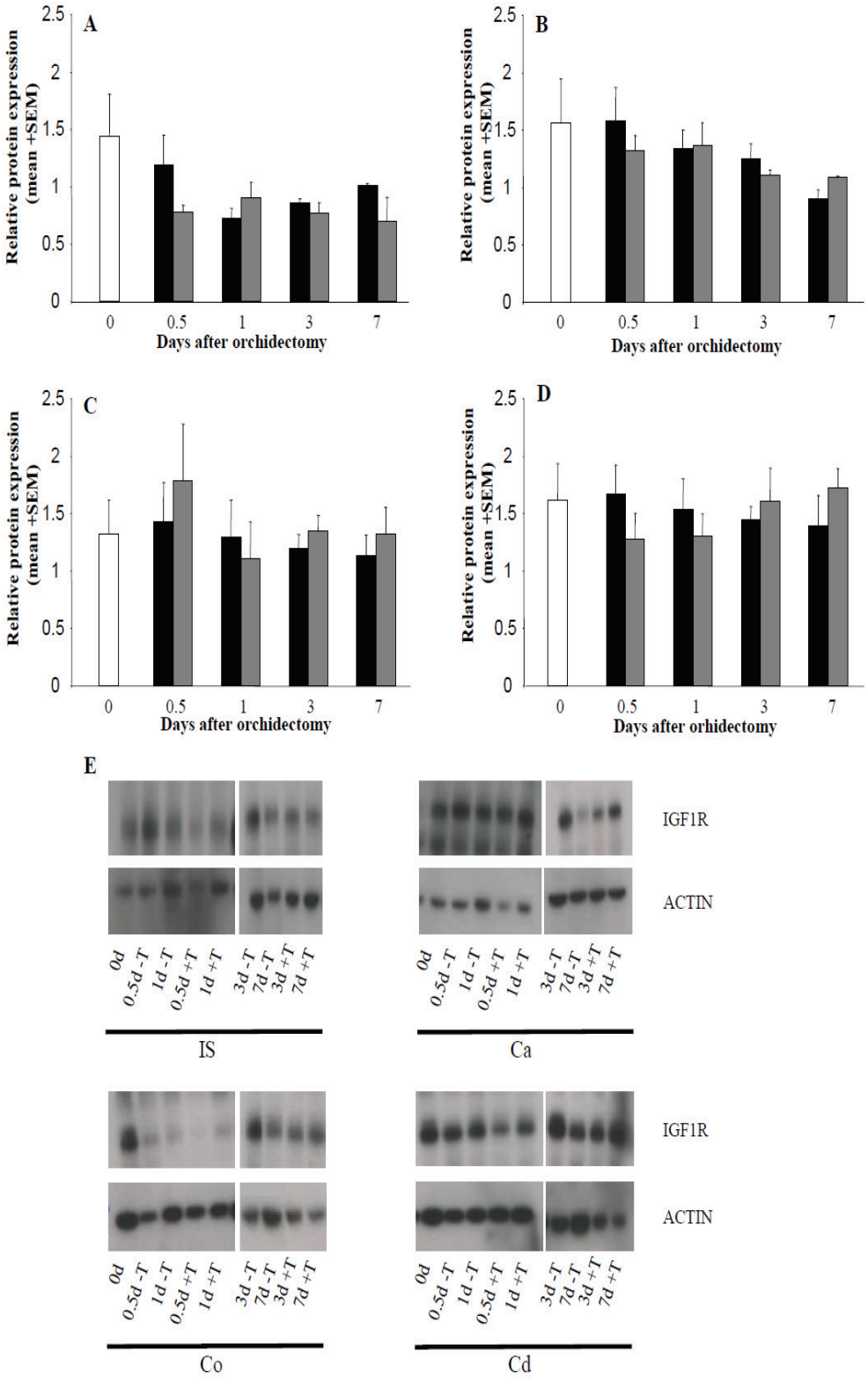

A
B
C
D
E
Relative protein expression (mean +SEM)
Days after orchidectomy
Days after orhidectomy
IGF1R
ACTIN
0d
0.5d -T
1d -T
0.5d +T
1d +T
3d -T
7d -T
3d +T
7d +T
IS
Ca
Co
Cd

Figure 12: Effects of orchidectomy with or without testosterone replacement on BIRC5 protein expression in the epididymis. Rats were orchidectomized with empty (black bars) or testosterone-filled (grey bars) implants and sacrificed 0.5, 1, 3, and 7 days after surgery. BIRC5 expression was determined by western blots in the IS (A), Ca (B), Co (C), and Cd (D). (E) Representative films for the different epididymal regions. Expression of BIRC5 was quantified and normalized relative to ACTIN expression. Day 0 corresponds to sham-operated animals (white bars). Data are presented as mean ±SEM (n=5/group). Significant effects ($p<0.05$) of treatment on expression are depicted by (**) and significant changes as compared to sham-operated are depicted by (*). n.d. is for non-detectable.

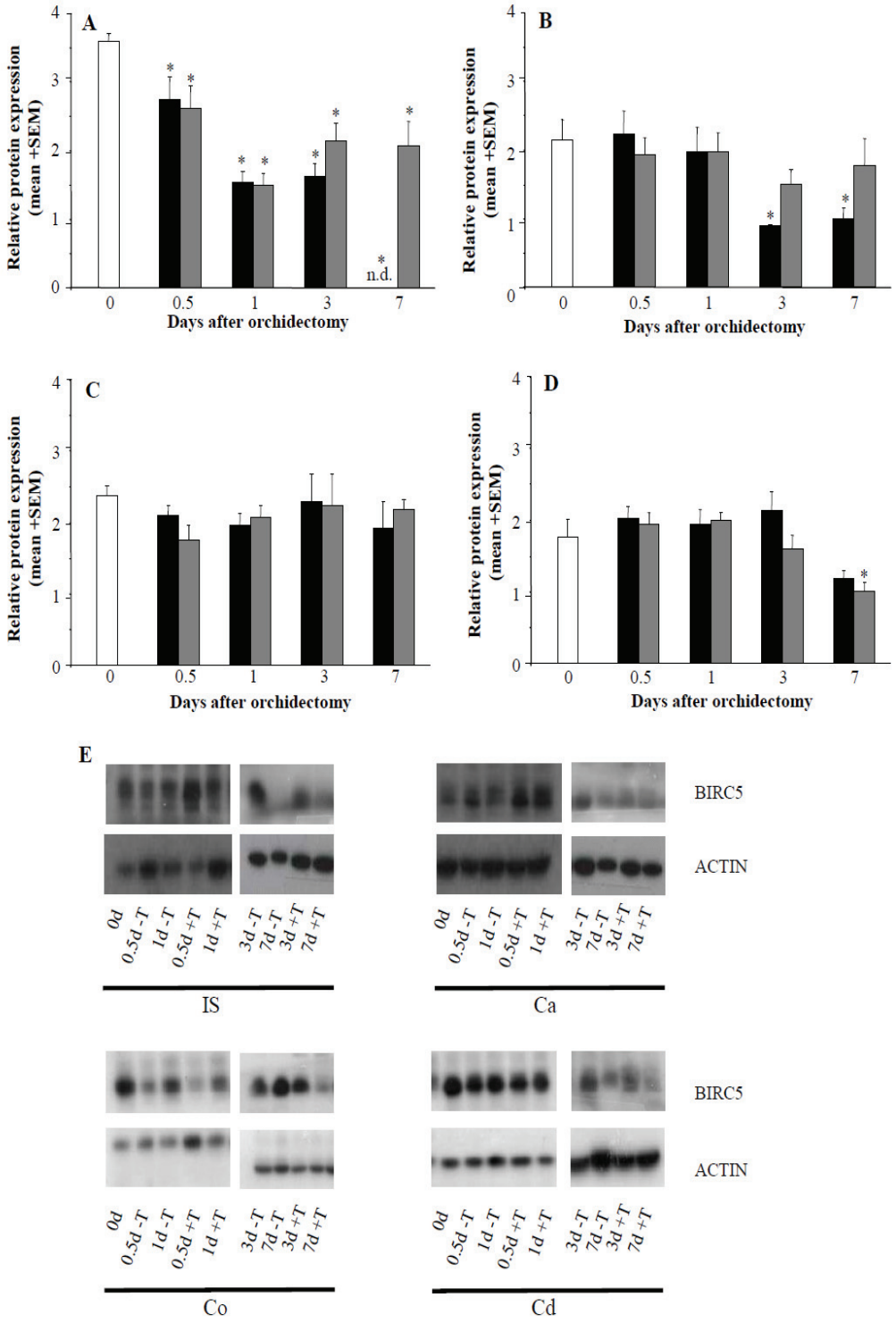

A
B
C
D
E
Relative protein expression (mean +SEM)
Days after orchidectomy
0
0.5
1
3
7
n.d.
BIRC5
ACTIN
0d
0.5d -T
1d -T
0.5d +T
1d +T
3d -T
7d -T
3d +T
7d +T
IS
Ca
Co
Cd

Figure 13: Patterns of changes in mRNA expression for *Igf1*, *Igf1r*, *Igfbp3*, and *Ide* in the epididymis. Patterns of changes in expression are represented for *Igf1* (black line), *Igf1r* (purple line), *Igfbp3* (light blue line), and *Ide* (orange line) after orchidectomy without and with testosterone replacement in the different epididymal regions.

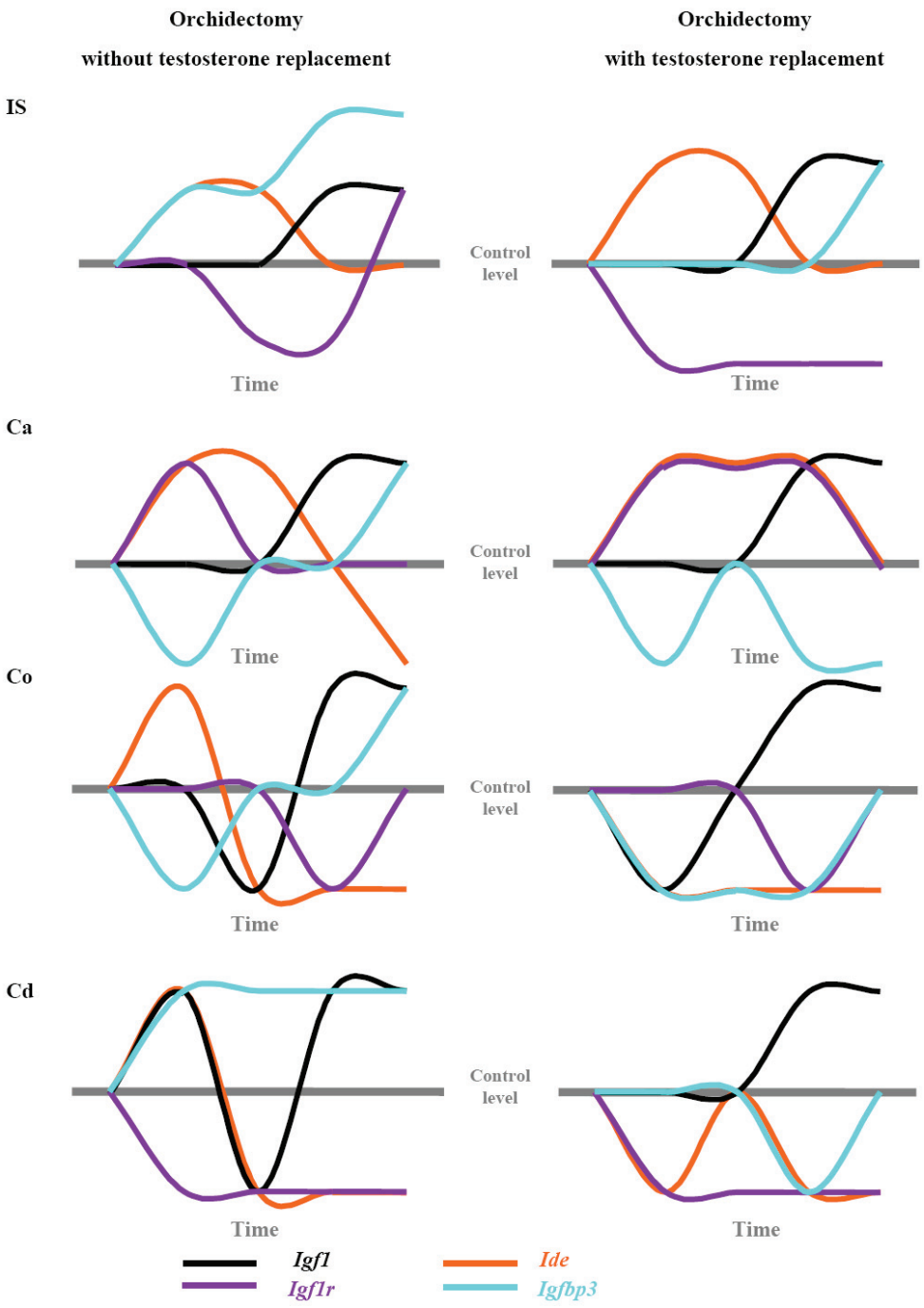

Orchidectomy
without testosterone replacement
Orchidectomy
with testosterone replacement
IS
Ca
Co
Cd
Control level
Time
Igf1
Igf1r
Ide
Igfbp3

Figure 14: Patterns of changes in mRNA expression for *Birc5* and *Diablo* in the epididymis. Patterns of changes in expression are represented for *Birc5* (pink line) and *Diablo* (green line) after orchidectomy without and with testosterone replacement in the different epididymal regions.

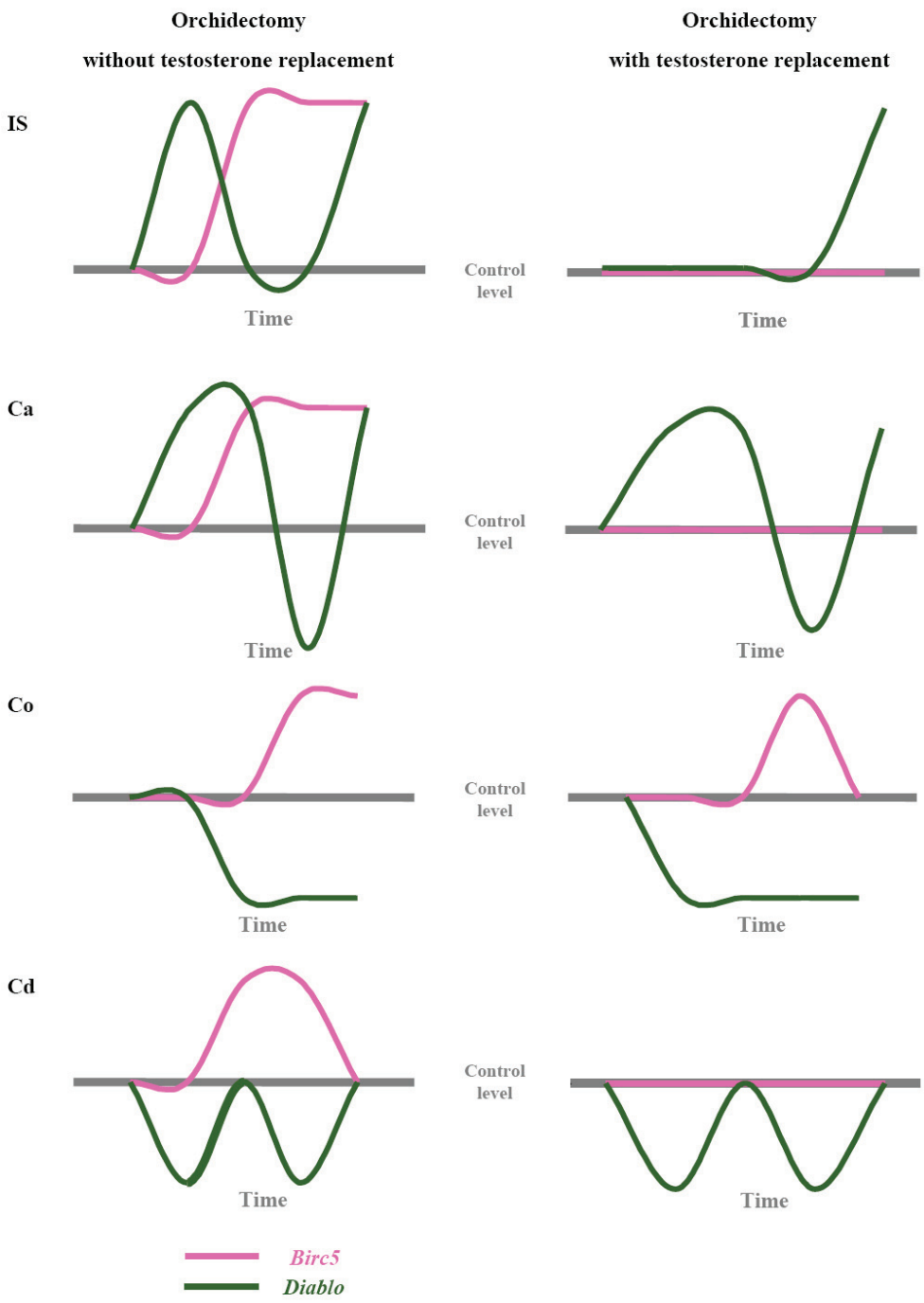
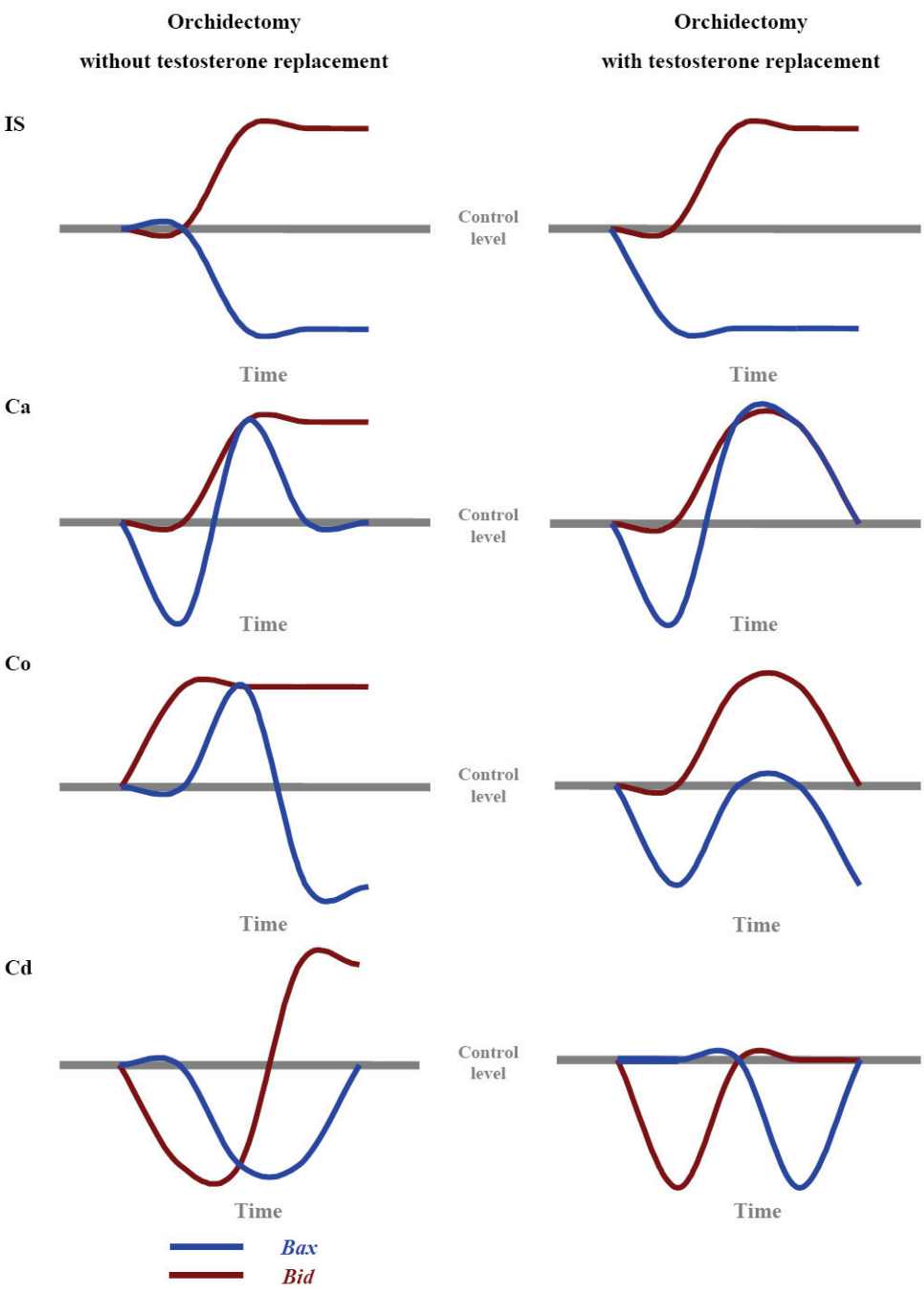

Orchidectomy
without testosterone replacement
Orchidectomy
with testosterone replacement
IS
Ca
Co
Cd
Control level
Time
Birc5
Diablo

Figure 15: Patterns of changes in mRNA expression for *Bax* and *Bid* in the epididymis. Patterns of changes in expression are represented for *Bax* (dark blue line) and *Bid* (brown line) after orchidectomy without and with testosterone replacement in the different epididymal regions.


Orchidectomy
without testosterone replacement
Orchidectomy
with testosterone replacement
IS
Ca
Co
Cd
Control level
Time
Bax
Bid

CONNECTING TEXT

In chapter 3, members of the IGF1 survival signaling pathway showed time-dependent and region-specific changes in transcription after orchidectomy with or without testosterone replacement. This suggested an involvement of this pathway in the response of the epididymis to androgen withdrawal. In order to confirm the role of this pathway in the response of the epididymis to androgen withdrawal, the effects of androgen withdrawal and blockade on two epididymal cell lines will be characterized in the next chapter.

CHAPTER 4

Effects of Androgen Withdrawal on the PC-1 and DC-3 Mouse Epididymal Cell Lines

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1. Abstract

The epididymis, the tissue responsible for the proper maturation and storage of spermatozoa, is regulated by androgens. However, androgen withdrawal by orchidectomy causes little apoptosis. The IGF1 signaling pathway is a candidate survival pathway in the response of the epididymis to androgen withdrawal. To investigate the signaling cascades activated after androgen withdrawal, we used the PC-1 and DC-3 mouse epididymal cell lines and assessed their response to androgen withdrawal and/or blockade. This response was characterized through evaluation of cell survival, changes in *Birc5*, *Igf1*, and *Igf1r* mRNA expressions by qRT-PCR, and changes in IGF1 concentrations by ELISA. We found that androgen withdrawal and/or blockade had no effect on cell survival or IGF1 concentration in the media for both PC-1 and DC-3 cells. However, duration of pre-treatment in charcoal-filtered FBS and DHT changed IGF1 concentration in the media; pre-treatment for 24h gave the highest IGF1 concentrations. In addition, PC-1 cells released more IGF1 into the media than DC-3 cells. In the PC-1 cells, androgen withdrawal and/or blockade did not change transcript expression of *Birc5*, *Igf1*, and *Igf1r*, whereas it increased *Birc5* and *Igf1* in the DC-3 cells. These results suggest that DC-3 cells may be a better model system to study androgen action.

2. Introduction

The epididymis, a single highly convoluted tubule that links the efferent ducts of the testis to the vas deferens, is responsible for the proper maturation and storage of spermatozoa (1). It is morphologically and functionally divided into four regions: initial segment (IS), caput (Ca), corpus (Co), and cauda (Cd); at the epithelial level, it is composed of four major cell types (principal, basal, halo, and clear cells) (2;3). Although the primary regulators of epididymal structure and functions are androgens, in particular testosterone (T) and its 5α–reduced metabolite dihydrotestosterone (DHT), other factors such as estrogens (4), growth factors (5), and testicular factors [basic fibroblast growth factor (FGF2) (6) and androgen binding protein (ABP) (7)] are also important for the regulation of the epididymis (1).

Androgen withdrawal by orchidectomy causes a decrease in epididymal weight due to the removal of spermatozoa and luminal fluid as well as a decrease in epididymal cell height (8;9). However, there is little apoptosis associated with androgen withdrawal (10); this is in sharp contrast with another androgen-dependent tissue, the prostate, where 80% of the cells are lost by apoptosis within 10 days of castration (11). Despite the identification of apoptotic and cell survival genes, in particular the anti-apoptotic genes *baculoviral IAP repeat-containing 5* (*Birc5*), *insulin-like growth factor 1* (*Igf1*), *Igf1r*, and *myeloid cell differentiation protein 1* (*Mcl1*), that show changes in expression in the epididymis after orchidectomy with or without testosterone replacement (chapter 2), little is known regarding the signaling cascades activated after androgen withdrawal. The IGF1 signaling cascade is a candidate survival pathway. IGF1 promotes cell survival through binding to its receptor, IGF1R, and the activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway (12;13). Activation of this pathway can lead to the activation of BIRC5, an inhibitor of apoptosis protein (IAP) that inhibits caspase-3 activity (14).

Determining the signaling cascades activated after androgen withdrawal is best done using immortalized epididymal cell lines. Although several immortalized rodent epididymal cell lines have been created (15-19), the PC-1

mouse epididymal cell line (18) is the only one that has demonstrated changes in gene expression after androgen withdrawal (20). In addition to the PC-1 cell line, Araki et al. (18) have also developed the DC-3 mouse epididymal cell line that might be more androgen-responsive than the PC-1 cell line (personal communication). In fact, DC-3 cells arise from cells of the distal Ca, whereas PC-1 cells have been isolated from cells of the proximal Ca. PC-1 and DC-3 cells maintain morphological features of epididymal cells *in vivo* as well as expression of genes present in the Ca epididymidis and in particular of the androgen receptor (AR) (18). In addition, Seenundun and Robaire (20) have shown that IGF1 is central to the response of the PC-1 cells to androgen withdrawal.

The objective of this study was to assess the response of the PC-1 and DC-3 mouse epididymal cell lines to androgen withdrawal. We found that androgen withdrawal had no effect on PC-1 and DC-3 cell viability, but that DHT supplementation increased *Birc5* and *Igf1r* mRNA expression in the DC-3 cells.

3. Materials and Methods

3.1. Chemicals

DHT (5α-androstan-17β-ol-3-one), estradiol (E2, 1,3,5(10)-estrien-3,17β-diol) were purchased from Steraloids Inc. (Newport, RI). Hydroxyflutamide (HF, CAS number 52806-53-8) was purchased from Toronto Research Chemicals Inc. (North York, ON). E2, DHT, and HF were dissolved in ethanol. All cell culture reagents were purchased from Wisent Inc. (St-Bruno, QC).

3.2. Cell culture

The mouse proximal caput epididymis PC-1 cell line and the mouse distal caput epididymis DC-3 cell line (kindly provided by Dr. M.-C. Orgebin-Crist, Department of Obstetrics and Gynecology, Vanderbilt University School of Medicine, Nashville, TN) (passage number <12) were grown in Iscove modified Dulbecco medium (IMDM) supplemented with 10% fetal bovine serum (FBS), 1mM sodium pyruvate, 0.1mM nonessential amino acids, 4mM glutamine, penicillin-streptomycin (25 000 U penicillin G sodium, 25 mg streptomycin

sulfate), and 1nM DHT. DHT was used instead of testosterone because epididymal cells respond to DHT and to bypass the conversion of testosterone to DHT by 5α-reductase. PC-1 and DC-3 cells were cultured at 33^0C with 5% CO_2. Cells were plated in 75cm^2 flasks in phenol-red negative IMDM where FBS was replaced by charcoal-filtered FBS (CF-FBS) supplemented with DHT for two passages (4 days; experiment 1), one passage (48h, experiment 2), or 24h (experiment 3) before treatment. The cells were then exposed to 4 different conditions (n=3/group): CF-FBS + vehicle (ethanol) (1), CF-FBS + 1nM DHT (2), CF-FBS + 1μM HF (3), and CF-FBS + 1nM DHT + 1μM HF (4). After 24h of treatment, media were collected for IGF1 ELISA analysis. To assess changes in gene transcription, PC-1 and DC-3 cells were pre-cultured for 24h in CF-FBS + 1nM DHT and then exposed to the 4 previously described conditions.

3.3. Cell viability assay

PC-1 and DC-3 cells were seeded on 96-well plates to determine the effects of androgen treatment, withdrawal and/or blockade on their viability. Cells to be collected after 1 day were seeded at a density of 25 000 cells/well, after 2 days at 12 500 cells/well, and after 4 days at 6250 cells/well. These numbers were chosen because cells double every 48h so that the final number of cells in each well should be identical if there was no treatment effect on growth. Fresh media were added every day. Cells were exposed to 4 different conditions (n=5/group): CF-FBS + vehicle (ethanol) (1), CF-FBS + 1nM DHT (2), CF-FBS + 1μM HF (3), and CF-FBS + 1nM DHT + 1μM HF (4). After 1, 2, and 4 days of treatment, cell viability was assessed using the CellTiter-Glo luminescent cell viability assay (Promega, Madison, WI) following the manufacturer's instructions. Luminescence was read with the Orion II microplate luminometer (Berthold Detection Systems, Huntsville, AL); the value of the instrument background was subtracted from each obtained measure. Cell numbers from luminescence signals were determined using a standard curve.

3.4. RNA extraction

RNA was extracted from PC-1 and DC-3 cells following treatment (n=3/group) using Qiagen Plus Mini-prep (Qiagen Inc., Mississauga, ON) following manufacturer's instructions. Concentration and quality of RNA were verified with a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

3.5. Quantitative Real-Time RT-PCR

Real-Time RT-PCR was done to quantify changes in expression for *Birc5*, *Igf1*, and *Igf1r* (Table 1) using the QuantiTect RT-PCR SybrGreen kit (Qiagen Inc.) and the LightCycler system (Roche Applied Science, Laval, QC) as described elsewhere (20). Each sample was assayed in duplicate. Expressions were normalized against *peptidylprolyl isomerase A* (*Ppia*, *cyclophilin A*) expression. *Ppia* is a housekeeping gene; its mRNA expression is not affected by androgen manipulation (21).

3.6. IGF1 ELISA

Quantification of IGF1 concentrations in the media (n=3/group) was done using the mouse IGF1 quantikine immunoassay (R&D Systems Inc., Minneapolis, MN) following the manufacturer's instructions. Every sample was evaluated in duplicate. The minimum detectable amount was 3.5pg/ml. Intra-assay CV was 4.3% and inter-assay CV was 6%.

3.7. Statistical analysis

Significant differences between the different treatments on mRNA expression, IGF1 concentration, and cell viability were assessed by two-way ANOVA followed by the Holm-Sidak *post-hoc* test. Significance was set at $p<0.05$.

4. Results

4.1. Androgen treatment, withdrawal and/or blockade had no effect on PC-1 and DC-3 cell viability

In order to assess the effects of androgen withdrawal on PC-1 and DC-3 cell viability, cells were cultured in the absence or presence of DHT; effects of androgen blockade were assessed by culturing the cells in the presence of hydroxyflutamide (HF), the active metabolite of flutamide, a non-steroidal full antagonist of the androgen receptor (AR) (22). For both PC-1 (fig. 1A,C) and DC-3 (fig. 1B,D) cells, androgen treatment, withdrawal and/or blockade had no effect on the numbers of viable cells.

4.2. Effects of androgen treatment, withdrawal and/or blockade on *Igf1*, *Igf1r*, and *Birc5* mRNA expression

Given that *Igf1*, *Igf1r*, and *Birc5* mRNA expression have been shown to be regulated by androgens in the epididymis (chapter 3), we determined the effects of androgen treatment, withdrawal and/or blockade on *Igf1*, *Igf1r*, and *Birc5* transcription in PC-1 (fig. 2A) and DC-3 (fig. 2B) cells. Cells were cultured in the absence or presence of DHT and/or HF and changes in mRNA for *Igf1*, *Igf1r*, and *Birc5* were assessed by qRT-PCR. The treatments had no effect on transcription of *Igf1*, *Igf1r*, and *Birc5* mRNA in the PC-1 cells (fig. 2A). In the DC-3 cells, presence of DHT significantly ($p<0.05$) increased *Ig1r* mRNA expression, even in the presence of HF. DHT also increased *Birc5* mRNA expression, even in the presence of HF (fig. 2B). This suggested that DC-3 cells were more sensitive to androgens than PC-1 cells.

4.3. Androgen withdrawal and/or blockade had no effect on IGF1 concentration

In order to determine if the lack of cell death after androgen withdrawal and/or blockade was associated with an increased expression of IGF1, we measured IGF1 concentration in media of PC-1 and DC-3 cells. Before the beginning of the experiment, PC-1 (fig. 3A-C) and DC-3 (fig. 3D-F) cells were

cultured in CF-FBS with DHT for different periods of time to assess if the duration of pre-treatment in CF-FBS and DHT had an effect on IGF1 concentration. Previously, Seenundun and Robaire (20) had pre-treated the cells for 2 passages (4 days) in CF-FBS (experiment 1; fig. 3A, C). We also pre-treated the cells for 1 passage (2 days, experiment 2; fig. 3B, D) and 24h (experiment 3; fig. 3C, E). In general, IGF1 concentrations for PC-1 cells (80-225pg/ml) were higher than for DC-3 cells (70-120pg/ml). For PC-1 cells, pre-treatment for 1 passage in CF-FBS with DHT (fig. 3B) gave the lowest concentration of IGF1 in the media (80pg/ml) and pre-treatment for 24h (fig. 3C) the highest concentration (225pg/ml). For DC-3 cells, pre-treatment for 24h in CF-FBS (fig. 3F) also gave the highest concentration (120pg/ml), whereas pre-treatment for 2 passages (fig. 3D) or 1 passage (fig. 3E) gave similar concentrations of IGF1 (50-80pg/ml). There was no effect of treatment on the concentration of IGF1 in the media. These data showed that PC-1 cells secreted more IGF1 than DC-3 cells. In addition, it suggested that duration of pre-treatment in CF-FBS and DHT could have an impact on IGF1 concentration.

5. Discussion

Although mRNA expression of *Igf1*, *Igf1r*, and *Birc5* are changed after orchidectomy with or without testosterone replacement, as well as expression of BIRC5 (chapter 3), suggesting a participation of the IGF1 signaling pathway in the response of the epididymis to androgen withdrawal, little is known about its functional involvement in the response of the epididymis to androgen withdrawal. In order to determine the signaling cascades activated after androgen withdrawal, the use of immortalized epididymal cell lines was warranted. Two immortalized epididymal cell lines, PC-1 and DC-3, were assessed for their response to androgen treatment, withdrawal and/or blockade; androgen blockade was achieved using HF. Androgen blockade allowed us to distinguish between transcriptional and potential non-transcriptional effects of androgens.

For both PC-1 and DC-3 cells, androgen treatment, withdrawal and/or blockade had no effect on the number of viable cells, a lack of effect that has been

previously reported for the PC-1 cells (20). These results mimick the lack of cell death observed *in vivo* after androgen withdrawal (23), as well as the lack of cellular proliferation in the epididymis in the presence of T (24). This suggests that both PC-1 and DC-3 cells were similar to epididymal cells *in vivo* and hence they could be good model systems to study the effects of androgen withdrawal on the epididymis.

After androgen treatment, withdrawal and/or blockade, there was no change in gene expression for *Birc5*, *Igf1*, *Igf1r*, and *Mcl1* in the PC-1 cells, whereas *Birc5* and *Igf1r* were increased in the presence of DHT in the DC-3 cells; the latter effect was not prevented by the presence of HF. The lack of effect of androgen withdrawal on *Igf1* expression in the PC-1 cells is different from the previous increase in *Igf1* expression observed 4 days after androgen withdrawal (20). These differential responses could be explained by differences in treatments and times of measurement: we pre-cultured the cells for 24h in CF-FBS and DHT instead of 2 passages and we measured changes in mRNA after 24h of treatments instead of 2 days, 4 days, and 6 days. In the DC-3 cells, the observed increase in *Igf1r* mRNA after DHT supplementation resembles the increase observed in the Ca epididymidis after T replacement (chapter 3). On the other hand, the increase in *Birc5* mRNA after DHT supplementation does not resemble the decreased expression observed in the Ca epididymidis after T replacement (chapter 3). This shows a differential regulation of *Birc5* in the DC-3 cells compared to the epididymis. In addition, these changes in *Birc5* and *Igf1r* mRNA expression by DHT supplementation were not prevented by the presence of HF suggesting that these changes are potentially not associated with transcriptional activity of AR. In fact, AR can activate signaling cascades independently of its transcriptional activity (25). Alternatively, it has been shown that HF by itself can activate the Ras/MAPK pathway leading to changes in gene transcription (26). Together, these data suggest that the DC-3 cells are more sensitive than PC-1 cells to androgens for the markers studied, making them a potentially better model system to study androgen actions.

Although androgen treatment, withdrawal and/or blockade had no effect on IGF1 concentration in the media of both PC-1 and DC-3 cultures, which matched the lack of change in *Igf1* transcripts, duration of pre-treatment in CF-FBS and DHT impacted the total amount of IGF1. This shows that experimental conditions could have an impact on measured outcomes suggesting that proper experimental conditions should be carefully determined.

We have shown that androgen treatment, withdrawal and/or blockade have no effect on PC-1 and DC-3 cell survival as well as on IGF1 concentration. Although androgen treatment, withdrawal and/or blockade do not change expression of *Birc5*, *Igf1*, and *Igf1r* in the PC-1 cells, DHT increases *Birc5* and *Igf1* expression in the DC-3 cells. This study suggests that DC-3 cells may be a better model system than PC-1 cells to study androgen actions.

6. Acknowledgements

We would like to thank Dr. Eddy Rijntjes for his help with the collection of samples and IGF1 ELISA assays. We are very grateful to Dr. Marie-Claire Orgebin-Crist for providing us with the PC-1 and DC-3 mouse epididymal cell lines.

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Table 1: Real-Time RT-PCR primers

Gene name	Gene symbol	Genbank accession no.	Forward primer sequence (5'→ 3')	Reverse primer sequence (5'→ 3')
Peptidylprolyl isomerase A (Cyclophilin A)	*Ppia*	NM_017101	GTGGTCTTTGGG AAGGTGAA	GTTGTCCACAGT CGGAGATG
Baculoviral IAP repeat-containing 5	*Birc5*	NM-009689	QuantiTect Primer Assay (Qiagen Inc.) QT00113379	
Insulin-like growth factor 1	*Igf1*	NM_010512	TCATGTCGTCTT CACACCTCTTCT	CCACACACGAAC TGAAGAGCAT
Insulin-like growth factor 1 receptor	*Igf1r*	NM_010513	GACACTTGGCAT CCTGCTCT	CCCAGACCGACA ACTCATCT

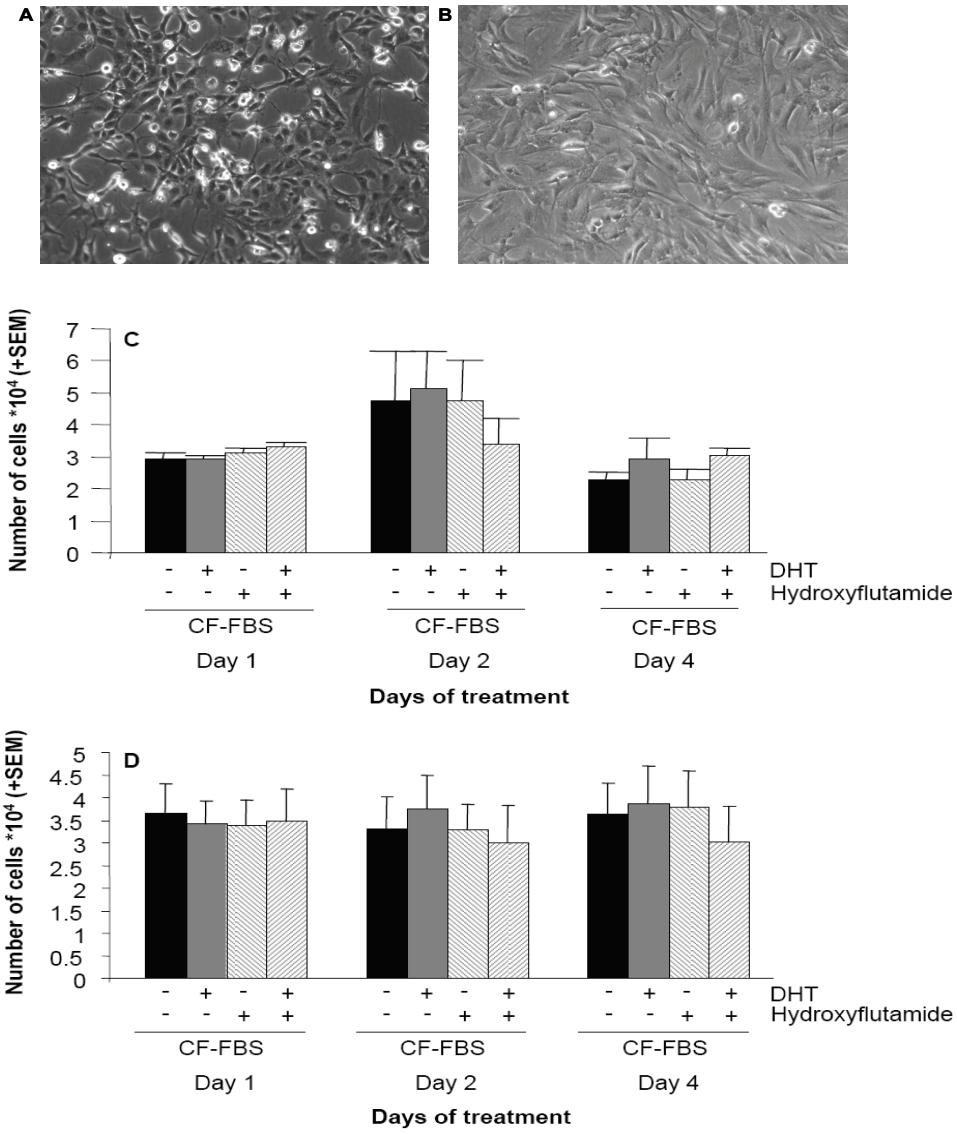

Figure 1: Effects of androgen treatment, withdrawal and/or blockade on PC-1 and DC-3 cell viability. PC-1 (A,C) and DC-3 (B,D) cells were cultured (n=5/group) in CF-FBS in the absence (-) of DHT without (black bars) or with (left-sided stripped bars) hydroxyflutamide and in the presence (+) of DHT without (grey bars) or with (right-sided stripped bars) hydroxyflutamide for 1, 2, and 4 days and numbers ($*10^4$) of viable cells were determined by the CellTiter-Glo luminescent cell viability assay. Data are presented as mean +SEM. Normal morphology of PC-1 (A) and DC-3 (B) cells are also shown.

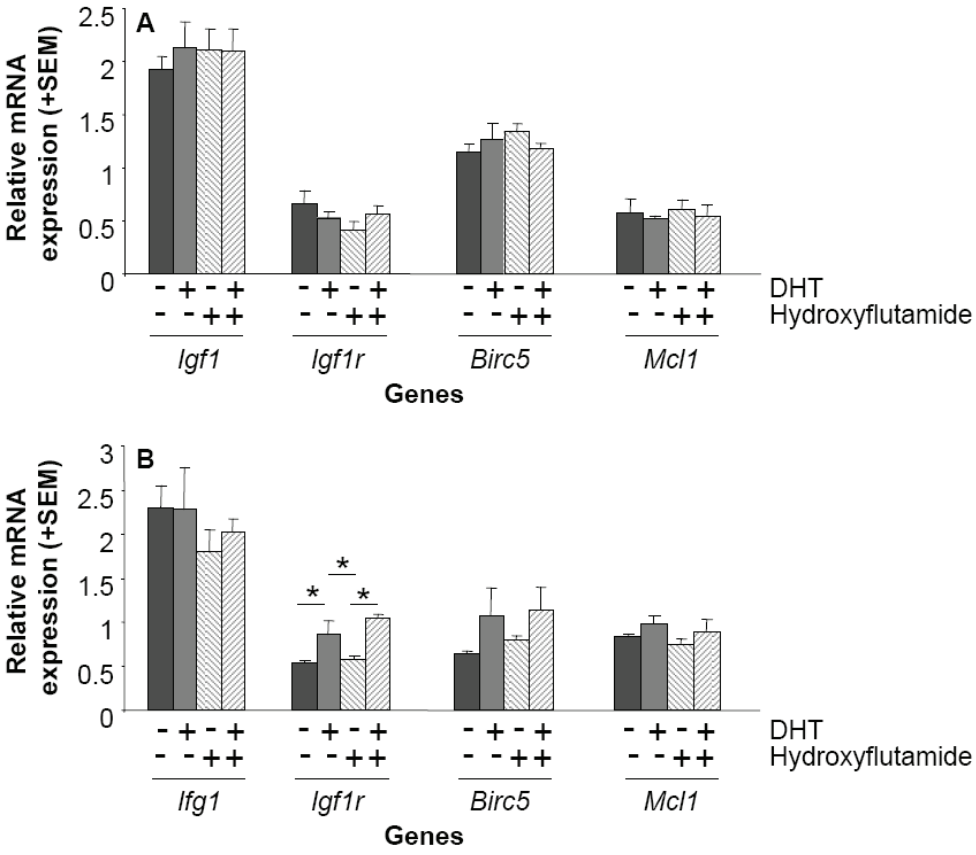

Figure 2: Effects of androgen treatment, withdrawal and/or blockade on *Ifg1*, *Igf1r*, and *Birc5* mRNA expression. PC-1 (A) and DC-3 (B) cells were cultured (n=3/group) in CF-FBS in the absence (-) of DHT without (black bars) or with (left-sided stripped bars) hydroxyflutamide and in the presence (+) of DHT without (grey bars) or with (right-sided stripped bars) hydroxyflutamide for 1 day. Changes in *Igf1*, *Igf1r*, and *Birc5* mRNA expression were assessed by qRT-PCR and normalized to *Ppia* (*cyclophilin A*) expression. Data are presented as mean +SEM. Significant effects ($p<0.05$) of treatments on mRNA expression are depicted by (*).

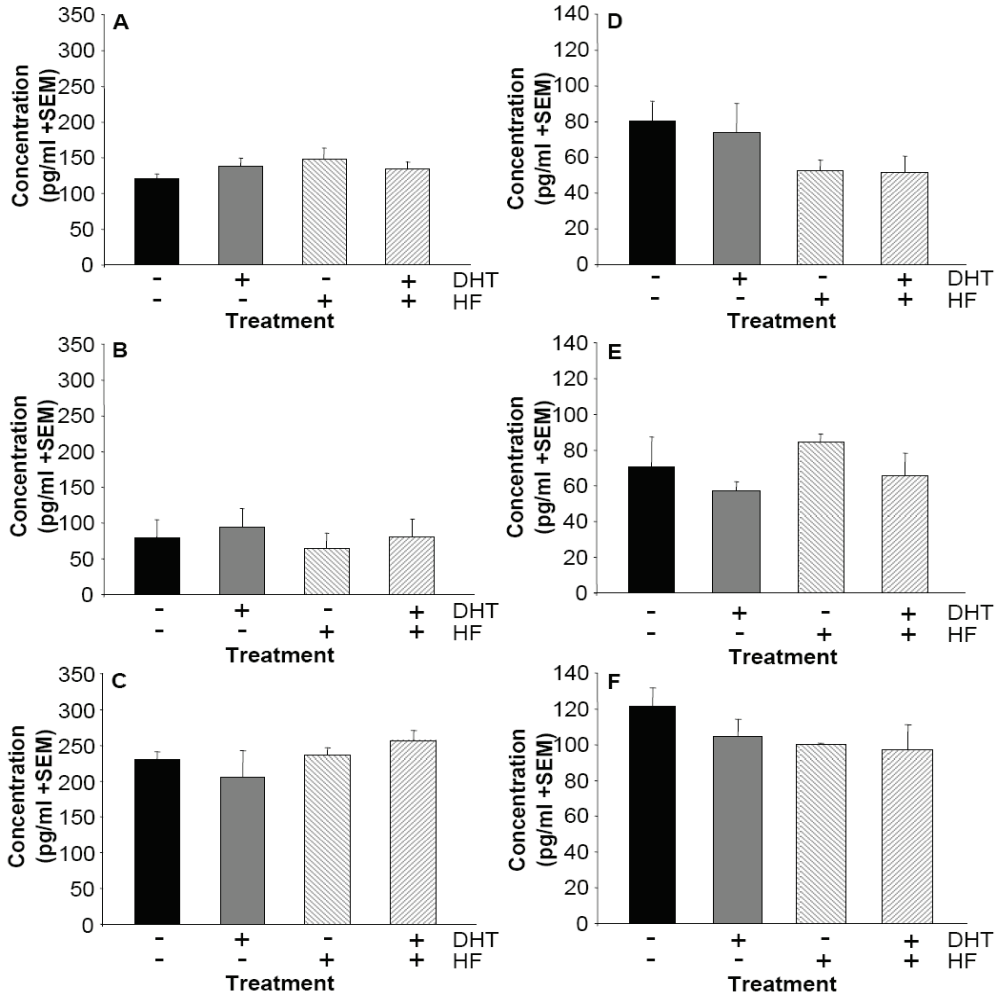

Figure 3: Effects of androgen treatment, withdrawal and/or blockade on IGF1 concentration. PC-1 (A-C) and DC-3 (D-F) cells were cultured (n=3/group) in CF-FBS with DHT for 2 passages (4 days, experiment 1; A, D), 1 passage (48h, experiment 2; B, E) or 24h (experiment 3; C, F). Then the cells were cultured for 24h in CF-FBS in the absence (-) of DHT without (black bars) or with (left-sided stripped bars) hydroxyflutamide (HF) and in the presence (+) of DHT without (grey bars) or with (right-sided stripped bars) HF. IGF1 concentrations (pg/ml) in the media were measured using the IGF1 mouse quantikine ELISA assay; every measure was done in duplicate.

CHAPTER 5

Discussion

Taking into account that androgen withdrawal by orchidectomy causes little apoptosis in the epididymis (1), this thesis aimed at understanding the molecular mechanisms underlying epididymal resistance to apoptosis triggered by androgen withdrawal. As androgens act through the androgen receptor (AR) to regulate gene transcription (2), this thesis assessed changes in transcription of apoptotic and cell survival genes as well as on members of the IGF1 survival signaling pathway after androgen withdrawal and/or replacement. In addition, in order to target specific signaling pathways, in particular the IGF1 signaling pathway, the effects of androgen withdrawal on two *in vitro* model systems were investigated.

This chapter will discuss the validity of the models used, some of the key findings from the studies undertaken in this thesis, and future research directions.

1. Androgen regulation of apoptosis and cell survival in the epididymis

Although in the epididymis, androgens are essential to regulate gene expression, structure, and functions [reviewed in (3)], androgen withdrawal by orchidectomy causes little apoptosis (1;4). The few studies that have investigated the molecular mechanisms triggered after orchidectomy have focused on explaining the apoptosis (5-7), but no one has examined the molecular mechanisms explaining the resistance of the epididymis to apoptosis triggered by androgen withdrawal.

1.1. Regulation of apoptosis and cell survival genes in the epididymis

As mentioned in section 2.4 of the introduction, many studies have been undertaken to understand changes in gene expression in the epididymis. However, the studies described in this thesis are the first ones to specifically assess androgen regulation of apoptosis and cell survival genes.

In chapter 2, apoptosis-focused arrays were used to identify specific apoptotic and cell survival genes differentially regulated after orchidectomy with or without testosterone replacement. The presence and regulation by androgens of *Birc5*, an IAP, was not expected in the epididymis due to the generally perceived

restricted expression pattern of BIRC5 in mitotically active tissues and cancers (8); the epididymis is a terminally-differentiated tissue with very rare cases of cancer (3). In the androgen-responsive prostate cancer LNCaP cell line, BIRC5 is highly expressed and shows increased expression after DHT supplementation (9). In the epididymis, *Birc5* expression was increased after orchidectomy and repressed by T replacement. The differential regulation of the same gene by androgens suggests the presence of specific coregulators (2) modulating *Birc5* expression differently in the prostate and the epididymis.

In chapter 3, members of the IGF1 signaling pathway were shown to be differentially regulated in the epididymis after androgen withdrawal and/or replacement. Androgen withdrawal caused an increase in expression of *Igf1*, *Igf1r*, and *Igfbp3*, which was prevented for *Igf1r* and *Igfbp3* by T replacement. In LNCaP cells, IGF1 promotes cell survival in an androgen-deprived state (9), hence increased expression of members of the IGF1 signaling pathway in the epididymis parallels changes observed in prostate cancer cells. However, in prostate cancer, *Igfbp3* expression is associated with apoptosis (10). Taking into account the little apoptosis observed in the epididymis after androgen withdrawal (1), it is unlikely that increased *Igfbp3* expression would be associated with apoptosis. In fact, IGFBP3 promotes cell survival in breast cancer cells (11) and it is possible that it has the same role in the epididymis.

1.2. Orchidectomy and testosterone replacement as a model system

All the studies that have specifically assessed the effects of androgen withdrawal on apoptosis in the epididymis have used orchidectomy with or without testosterone (T) replacement as their model system (1;4-7), with the exception of Fan and Robaire (1) who have also used efferent duct ligation. Given that orchidectomy was the model of choice in preceding studies, its use for the experiments presented in chapters 2 and 3 of this thesis was justified.

In order to determine the direct effects of androgens on the measured endpoints, T implants designed to maintain serum T concentration at control levels were used (12). Testosterone implants were selected instead of DHT

implants to mimic the control situation where testosterone is the circulating androgen. However, the epididymis receives T from both the circulation and the testis via the lumen (3) and luminal T concentration is ten to twelve times greater than that of circulation (13). It is then reasonable to ask if using implants that would mimic the high intraluminal T concentration would differentially affect the chosen endpoints. Robaire et al. (14) have shown that a higher circulating T concentration does not increase epididymal weight above the one measured with a control serum T concentration. In addition, the control T concentration is sufficient to maintain epididymal structure in all regions except the initial segment where a ten-fold higher circulating T concentration does not prevent regressive changes; epididymal 3-alpha hydroxysteroid dehydrogenase activity that decreases after orchidectomy, is maintained by a circulating T concentration and is not further increased by a ten-fold higher T concentration (14). This is due to the dependence of the initial segment on testicular factors to maintain its functions (3). However, DHT is the main androgen found in the epididymis (15;16) and arises from the reduction of T by 5α-reductase, the rate-limiting enzyme in this process (17). In fact, 5α-reductase can convert T to DHT at a rate of 7.5nmoles/min up to a substrate concentration of 0.5μmoles, after which production of DHT stays constant (17). Hence, it is fair to assume that different concentrations of circulating T concentrations could cause different responses and, in particular, different gene expression profiles. For example, high circulating T concentration is necessary to maintain control expression of 5α-reductase type 1 mRNA (18).

1.3. Other models of androgen blockade

As mentioned in section 4.4 of the introduction, other methods exist to block androgen action in the epididymis; these include treatment with AR antagonists, 5α-reductase inhibitors, and GnRH antagonists.

AR antagonists. AR antagonists that have been used to study epididymal functions include cyproterone acetate, flutamide, and bicalutamide (casodex). Cyproterone acetate, a progestin (19) is not only a potent androgen antagonist, but

also a weak antagonist; at a low concentration, it inhibits T-stimulated transcription, but at high concentrations, it promotes it (20). Cyproterone acetate has been shown to decrease epididymal weight and affect epididymal functions (21). From these studies, cyproterone acetate seems to be a potential alternative to orchidectomy; however, its partial agonist activity can confound results obtained with that compound. Flutamide and bicalutamide are pure antiandrogens; however, flutamide causes a compensatory rise of serum androgens (22). Most studies done on the epididymis with those two compounds show that they have no effect on the epididymis of the adult rat, except after orchidectomy followed by T replacement (23-26). However, a recent study by Obregon and Esponda (27) suggests that flutamide can induce a small degree of apoptosis in the epididymis, whereas Carvelli et al. (28) have shown that flutamide treatment increases the cation-dependent mannose-6-phosphate receptor, a similar outcome as observed when rats are orchidectomized. The main difference between the studies that show no effect of flutamide and the ones that do is the route of administration; when flutamide is given orally, there is no effect, whereas there is an effect when it is injected intraperitoneally. Although the recent studies indicate that flutamide could be an alternative to orchidectomy, the rise in serum androgens that it triggers can confound the results.

5α-reductase inhibitors. Finasteride is a 5α-reductase type 2 inhibitor (29), whereas dutasteride (30), PNU157706 (31), and FK143 (32) are dual 5α-reductase inhibitors. Although finasteride, dutasteride, and PNU157706 inhibit intraprostatic DHT concentration, they increase intraprostatic T concentration (33;34). They also do not decrease ventral prostate weight as efficiently as castration (34;35). However, PNU157706 decreases epididymal weight (34). Treatment with PNU157706 and FK143 affects the expression of genes involved in different cellular processes important for the creation of optimal luminal microenvironments [(36;37), reviewed in (38)]. PNU157706 and FK143 treatments have no effect on *Igf1r* expression in the epididymis, but decrease *Igf1* expression in the distal regions (37). The latter findings do not correspond to the findings reported in chapter 3 of this thesis, where orchidectomy increased *Igf1*

expression and decreased *Igf1r* expression. The discrepancy between the obtained results can be explained by the mode of action of PNU157706 and FK143, which inhibit conversion of T to DHT (31;32) and hence do not remove all androgens as opposed to orchidectomy. Taken together, treatment with 5α-reductase inhibitors would not be an appropriate model for the type of studies undertaken in this thesis.

GnRH antagonists. GnRH antagonists decrease serum T concentration to castrate concentration by inhibiting secretions of LH and FSH (39). Treatment with a GnRH antagonist decreases epididymal weight (40), but only reduces by 50% androgens present in the epididymis (41) and does not change the nuclear localization of AR (26). Thus, using GnRH antagonists would not be an alternative to orchidectomy for the type of studies described in this thesis.

1.4. *In vitro* model systems

Using *in vitro* model systems allows the characterization of specific signaling pathways under controlled conditions. In order to confirm the involvement of the IGF1 survival signaling pathway in the response of the epididymis to androgen withdrawal, chapter 4 of this thesis assessed the effects of androgen treatment, withdrawal and blockade on the PC-1 and DC-3 mouse epididymal cell lines. PC-1 and DC-3 cells maintain morphological features of epididymal cells *in vivo* and expression of genes present in the caput epididymidis, in particular *Ar*. Although they are derived from the same region, PC-1 cells are derived from cells of the proximal caput, whereas DC-3 are derived from cells of the distal caput (42), which could affect their response to androgen withdrawal. In fact, as spermatozoa move down the epididymal lumen, they are exposed to different microenvironments created by the secretion of specific proteins (43) and hence PC-1 and DC-3 gene expression and protein secretion profiles are likely to be different. There are other available rodent epididymal cell lines (44-47), however, the PC-1 cell line is the only one that has shown changes in gene expression after androgen withdrawal and in particular for *Igf1* (48); DC-3 cells were used for the first time, beyond their initial report, in this study.

In chapter 4, PC-1 and DC-3 cells were further characterized as model systems. Androgen treatment, withdrawal and/or blockade did not decrease PC-1 and DC-3 cell viability, an outcome that mimics the low cell death observed *in vivo* after orchidectomy (1). However, androgen supplementation only increased *Igf1r* and *Birc5* expression in DC-3 cells suggesting a higher sensitivity of DC-3 cells to androgens as well as differences in gene expression between the two cell lines. In addition, DC-3 cells express not only *Igf1*, *Igf1r*, and *Birc5*, but also *Tnfrsf11b* (appendix 3), a gene uncharacterized in the epididymis. Together, this indicates that the DC-3 cell line might be a better model system than the PC-1 cell line.

2. Future directions

2.1. IGF1 survival signaling pathway in the response of the epididymis to androgen withdrawal

In chapter 3, the involvement of the IGF1 survival signaling pathway in the response of the epididymis to androgen withdrawal was suggested. However, a functional link needs to be established between the IGF1 survival pathway and the low level of apoptosis observed in the epididymis after androgen withdrawal. Although, *Igf1* null mice have been engineered (49), they are unsuitable for this type of studies because they already have a low concentreation of T and their distal epididymis is underdeveloped. An alternative approach would be to block IGF1R *in vivo* using an antibody (50) or more recently derived inhibitors (51-53). In addition, it would be possible to transfer by electroporation small interfering RNA (siRNA), short hairpin RNA (shRNA) or dominant-negative constructs targeting IGF1R into a specific epididymal region. This approach has been used by Fox et al. (54) to introduce dominant-negative plasmids of FGF receptor 1 and ETS translocation variant 5 (ETV5) into the initial segment to study the effects of their inhibition on downstream genes. Once IGF1R is blocked or inhibited, orchidectomy with or without T replacement can be done and the levels of apoptosis measured.

2.2. Developing a good *in vitro* model system to study the IGF1 signaling pathway

In chapter 4, androgen withdrawal was obtained by culturing PC-1 and DC-3 cells in charcoal-stripped FBS medium. This culture condition led to no increase in IGF1 secretion. Assessment of the IGF1 signaling pathway requires specific culture conditions, and in particular serum-free conditions; serum is known to activate the IGF1 signaling pathway. In order to remove the influence of FBS on the IGF1 pathway, other groups have used serum-starved cells from 4hrs (55) to 48hrs (56). For PC-1 and DC-3 cells, the number of hours of serum-starvation to give the best response will need to be determined empirically.

Once the optimal culture conditions will be defined, the involvement of the IGF1 signaling cascade in the response of the PC-1 and DC-3 cells can be assessed by inhibiting different members of the signaling cascade. It is possible to use inhibitors to block PI3K (LY294002 and/or wortmannin), Akt (Akt inhibitor IV) (57), and IGF1R (IGF1R inhibitor, PPP) (51); IGF1R activation can also be blocked by an IGF1R antibody (58).

2.3. Assessing the role of BIRC5 in the epididymis

It has been shown that BIRC5 localization in a cell, in the cytoplasm or the nucleus, indicates its function; when BIRC5 localizes to the cytoplasm, it acts as an anti-apoptotic protein, whereas in the nucleus, it participates in cell division (59). In appendix 2, BIRC5 was localized in the cytoplasm of epididymal cells, which suggested a role as an anti-apoptotic protein (59). In order to determine the role of BIRC5 in the epididymis, two approaches can be taken. The first one uses *in vitro* model systems, PC-1 and/or DC-3 cells, to assess the role of BIRC5 in the response of the cells to androgen withdrawal. Functions of BIRC5 in apoptosis and cell division have been assessed using knockdown by siRNA, shRNA (60) or dominant negative constructs (61). However, these techniques cause BIRC5 knockdown in both the cytoplasm and nucleus rendering the distinction between BIRC5 anti-apoptotic and proliferative functions difficult. To solve that issue,

Colnaghi et al. (62) have designed constructs expressing BIRC5 with point mutations in its nuclear export signal causing BIRC5 accumulation in the nucleus. This allows the specific assessment of BIRC5 role as an anti-apoptotic protein. The second approach uses *in vivo* knockdown of BIRC5. Since BIRC5 knockout mice are embryonic lethal (63), BIRC5 has to be specifically knocked down in the epididymis to assess its functions. It is possible to engineer transgenic mice with BIRC5 exclusively knocked down in the initial segment (64) or caput (65) and then assess the effects of orchidectomy on those two regions.

2.4. Androgen-dependence of *Birc5* and regulation of BIRC5

In chapter 3, orchidectomy caused an increase in *Birc5* mRNA expression, which was suppressed by T replacement suggesting transcriptional regulation. This transcriptional regulation could occur through 5 putative androgen response elements (AREs) identified in the 3kb upstream promoter region of *Birc5*. In order to confirm the functionality of these AREs, fragments containing 0 to 5 AREs can be isolated from a rat cDNA library, cloned into vectors, and binding by AR assessed with a reporter assay. Another method, cloning by PCR amplification, is not viable because it is impossible to design good primers to amplify *Birc5* promoter region (appendix 2). Alternatively, reporter vectors containing different lengths of the human *Birc5* promoter are available (66); *in silico* analysis of this promoter would allow for the localization of putative AREs.

In chapter 3, although orchidectomy with or without T replacement regulated transcriptionally *Birc5* expression in all regions, only the proximal regions showed a decreaed BIRC5 protein expression after orchidectomy that was only prevented by T replacement in the Ca epididymidis. As BIRC5 expression is regulated by the ubiquitin-proteasome pathway (67), it is possible to assess the amount of BIRC5 bound to ubiquitin using co-immunoprecipitation. Another possibility is that translation of BIRC5 is decreased after orchidectomy in the proximal regions. Translation of BIRC5 can be determined by measuring the amount of BIRC5 bound by either monosomes or polysomes; untranslated proteins are bound by monosomes, whereas translated proteins are bound by

polysomes. Monosomes and polysomes can be isolated by sucrose gradient and the bound RNA extracted and identified by northern blot (71).

2.5. TNFRSF11B, a protein with a new role in the epididymis?

In chapter 2, *Tnfrsf11b*, *Tnfrsf11a* (receptor activator of NF-kappaB; RANK), and *Tnfsf11* (RANKL) were identified in the epididymis. Given the multiple roles of TNFRS11A/TNFRSF11B/TNFSF11 in osteogenesis (67) and immunity (68) as well as the inhibition of TNFSF10 (TNF-related apoptosis-inducing ligand; TRAIL)-induced apoptosis in prostate cancer by TNFRSF11B (69), one can speculate that there is a dual role of TNFRSF11B in immunity in the control epididymis and as an anti-apoptotic protein after androgen withdrawal.

In order to assess the role of TNFRSF11B in the epididymis in immunity and/or cell survival, one can use TNFRSF11B knockout mice (3). First, the fertility of these mice should be determined by assessing sperm motility by computer-assisted sperm analysis (CASA), sperm quality by comet assay, and progeny outcome. In addition, morphology of the epididymis and composition of the epididymal epithelium should be established. Second, the role of TNFRSF11B as a survival protein could be determined by assessing the effects of androgen withdrawal on the epididymis of TNFRSF11B knockout mice.

3. Final conclusions

In this thesis, *in vivo* and *in vitro* model systems were used to understand androgen regulation of apoptosis and cell survival in the epididymis. These studies not only identified novel androgen-regulated apoptotic and cell survival genes, but also identified the IGF1 survival signaling pathway as a potential pathway involved in the response of the epididymis to androgen withdrawal. They also identified the DC-3 cell line as a better *in vitro* model system to study androgen actions.

This thesis, as a whole, provides novel insights into androgen regulation of apoptotic and cell survival genes in the epididymis as well as into the molecular mechanisms underlying epididymal resistance to apoptosis triggered by androgen

withdrawal. Understanding how androgens regulate survival while maintaining homeostasis in a tissue with very limited cancer occurrences could help identify differential regulatory mechanisms by androgens. This, in turn, could offer ways to modulate disregulated proteins in androgen-associated pathologies such as prostate cancer.

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LIST OF ORIGINAL CONTRIBUTIONS

1. Characterized the Brown Norway rat model system in terms of the effects of androgen withdrawal and/or replacement on serum testosterone concentrations, weights of empty seminal vesicles, ventral prostate, and epididymis.

2. Adapted an apoptosis-focused microarray experiment, which showed that apoptotic and cell survival genes are regulated in a region-specific manner after orchidectomy with or without testosterone replacement.

3. Identified 8 genes with known regulatory relationships with testosterone or the androgen receptor and uncovered 21 apoptotic and cell survival genes potentially regulated at the transcriptional level by the androgen receptor through the identification of putative androgen response elements in their promoter region.

4. Identified a previously uncharacterized gene in the epididymis, *Tnfrsf11b*, as well as two interacting proteins, *Tnfsf11* and *Tnfrsf11a*.

5. Testosterone replacement represses *Tnfrsf11b* expression indicating that it is regulated by androgens.

6. The epididymis expresses *Tnfrsf11b* at an average level as compared to other rat tissues.

7. TNFRSF11B immunolocalizes to epididymal principal cells and shows a different cellular localization between proximal and distal regions.

8. *Rad52* expression decreases after orchidectomy with testosterone replacement. This is the first study to identify a repair protein as being regulated by androgens.

9. *Mcl1* expression is repressed by testosterone replacement in the proximal regions, but increased in the distal regions.

10. *Bmf* has the highest induction of transcription in the initial segment after orchidectomy and testosterone replacement first represses *Bmf* expression, but then increases it.

11. *Igf1* expression increases after orchidectomy in the epididymis and testosterone replacement potentiates this increase.

12. *Igf1r* expression is repressed by orchidectomy and testosterone replacement increases its expression, except in the cauda epididymis where it stays repressed.

13. *Ide* expression is rapidly increased after orchidectomy and this increase is not prevented by testosterone replacement.

14. *Igbp3* expression increases after orchidectomy and this increase is prevented by testosterone replacement.

15. *Birc5* expression is increased after orchidectomy and repressed by testosterone replacement indicating that *Birc5* is regulated by androgens.

16. There are five putative androgen response elements in the promoter region of rat *Birc5*.

17. BIRC5 localizes to the cytoplasm of principal cells suggesting that it plays a role as an anti-apoptotic protein in the epididymis.

18. BIRC5 expression is decreased after orchidectomy in the proximal regions; this decrease is prevented by testosterone replacement in the caput epididymidis, but not in the initial segment. This suggests post-transcriptional regulations of BIRC5.

19. The epididymis has the second highest expression of *Birc5* in rat tissues.

20. *Diablo* expression is increased after orchidectomy in the proximal regions, but decreased in the distal regions; these changes cannot be prevented by testosterone replacement.

21. *Bax* expression is repressed by testosterone replacement.

22. *Bid* expression increases after orchidectomy and this increase is not prevented by testosterone replacement.

23. Members of the IGF1 signaling pathway are modulated differently after orchidectomy with or without testosterone replacement with respect to time, region of the epididymis and individual components of the pathway; this could lead to a fine-tuned response of the epididymis.

24. Androgen withdrawal and blockade have no effect on PC-1 and DC-3 cell viability, an outcome similar to what is observed *in vivo*.

25. DC-3 cells express both *Tnfrsf11b* and *Birc5*, whereas PC-1 cells only express *Birc5*.

26. BIRC5 localizes to both the cytoplasm and nucleus of PC-1 cells.

27. PC-1 cells secrete more IGF1 than DC-3 cells under the same conditions.

28. Duration of pre-treatment of cells in charcoal-filtered FBS with DHT supplementation has an effect on the amount of IGF1 secreted by PC-1 and DC-3 cells.

29. DHT supplementation increases *Igf1r* and *Birc5* expression in DC-3 cells, but not in PC-1 cells. These changes are not prevented by the presence of hydroxyflutamide suggesting a potential non-transcriptional regulation of expression.

30. The number of passages has little effect on the survival response of PC-1 cells to androgen withdrawal.

31. Androgen treatment, withdrawal and blockade have no effect on *Mcl1* transcription in PC-1 and DC-3 cells.

32. The DC-3 cell line, a previously uncharacterized *in vitro* model, is more sensitive to androgens than PC-1 cells in terms of the markers studied, which suggests that it could be a better model system to study androgen action.

APPENDIX 1

1. Materials and Methods

All procedures for the animal study were as described in chapter 2 with the addition of adult male Sprague-Dawley (SD) rats (3-4 months old) obtained from Charles River Canada (Saint-Constant, QC).

Procedures for orchidectomy, serum testosterone analysis, RNA extraction, and Real- Time RT-PCR were as described in chapter 2.

1.1. K-means cluster analysis

In order to visualize the expression profiles of transcripts on the same vertical axis, K-means cluster analysis was done on the total number of normalized probes as described elsewhere (1). Number of clusters selected varied between 2 to 5 clusters.

2. Results and Discussion

2.1. The Brown Norway rat strain responded similarly to orchidectomy as the Sprague-Dawley rat strain

To make sure that the Brown Norway (BN) rat strain was a suitable model to study the effects of orchidectomy, we compared changes in serum T concentrations and weights of ventral prostate, empty seminal vesicles, and epididymis between BN rats and SD rats (fig. 1). We found that orchidectomy caused a significant ($p<0.05$) decrease in serum T concentrations as early as 0.5 day after surgery in both BN and SD rats (fig. 1A). In terms of changes in ventral prostate (fig. 1B), empty seminal vesicles (fig. 1C), and epididymis (fig. 1D) weights both BN and SD followed a similar pattern of decrease. For the ventral prostate and empty seminal vesicles, the first significant ($p<0.05$) decreases in weights were observed at 3 days and 7 days for both strains (fig. 1B-C). However, epididymal weight for the SD was significantly ($p<0.05$) decreased from 3 days to 7 days, whereas for the BN, the first significant ($p<0.05$) decrease was observed at 7 day after orchidectomy (fig. 1D). Together, these data showed that in terms of serum T concentrations and weights of sex accessory tissues, BN and SD rats

behave similarly after orchidectomy. This made the BN strain a suitable model to study the effects of orchidectomy on the epididymis.

2.2. Treatment- and region-specific changes in the number of affected transcripts

Comparisons of the numbers of transcripts affected after orchidectomy with or without testosterone replacement within each epididymal region showed treatment-specific changes, except in the Cd in the 1 day (+T) group (supp. fig. 2). The IS and Ca were the only two regions to have 3 and 2 transcripts, respectively, commonly affected in all treatment groups. The Co had the highest number (total of 9 transcripts) of commonly affected transcripts, especially between the (-T) 0.5 day and 1 day groups (7 common transcripts), whereas the Cd had the least with 2 common transcripts between (-T) 0.5 day and 1 day (fig. 2).

Comparisons of the numbers of transcripts affected after orchidectomy with or without testosterone replacement within each treatment group showed region-specific changes with only 1 transcript commonly affected in all regions at 0.5 day and 1 day without T replacement (fig. 3). At 0.5 day after orchidectomy without T replacement, all regions had similar numbers of affected transcripts. With time, there was a decrease in the number of affected transcripts in the IS (from 11 to 9), but an increase in the Cd (from 12 to 17). T replacement at 0.5 day decreased the number of affected transcripts in the IS (from 11 to 5) and Co (from 15 to 4), whereas at 1 day, the Co and Cd had decreased numbers of affected transcripts (from 15 to 8 and from 17 to 5, respectively).

Together, these data showed treatment- and region-specific changes in the numbers of affected transcripts.

2.3. Effects of tesoterone replacement on the number of affected transcripts within each region

Numbers of transcripts differentially affected between (-T) and (+T) groups at 0.5 day and 1 day after orchidectomy were determined for IS (fig. 4A), Ca (fig. 4B), Co (fig. 4C), and Cd (fig. 4D). At 0.5 day, IS (fig. 4A) and Ca (fig.

4B) had similar numbers of up- and down-regulated transcripts (2-3 transcripts), whereas Co (fig. 4C) had the highest number of down-regulated transcripts (10 transcripts). At 1 day, both IS (fig. 4A) and Co (fig. 4C) had 10 transcripts up-regulated, whereas Ca (fig. 4B) and Cd (fig. 4D) had similar numbers of up-regulated transcripts (5 and 6 transcripts, respectively). In addition, the IS (fig. 4A) was the only region with no down-regulated transcripts at 1 day. These data demonstrated that T replacement changed gene expression in the different regions of the epididymis.

2.4. Orchidectomy with or without testosterone replacement similarly affected pro- and anti-apoptotic genes

Using K-means cluster analysis, we grouped changes in gene expression after orchidectomy without (fig. 5) and with (fig. 6) T replacement in 2 to 5 groups representing different patterns of expression; the names of the affected genes and their classification can be found in tables 1-8. In all groups and treatment, the pro-apoptotic genes represented the majority of affected genes. Without T replacement (fig. 5), the Co had the highest number of classified affected genes (77 genes), whereas the IS had the lowest (59 genes); the Ca and Cd showed similar numbers of affected genes with 67 and 66 genes, respectively. In addition, the IS (fig. 5A) and Co (fig. 5C) had similar number of genes spread across the different groups. On the other hand, in the group with genes showing a transient increase of expression, both the Ca (fig. 5B) and Cd (fig. 5D) had the lowest number of genes. With T replacement (fig. 6), the Cd had the highest number of classified affected genes (86 genes), whereas the other regions showed similar numbers (73, 69, and 74 genes for the IS, Ca, and Co, respectively). In all regions, except the Co (fig. 6C), there were similar numbers of affected genes spread across the different groups; in the Co, there were less genes increased at 1 day after orchidectomy compared to the other two groups. This demonstrated that genes with different functions were similarly spread across the different patterns of expression indicating that specific gene functions did not group into a particular trend.

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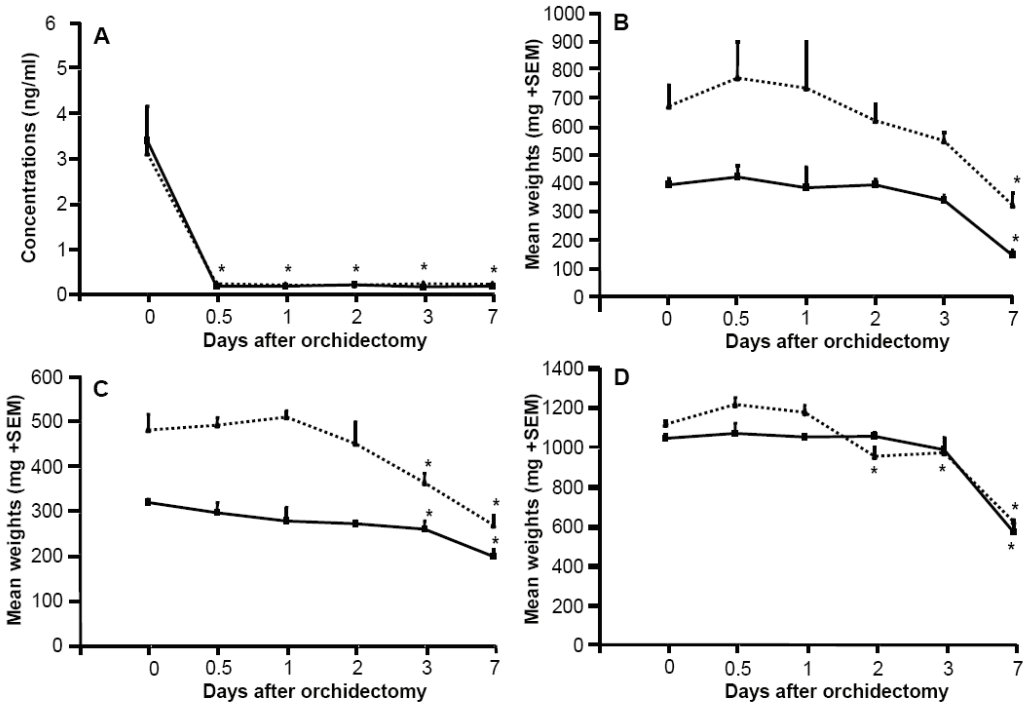


Figure 1: Comparisons of the effects of orchidectomy on serum testosterone concentration and weights of ventral prostate, empty seminal vesicles, and epididymis between the Brown Norway and Sprague-Dawley rat strains. Brown Norway (solid line) and Sprague-Dawley (dashed line) rats were orchidectomized and sacrificed 0.5, 1, 2, 3, and 7 days after surgery. Serum testosterone concentration (A) was measured by ELISA, whereas weights of ventral prostate (B), empty seminal vesicles (C), and epididymis (D) were recorded. Day 0 corresponds to sham-operated animals. Data are presented as mean ± SEM (n=5-6/group). Significant effects ($P<0.05$) of time on serum testosterone concentration and tissue weights are depicted by (*).

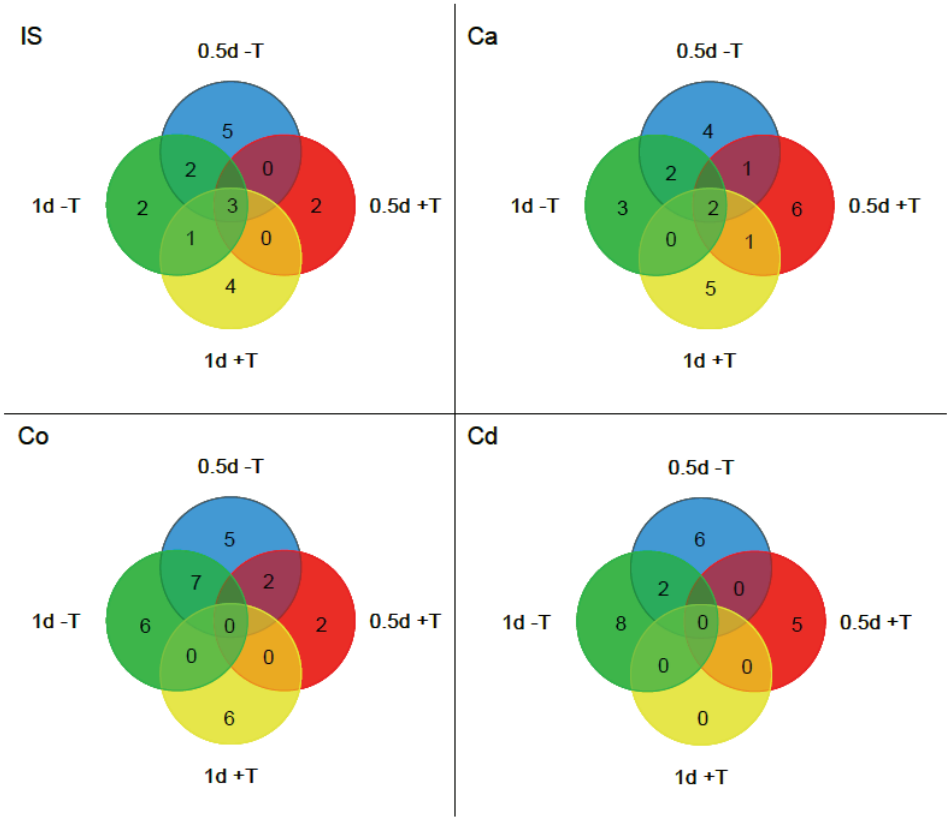


Figure 2: Numbers of differentially affected transcripts in the different regions of the epididymis at 0.5 day and 1 day after orchidectomy with or without testosterone replacement. The number of transcripts changing by at least 1.5 fold as compared to sham-operated were determined in the IS, Ca, Co, and Cd at 0.5 day after orchidectomy without testosterone replacement (blue circle), with testosterone replacement (red circle) and at 1 day after orchidectomy with testosterone replacement (yellow circle), without testosterone replacement (green circle).

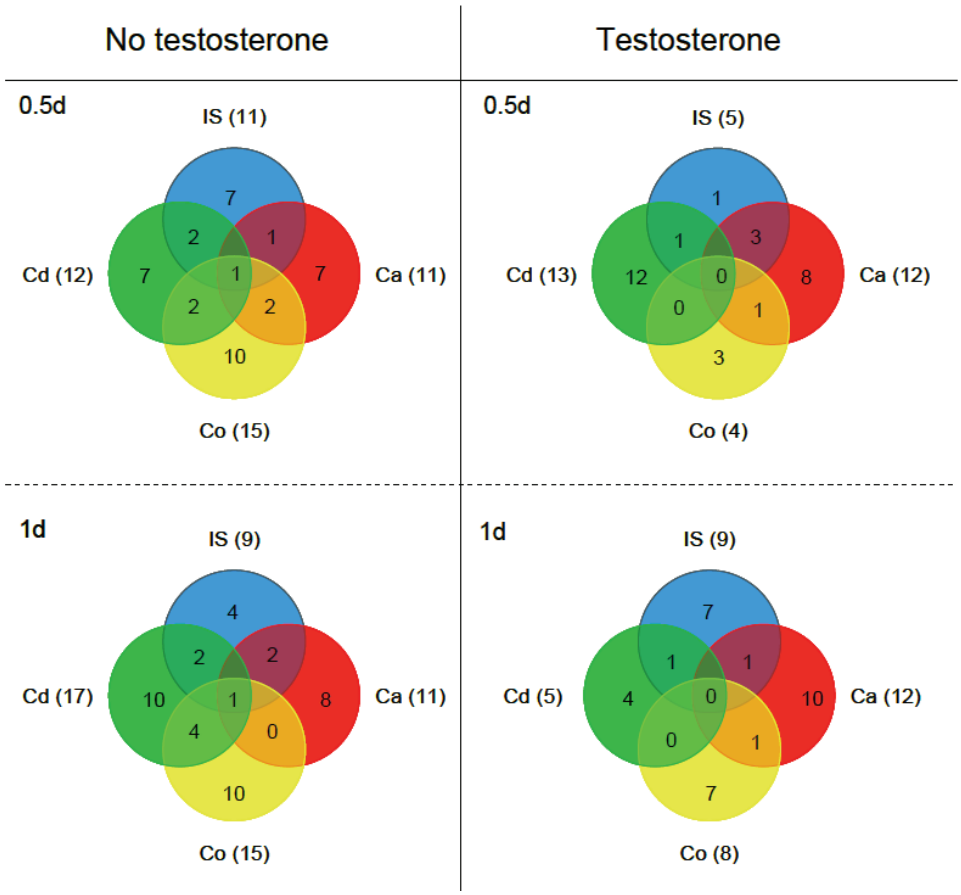


Figure 3: Numbers of differentially affected transcripts at 0.5 day and 1 day after orchidectomy with or without testosterone replacement in the different regions of the epididymis. The number of transcripts changing by at least 1.5 fold as compared to sham-operated were determined at 0.5 day and 1 day after orchidectomy with or without testosterone replacement for the IS (blue circle), Ca (red circle), Co (yellow circle), and Cd (green circle). Numbers in parentheses are total numbers of affected transcripts in each region.

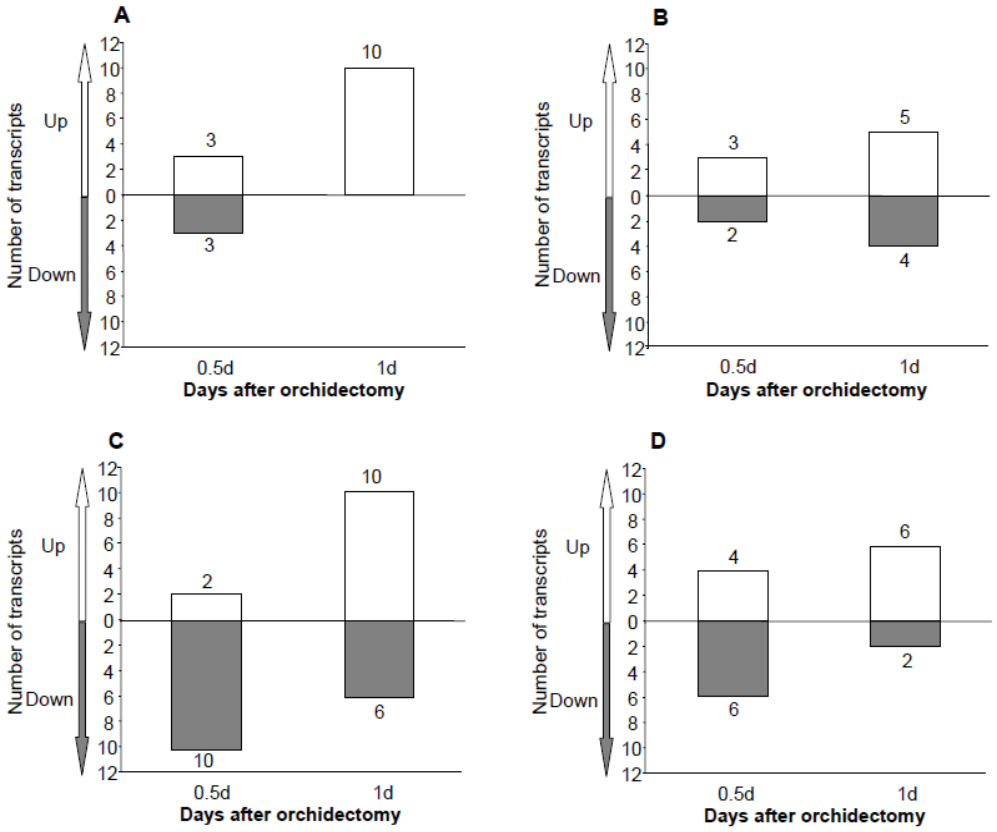
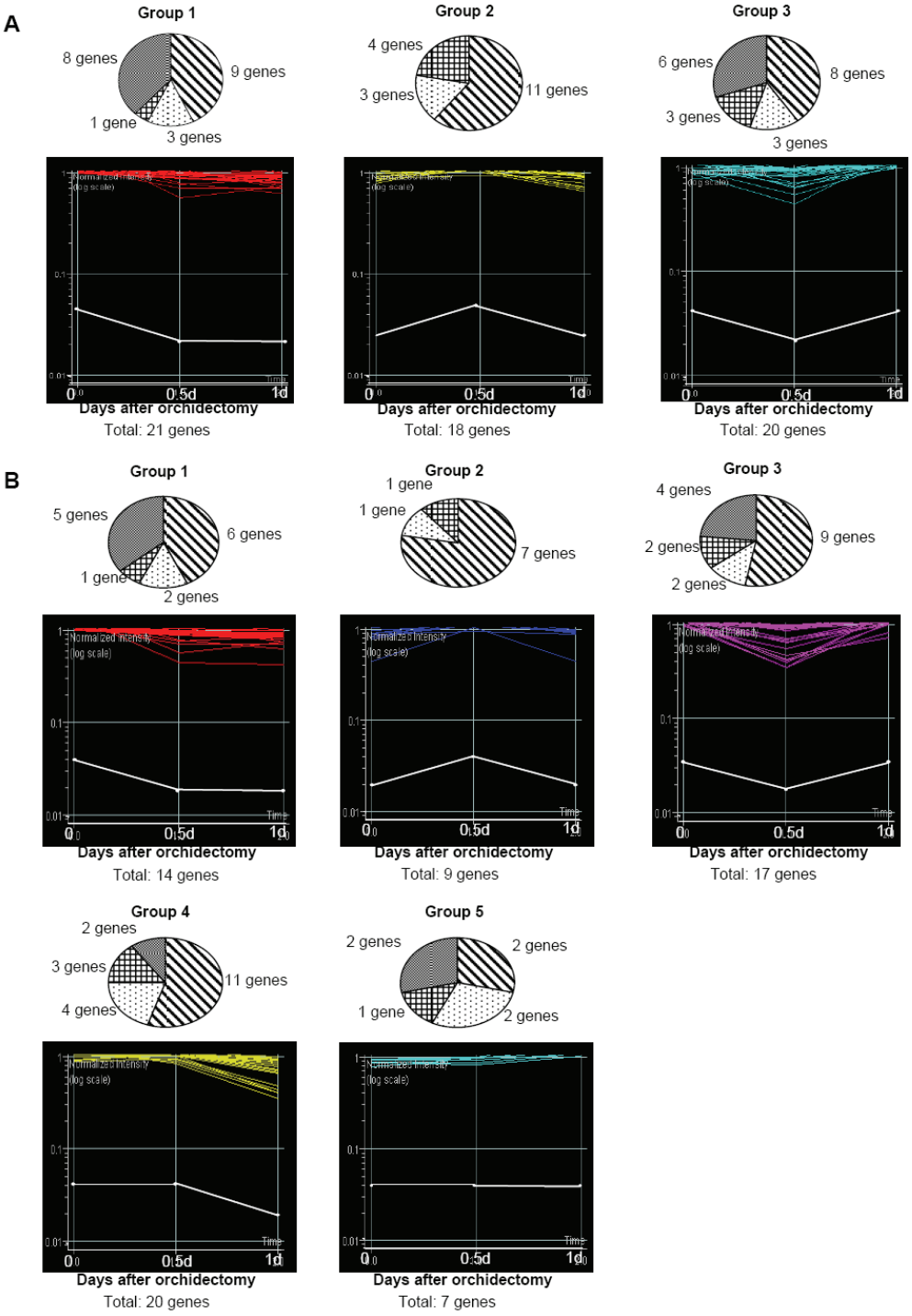


Figure 4: Number of transcripts changing at 0.5 day and 1 day after orchidectomy between the without testosterone replacement group and the with testosterone replacement group in the different regions of the epididymis. The number of transcripts changing by at least 1.5 fold in either direction (50% increase or 33% decrease) (vertical axis) was determined for the IS (A), Ca (B), Co (C), and Cd (D). Fold change was determined at 0.5 day and 1 day after orchidectomy (horizontal axis) between the "without testosterone replacement group" relative to the "with testosterone replacement group". The white bars indicate the number of transcripts increasing in expression (above x-axis), whereas the grey bars indicate transcripts decreasing in expression (below the x-axis). Each number was obtained independently at each treatment time.


A
Group 1
8 genes
9 genes
1 gene
3 genes
Group 2
4 genes
3 genes
11 genes
Group 3
6 genes
8 genes
3 genes
3 genes
Normalized Intensity (log scale)
0.1
0.01
Time
0
0.5d
1d
Days after orchidectomy
Total: 21 genes
Total: 18 genes
Total: 20 genes
B
Group 1
5 genes
6 genes
1 gene
2 genes
Group 2
1 gene
1 gene
7 genes
Group 3
4 genes
2 genes
9 genes
2 genes
Total: 14 genes
Total: 9 genes
Total: 17 genes
Group 4
2 genes
3 genes
11 genes
4 genes
Group 5
2 genes
2 genes
1 gene
2 genes
Total: 20 genes
Total: 7 genes

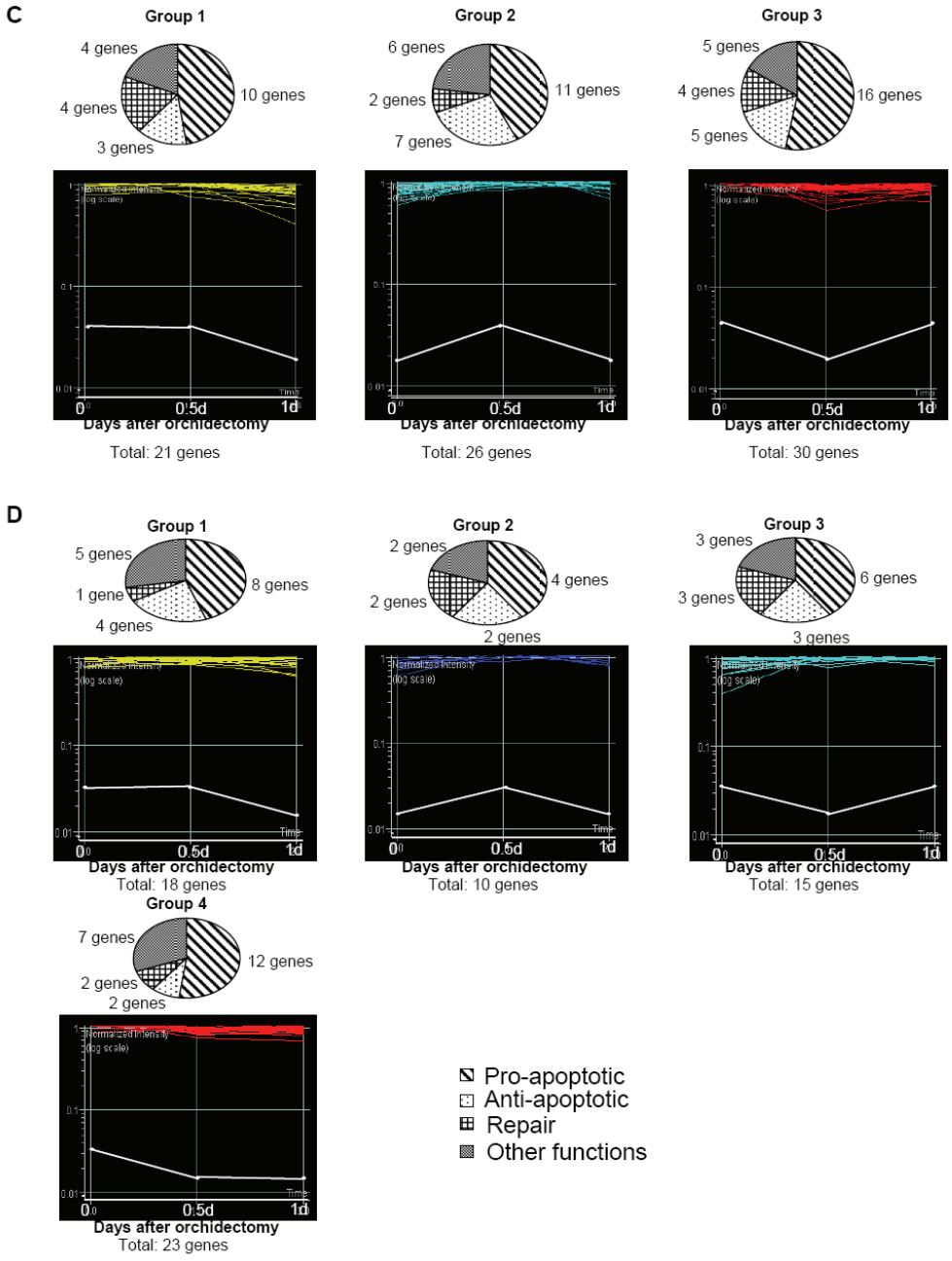
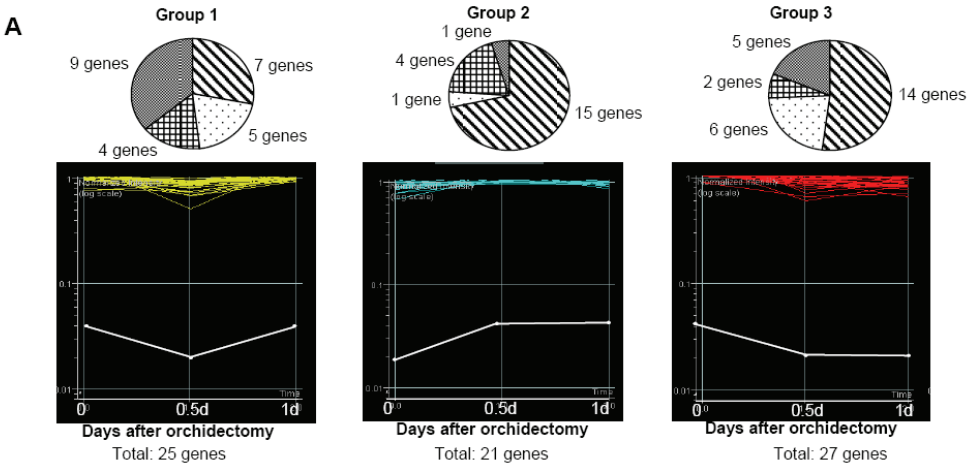
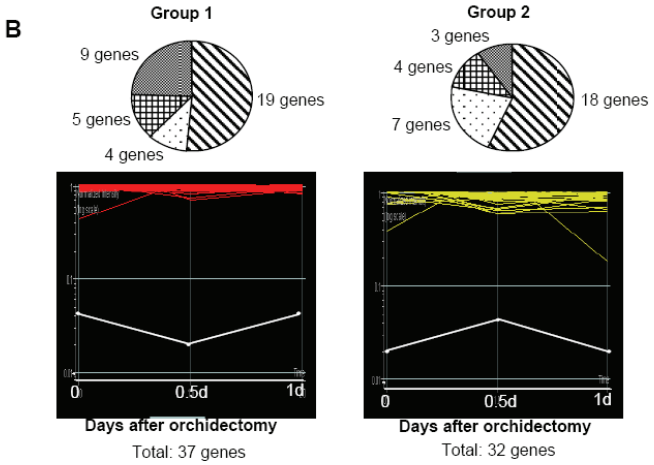
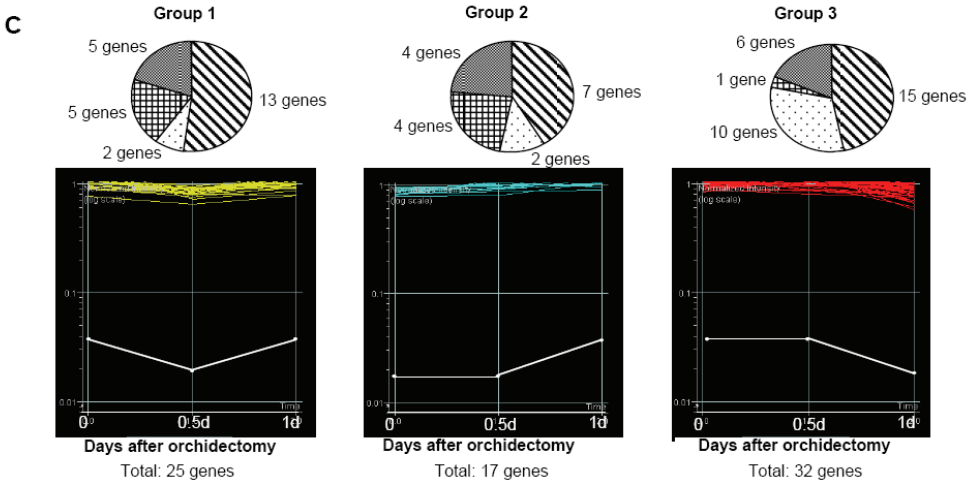


Figure 5: Effects of orchidectomy on overall gene expression in the different regions of the epididymis. Patterns of changes in gene expression were determined by K-means analysis at 0 day, 0.5 day, and 1 day after orchidectomy (horizontal axis) in the IS (A), Ca (B), Co (C), and Cd (D). Day 0 corresponds to sham-operated values. Genes affected in each group were classified as pro-apoptotic, anti-apoptotic, repair genes or genes with other functions.


A
Group 1
9 genes
7 genes
5 genes
4 genes
Group 2
1 gene
4 genes
1 gene
15 genes
Group 3
5 genes
2 genes
14 genes
6 genes
0
0.5d
1d
Days after orchidectomy
Total: 25 genes
Total: 21 genes
Total: 27 genes


B
Group 1
9 genes
19 genes
5 genes
4 genes
Group 2
3 genes
4 genes
18 genes
7 genes
0
0.5d
1d
Days after orchidectomy
Total: 37 genes
Total: 32 genes


C
Group 1
5 genes
13 genes
5 genes
2 genes
Group 2
4 genes
7 genes
4 genes
2 genes
Group 3
6 genes
1 gene
15 genes
10 genes
0
0.5d
1d
Days after orchidectomy
Total: 25 genes
Total: 17 genes
Total: 32 genes

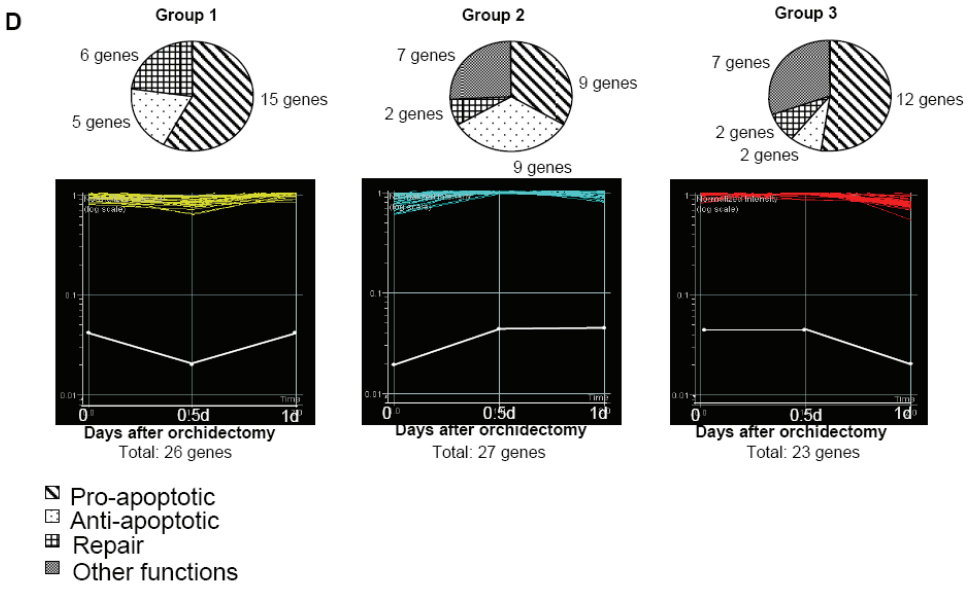


Figure 6: Effects of orchidectomy with testosterone replacement on overall gene expression in the different regions of the epididymis. Patterns of changes in gene expression were determined by K-means analysis at 0 day, 0.5 day, and 1 day after orchidectomy with testosterone replacement (horizontal axis) in the IS (A), Ca (B), Co (C), and Cd (D). Day 0 corresponds to sham-operated values. Genes affected in each group were classified as pro-apoptotic, anti-apoptotic, repair genes or genes with other functions.

Table 1: IS - K-means analysis: orchidectomy without testosterone replacement

Group 1	**Total number of genes: 19**		
Gene symbol	**Common gene name**	**Family**	**Role in apoptosis**
Apaf1	Apoptotic peptidase activating factor 1	CARD domain	Pro-apoptotic
Pycard	Apoptosis-associated speck-like protein containing a CARD	CARD domain	Pro-apoptotic
Bcl10	B-cell CLL/lymphoma 10	CARD domain	Pro-apoptotic
Becn1	Beclin 1 (coiled-coil, myosin-like BCL2-interacting protein)	Bcl2	Anti-apoptotic
Birc5	Baculoviral IAP repeat-containing 5	IAP	Anti-apoptotic
Bnip3	BCL2/adenovirus E1B 19 kDa-interacting protein 3	Bcl2	Pro-apoptotic
Bnip3l	BCL2/adenovirus E1B 19 kDa-interacting protein 3-like	Bcl2	Pro-apoptotic
Casp8ap2	Midasin homolog (yeast) (predicted)	Death Effector domain	Pro-apoptotic
Cidea	Cell death-inducing DNA fragmentation factor, alpha subunit-like effector A (predicted)	CIDE domain	Pro-apoptotic
Ngfrap1	Nerve growth factor receptor associated protein 1	TNFR	Pro-apoptotic
Tnfrsf11b	Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	TNFR	Anti-apoptotic
Tnfsf10	Tumor necrosis factor (ligand) superfamily, member 10	TNF ligand	Pro-apoptotic
Tp53	Tumor protein p53	p53 and ATM	Repair
Zranb1	Zinc finger, RAN-binding domain containing 1 (predicted)	TRAF	Other related function
Ube1c	Ubiquitin-activating enzyme E1C	Other related genes	Other related function
Ube2d2	Ubiquitin-conjugating enzyme E2D 2	Other related genes	Other related function
Ube2d3	Ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast)	Other related genes	Other related function
Ube2i	Ubiquitin-conjugating enzyme E2I	Other related genes	Other related function
Ube2n	Ubiquitin-conjugating enzyme E2N (homologous to yeast UBC13)	Other related genes	Other related function

Group 2	**Total number of genes: 18**		
Gene symbol	**Common gene name**	**Family**	**Role in apoptosis**
Bad	Bcl2-associated death promoter	Bcl2	Pro-apoptotic
Bak1	BCL2-antagonist/killer 1	Bcl2	Pro-apoptotic
Bax	Bcl2-associated X protein	Bcl2	Pro-apoptotic
Bnip1	BCL2/adenovirus E1B 19kDa-interacting protein 1	Bcl2	Anti-apoptotic
Casp4	Caspase 11	Caspase	Pro-apoptotic
Casp2	Caspase 2	Caspase	Pro-apoptotic
Casp6	Caspase 6	Caspase	Pro-apoptotic
Chek1	Checkpoint kinase 1 homolog (S. pombe)	p53 and ATM	Repair
E2f5	E2F transcription factor 5	p53 and ATM	Repair
Mcl1	Myeloid cell leukemia sequence 1	Bcl2	Anti-apoptotic
Myd88	Myeloid differentiation primary response gene 88	Death domain	Pro-apoptotic
Rad52	Similar to Rad52 protein	p53 and ATM	Repair
Chk2	Protein kinase Chk2	p53 and ATM	Repair

Group 2	Total number of genes: 18		
Gene symbol	**Common gene name**	**Family**	**Role in apoptosis**
Tnfrsf1a	Tumor necrosis factor receptor superfamily, member 1a	TNFR	Pro-apoptotic
Tnfsf13	Tumor necrosis factor ligand superfamily, member 13	TNF ligand	Pro-apoptotic
CD70	Similar to CD70 protein (CD27 ligand) (LOC301132), mRNA	TNF ligand	Pro-apoptotic
Traf4	Similar to TNF receptor associated factor 4 (LOC303285), mRNA	TRAF	Anti-apoptotic
Traip	TRAF-interacting protein (predicted)	TRAF	Pro-apoptotic

Group 3	Total number of genes: 20		
Gene symbol	**Common gene name**	**Family**	**Role in apoptosis**
Baiap2	Brain-specific angiogenesis inhibitor 1-associated protein 2	Other related genes	Other related function
Bcl2a1	B-cell leukemia/lymphoma 2 related protein A1	Bcl2	Anti-apoptotic
Cflar	CASP8 and FADD-like apoptosis regulator	Death Effector domain	Anti-apoptotic
Dap3	Death associated protein 3 (predicted)	Death domain	Pro-apoptotic
Dlk	Similar to Death-associated protein kinase 3	Death domain	Pro-apoptotic
Fadd	Fas (TNFRSF6)-associated via death domain	Death Effector domain	Pro-apoptotic
Gadd45a	Growth arrest and DNA-damage-inducible 45 alpha	p53 and ATM	Repair
Ltb	Lymphotoxin B	TNF ligand	Pro-apoptotic
Rad23a	Similar to UV excision repair protein RAD23 homolog A	p53 and ATM	Repair
Rad50	RAD50 homolog (S. cerevisiae)	p53 and ATM	Repair
Rfng	Radical fringe gene homolog (Drosophila)	Other related genes	Other related function
Ripk2	Similar to receptor-interacting protein 2 (LOC362491), mRNA	Death domain	Pro-apoptotic
Tank	TRAF family member-associated Nf-kappa B activator	TRAF	Pro-apoptotic
Tnfaip2	Similar to [Mouse primary response gene B94 mRNA, 3end.], gene product	Other related genes	Other related function
Tnfrsf1b	Tumor necrosis factor receptor superfamily, member 1b	TNFR	Anti-apoptotic
Tnfrsf8	Tumor necrosis factor receptor superfamily, member 8	TNFR	Pro-apoptotic
Tnfsf12	Tumor necrosis factor ligand superfamily member 12	TNF ligand	Pro-apoptotic
Tnfsf9	Tumor necrosis factor (ligand) superfamily, member 9	TNF ligand	Other related function
Tradd	TNFRSF1A-associated via death domain	TRAF	Other related function
Ube1x	Similar to ubiquitin-protein ligase (EC 6.3.2.19) E1 - mouse	Other related genes	Other related function

Unclassified	Total number of genes: 34		
Gene symbol	**Common gene name**	**Family**	**Role in apoptosis**
Atm	Ataxia telangiectasia mutated homolog (human)	p53 and ATM	Repair
Bcl2	B-cell leukemia/lymphoma 2	Bcl2	Anti-apoptotic
Bcl2l1	Bcl2-like 1	Bcl2	Anti-apoptotic
Bcl2l10	Bcl2-like 10	Bcl2	Anti-apoptotic
Bcl2l11	BCL2-like 11 (apoptosis facilitator)	Bcl2	Pro-apoptotic
Bcl2l2	Bcl2-like 2	Bcl2	Anti-apoptotic
Bid3	BH3 interacting (with BCL2 family) domain, apoptosis agonist	Bcl2	Pro-apoptotic
Biklk	Bcl2-interacting killer-like	Bcl2	Pro-apoptotic
Birc1b	Baculoviral IAP repeat-containing 1b	IAP	Anti-apoptotic

Unclassified	**Total number of genes: 34**		
Gene symbol	**Common gene name**	**Family**	**Role in apoptosis**
Birc3	Inhibitor of apoptosis protein 1	IAP	Anti-apoptotic
Bmf	Bcl-2 modifying factor	Bcl2	Pro-apoptotic
Bok	Bcl-2-related ovarian killer protein	Bcl2	Pro-apoptotic
Casp12	Caspase 12	Caspase	Pro-apoptotic
Casp7	Caspase 7	Caspase	Pro-apoptotic
Casp8	Caspase 8	Caspase	Pro-apoptotic
Casp9	Caspase 9	Caspase	Pro-apoptotic
Cntnap1	Contactin associated protein 1	Other related genes	Other related funciton
Cradd	CASP2 and RIPK1 domain containing adaptor with death domain (predicted)	Death domain	Pro-apoptotic
Dffa	DNA fragmentation factor, alpha subunit	CIDE domain	Pro-apoptotic
Dffb	DNA fragmentation factor, beta subunit	CIDE domain	Pro-apoptotic
E2f3	Similar to E2f3 protein (LOC291105), mRNA	p53 and ATM	Repair
E2f6	E2F transcription factor 6	p53 and ATM	Repair
Card9	Caspase recruitment domain protein 9	CARD family	Pro-apoptotic
Tnfsf5	Tumor necrosis factor (ligand) superfamily, member 5	TNF ligand	Pro-apoptotic
Ltbr	Lymphotoxin B receptor (predicted)	TNFR	Pro-apoptotic
Rad1	Similar to Rad1p (LOC294800), mRNA	p53 and ATM	Repair
Rrad	Ras-related associated with diabetes	Other related genes	Other related funciton
Tnfrsf26	Tumor necrosis factor receptor superfamily, member 26 (predicted)	TNFR	Other related funciton
Tnfrsf10b	Similar to TRAIL receptor2 KILLER/DR5 homologue (LOC364420), mRNA	TNFR	Pro-apoptotic
Tnfrsf12a	Tumor necrosis factor receptor superfamily, member 12a	TNFR	Pro-apoptotic
Tnfrsf4	Tumor necrosis factor receptor superfamily, member 4	TNFR	Anti-apoptotic
Tnfsf15	Tumor necrosis factor (ligand) superfamily, member 15	TNF ligand	Pro-apoptotic
Tnfaip3	TNFAIP3 interacting protein 2 (predicted)	Other related genes	Other related funciton
Traf2	Tnf receptor-associated factor 2 (predicted)	TRAF	Anti-apoptotic

Table 2: IS - K-means analysis: orchidectomy with testosterone replacement

Group 1	**Total number of genes: 25**		
Gene symbol	**Common gene name**	**Family**	**Role in apoptosis**
Pycard	Apoptosis-associated speck-like protein containing a CARD	CARD domain	Pro-apoptotic
Bcl10	B-cell CLL/lymphoma 10	CARD domain	Pro-apoptotic
Bcl2a1	B-cell leukemia/lymphoma 2 related protein A1	Bcl2	Anti-apoptotic
Becn1	Beclin 1 (coiled-coil, myosin-like BCL2-interacting protein)	Bcl2	Anti-apoptotic
Birc3	Inhibitor of apoptosis protein 1	IAP	Anti-apoptotic
Cidea	Cell death-inducing DNA fragmentation factor, alpha subunit-like effector A (predicted)	CIDE domain	Pro-apoptotic
Mcl1	Myeloid cell leukemia sequence 1	Bcl2	Anti-apoptotic
Rad1	Similar to Rad1p (LOC294800), mRNA	p53 and ATM	Repair
Rad23a	Similar to UV excision repair protein RAD23 homolog A (MHR23A)	p53 and ATM	Repair
Rad50	RAD50 homolog (S. cerevisiae)	p53 and ATM	Repair
Ripk2	Similar to receptor-interacting protein 2 (LOC362491), mRNA	Death domain	Pro-apoptotic
Tank	TRAF family member-associated Nf-kappa B activator	TRAF	Pro-apoptotic
Tnfaip2	Similar to [Mouse primary response gene B94 mRNA, 3end.], gene product	Other related genes	Other related function
Tnfrsf26	Tumor necrosis factor receptor superfamily, member 26 (predicted)	TNFR	Other related function
Tnfsf12	Tumor necrosis factor ligand superfamily member 12	TNF ligand	Pro-apoptotic
Tnfsf13	Tumor necrosis factor ligand superfamily, member 13	TNF ligand	Pro-apoptotic
Tnfsf9	Tumor necrosis factor (ligand) superfamily, member 9	TNF ligand	Other related function
Tnfaip3	TNFAIP3 interacting protein 2 (predicted)	Other related genes	Other related function
Tp53	Tumor protein p53	p53 and ATM	Repair
Tradd	TNFRSF1A-associated via death domain	TRAF	Other related function
Traf2	Tnf receptor-associated factor 2 (predicted)	TRAF	Anti-apoptotic
Ube1c	Ubiquitin-activating enzyme E1C	Other related genes	Other related function
Ube1x	Similar to ubiquitin-protein ligase (EC 6.3.2.19) E1 - mouse	Other related genes	Other related function
Ube2d3	Ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast)	Other related genes	Other related function
Ube2i	Ubiquitin-conjugating enzyme E2I	Other related genes	Other related function

Group 2	**Total number of genes: 21**		
Gene symbol	**Common gene name**	**Family**	**Role in apoptosis**
Baiap2	Brain-specific angiogenesis inhibitor 1-associated protein 2	Other related genes	Other related function
Bak1	BCL2-antagonist/killer 1	Bcl2	Pro-apoptotic
Bax	Bcl2-associated X protein	Bcl2	Pro-apoptotic
Bmf	Bcl-2 modifying factor	Bcl2	Pro-apoptotic
Casp4	Caspase 11	Caspase	Pro-apoptotic
Casp6	Caspase 6	Caspase	Pro-apoptotic
Casp9	Caspase 9	Caspase	Pro-apoptotic
Dlk	Similar to Death-associated protein kinase 3	Death domain	Pro-apoptotic

Group 2	Total number of genes: 21		
Gene symbol	**Common gene name**	**Family**	**Role in apoptosis**
E2f5	E2F transcription factor 5	p53 and ATM	Repair
Gadd45a	Growth arrest and DNA-damage-inducible 45 alpha	p53 and ATM	Repair
LOC64171	Caspase recruitment domain protein 9	CARD family	Pro-apoptotic
Tnfsf5	Tumor necrosis factor (ligand) superfamily, member 5	TNF ligand	Pro-apoptotic
Ltb	Lymphotoxin B	TNF ligand	Pro-apoptotic
Myd88	Myeloid differentiation primary response gene 88	Death domain	Pro-apoptotic
Rad52	Similar to Rad52 protein	p53 and ATM	Repair
Chek2	Protein kinase Chk2	p53 and ATM	Repair
Tnfrsf1a	Tumor necrosis factor receptor superfamily, member 1a	TNFR	Pro-apoptotic
Tnfrsf1b	Tumor necrosis factor receptor superfamily, member 1b	TNFR	Anti-apoptotic
Tnfrsf8	Tumor necrosis factor receptor superfamily, member 8	TNFR	Pro-apoptotic
CD70	Similar to CD70 protein (CD27 ligand) (LOC301132), mRNA	TNF ligand	Pro-apoptotic
Traip	TRAF-interacting protein (predicted)	TRAF	Pro-apoptotic

Group 3	Total number of genes: 27		
Gene symbol	**Common gene name**	**Family**	**Role in apoptosis**
Apaf1	Apoptotic peptidase activating factor 1	CARD domain	Pro-apoptotic
Atm	Ataxia telangiectasia mutated homolog (human)	p53 and ATM	Repair
Bad	Bcl2-associated death promoter	Bcl2	Pro-apoptotic
Bcl2	B-cell leukemia/lymphoma 2	Bcl2	Anti-apoptotic
Bid3	BH3 interacting (with BCL2 family) domain, apoptosis agonist	Bcl2	Pro-apoptotic
Biklk	Bcl2-interacting killer-like	Bcl2	Pro-apoptotic
Birc1b	Baculoviral IAP repeat-containing 1b	IAP	Anti-apoptotic
Bnip1	BCL2/adenovirus E1B 19kDa-interacting protein 1	Bcl2	Anti-apoptotic
Bnip3	BCL2/adenovirus E1B 19 kDa-interacting protein 3	Bcl2	Pro-apoptotic
Bnip3l	BCL2/adenovirus E1B 19 kDa-interacting protein 3-like	Bcl2	Pro-apoptotic
Bok	Bcl-2-related ovarian killer protein	Bcl2	Pro-apoptotic
Casp2	Caspase 2	Caspase	Pro-apoptotic
Casp8	Caspase 8	Caspase	Pro-apoptotic
Casp8ap2	Midasin homolog (yeast) (predicted)	Death Effector domain	Pro-apoptotic
Cflar	CASP8 and FADD-like apoptosis regulator	Death Effector domain	Anti-apoptotic
Chek1	Checkpoint kinase 1 homolog (S. pombe)	p53 and ATM	Repair
Cntnap1	Contactin associated protein 1	Other related genes	Other related function
Dap3	Death associated protein 3 (predicted)	Death domain	Pro-apoptotic
Fadd	Fas (TNFRSF6)-associated via death domain	Death Effector domain	Pro-apoptotic
Ngfrap1	Nerve growth factor receptor associated protein 1	TNFR	Pro-apoptotic
Rrad	Ras-related associated with diabetes	Other related genes	Other related function
Tnfrsf11b	Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	TNFR	Anti-apoptotic
Tnfsf10	Tumor necrosis factor (ligand) superfamily, member 10	TNF ligand	Pro-apoptotic
Zranb1	Zinc finger, RAN-binding domain containing 1 (predicted)	TRAF	Other related function

Group 3	**Total number of genes: 27**		
Gene symbol	**Common gene name**	**Family**	**Role in apoptosis**
Traf4	Similar to TNF receptor associated factor 4 (LOC303285), mRNA	TRAF	Anti-apoptotic
Ube2d2	Ubiquitin-conjugating enzyme E2D 2	Other related genes	Other related function
Ube2n	Ubiquitin-conjugating enzyme E2N (homologous to yeast UBC13)	Other related genes	Other related function

Unclassified	**Total number of genes: 18**		
Gene symbol	**Common gene name**	**Family**	**Role in apoptosis**
Bcl2l1	Bcl2-like 1	Bcl2	Anti-apoptotic
Bcl2l10	Bcl2-like 10	Bcl2	Anti-apoptotic
Bcl2l11	BCL2-like 11 (apoptosis facilitator)	Bcl2	Pro-apoptotic
Bcl2l2	Bcl2-like 2	Bcl2	Anti-apoptotic
Birc5	Baculoviral IAP repeat-containing 5	IAP	Anti-apoptotic
Casp12	Caspase 12	Caspase	Pro-apoptotic
Casp7	Caspase 7	Caspase	Pro-apoptotic
Cradd	CASP2 and RIPK1 domain containing adaptor with death domain (predicted)	Death domain	Pro-apoptotic
Dffa	DNA fragmentation factor, alpha subunit	CIDE domain	Pro-apoptotic
Dffb	DNA fragmentation factor, beta subunit	CIDE domain	Pro-apoptotic
E2f3	Similar to E2f3 protein (LOC291105), mRNA	p53 and ATM	Repair
E2f6	E2F transcription factor 6	p53 and ATM	Repair
Ltbr	Lymphotoxin B receptor (predicted)	TNFR	Pro-apoptotic
Rfng	Radical fringe gene homolog (Drosophila)	Other related genes	Other related function
Tnfrsf10b	Similar to TRAIL receptor2 KILLER/DR5 homologue (LOC364420), mRNA	TNFR	Pro-apoptotic
Tnfrsf12a	Tumor necrosis factor receptor superfamily, member 12a	TNFR	Pro-apoptotic
Tnfrsf4	Tumor necrosis factor receptor superfamily, member 4	TNFR	Anti-apoptotic
Tnfsf15	Tumor necrosis factor (ligand) superfamily, member 15	TNF ligand	Pro-apoptotic

Table3: Ca - K-means analysis orchidectomy without testosterone maintenance

Group 1 Total number of genes: 14

Gene symbol	Common gene name	Family	Role in apoptosis
Pycard	Apoptosis-associated speck-like protein containing a CARD	CARD domain	Pro-apoptotic
Bcl2l2	Bcl2-like 2	Bcl2	Anti-apoptotic
Bnip3l	BCL2/adenovirus E1B 19 kDa-interacting protein 3-like	Bcl2	Pro-apoptotic
Casp8ap2	Midasin homolog (yeast) (predicted)	Death Effector domain	Pro-apoptotic
Cidea	Cell death-inducing DNA fragmentation factor, alpha subunit-like effector A (predicted)	CIDE domain	Pro-apoptotic
Ngfrap1	Nerve growth factor receptor associated protein 1	TNFR	Pro-apoptotic
Tnfrsf11b	Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	TNFR	Anti-apoptotic
Tnfsf10	Tumor necrosis factor (ligand) superfamily, member 10	TNF ligand	Pro-apoptotic
Tp53	Tumor protein p53	p53 and ATM	Repair
Zranb1	Zinc finger, RAN-binding domain containing 1 (predicted)	TRAF	Other related function
Ube1c	Ubiquitin-activating enzyme E1C	Other related genes	Other related function
Ube2d2	Ubiquitin-conjugating enzyme E2D 2	Other related genes	Other related function
Ube2i	Ubiquitin-conjugating enzyme E2I	Other related genes	Other related function
Ube2n	Ubiquitin-conjugating enzyme E2N (homologous to yeast UBC13)	Other related genes	Other related function

Group 2 Total number of genes: 9

Gene symbol	Common gene name	Family	Role in apoptosis
Bak1	BCL2-antagonist/killer 1	Bcl2	Pro-apoptotic
Bmf	Bcl-2 modifying factor	Bcl2	Pro-apoptotic
Casp4	Caspase 11	Caspase	Pro-apoptotic
Casp2	Caspase 2	Caspase	Pro-apoptotic
Mcl1	Myeloid cell leukemia sequence 1	Bcl2	Anti-apoptotic
Myd88	Myeloid differentiation primary response gene 88	Death domain	Pro-apoptotic
Rad52	Similar to Rad52 protein	p53 and ATM	Repair
Tnfrsf1a	Tumor necrosis factor receptor superfamily, member 1a	TNFR	Pro-apoptotic
CD70	Similar to CD70 protein (CD27 ligand) (LOC301132), mRNA	TNF ligand	Pro-apoptotic

Group 3 Total number of genes: 17

Gene symbol	Common gene name	Family	Role in apoptosis
Birc3	Inhibitor of apoptosis protein 1	IAP	Anti-apoptotic
Bok	Bcl-2-related ovarian killer protein	Bcl2	Pro-apoptotic
Casp9	Caspase 9	Caspase	Pro-apoptotic
Cflar	CASP8 and FADD-like apoptosis regulator	Death Effector domain	Anti-apoptotic
Dap3	Death associated protein 3 (predicted)	Death domain	Pro-apoptotic
Dlk	Similar to Death-associated protein kinase 3	Death domain	Pro-apoptotic
Card9	Caspase recruitment domain protein 9	CARD	Pro-apoptotic
Rad1	Similar to Rad1p (LOC294800), mRNA	p53 and ATM	Repair

Group 3	**Total number of genes: 17**		
Gene symbol	**Common gene name**	**Family**	**Role in apoptosis**
Rad23a	Similar to UV excision repair protein RAD23 homolog A (MHR23A)	p53 and ATM	Repair
Rfng	Radical fringe gene homolog (Drosophila)	Other related genes	Other related function
Ripk2	Similar to receptor-interacting protein 2 (LOC362491), mRNA	Death domain	Pro-apoptotic
Rrad	Ras-related associated with diabetes	Other related genes	Other
Tank	TRAF family member-associated Nf-kappa B activator	TRAF	Pro-apoptotic
Tnfrsf8	Tumor necrosis factor receptor superfamily, member 8	TNFR	Pro-apoptotic
Tnfsf12	Tumor necrosis factor ligand superfamily member 12	TNF ligand	Pro-apoptotic
Tradd	TNFRSF1A-associated via death domain	TRAF	Other related function
Ube1x	Similar to ubiquitin-protein ligase (EC 6.3.2.19) E1 - mouse	Other related genes	Other related function

Group 4	**Total number of genes: 20**		
Gene symbol	**Common gene name**	**Family**	**Role in apoptosis**
Apaf1	Apoptotic peptidase activating factor 1	CARD family	Pro-apoptotic
Bad	Bcl2-associated death promoter	Bcl2	Pro-apoptotic
Bax	Bcl2-associated X protein	Bcl2	Pro-apoptotic
Bcl10	B-cell CLL/lymphoma 10	CARD family	Pro-apoptotic
Bcl2	B-cell leukemia/lymphoma 2	Bcl2	Anti-apoptotic
Becn1	Beclin 1 (coiled-coil, myosin-like BCL2-interacting protein)	Bcl2	Anti-apoptotic
Bid3	BH3 interacting (with BCL2 family) domain, apoptosis agonist	Bcl2	Pro-apoptotic
Biklk	Bcl2-interacting killer-like	Bcl2	Pro-apoptotic
Bnip1	BCL2/adenovirus E1B 19kDa-interacting protein 1	Bcl2	Anti-apoptotic
Bnip3	BCL2/adenovirus E1B 19 kDa-interacting protein 3	Bcl2	Pro-apoptotic
Casp6	Caspase 6	Caspase	Pro-apoptotic
Casp8	Caspase 8	Caspase	Pro-apoptotic
Chek1	Checkpoint kinase 1 homolog (S. pombe)	p53 and ATM	Repair
Cntnap1	Contactin associated protein 1	Other related genes	Other related function
E2f5	E2F transcription factor 5	p53 and ATM	Repair
Chek2	Protein kinase Chk2	p53 and ATM	Repair
Tnfsf13	Tumor necrosis factor ligand superfamily, member 13	TNF ligand	Pro-apoptotic
Traf4	Similar to TNF receptor associated factor 4 (LOC303285), mRNA	TRAF	Anti-apoptotic
Traip	TRAF-interacting protein (predicted)	TRAF	Pro-apoptotic
Ube2d3	Ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast)	Other related genes	Other related function

Group 5	**Total number of genes: 7**		
Gene symbol	**Common gene name**	**Family**	**Role in apoptosis**
Baiap2	Brain-specific angiogenesis inhibitor 1-associated protein 2	Other related genes	Other related function
Bcl2a1	B-cell leukemia/lymphoma 2 related protein A1	Bcl2	Anti-apoptotic
Fadd	Fas (TNFRSF6)-associated via death domain	Death Effector domain	Pro-apoptotic
Gadd45a	Growth arrest and DNA-damage-inducible 45 alpha	p53 and ATM	Repair

Group 5	**Total number of genes: 7**		
Gene symbol	**Common gene name**	**Family**	**Role in apoptosis**
Ltb	Lymphotoxin B	TNF ligand	Pro-apoptotic
Tnfaip2	Similar to [Mouse primary response gene B94 mRNA, 3end.], gene product	Other related genes	Other related function
Tnfrsf1b	Tumor necrosis factor receptor superfamily, member 1b	TNFR	Anti-apoptotic

Unclassified			
Gene symbol	**Common gene name**	**Family**	**Role in apoptosis**
Atm	Ataxia telangiectasia mutated homolog (human)	p53 and ATM	Repair
Bcl2l1	Bcl2-like 1	Bcl2	Anti-apoptotic
Bcl2l10	Bcl2-like 10	Bcl2	Anti-apoptotic
Bcl2l11	BCL2-like 11 (apoptosis facilitator)	Bcl2	Pro-apoptotic
Birc1b	Baculoviral IAP repeat-containing 1b	IAP	Anti-apoptotic
Birc5	Baculoviral IAP repeat-containing 5	IAP	Anti-apoptotic
Casp12	Caspase 12	Caspase	Pro-apoptotic
Casp7	Caspase 7	Caspase	Pro-apoptotic
Cradd	CASP2 and RIPK1 domain containing adaptor with death domain (predicted)	Death domain	Pro-apoptotic
Dffa	DNA fragmentation factor, alpha subunit	CIDE domain	Pro-apoptotic
Dffb	DNA fragmentation factor, beta subunit	CIDE domain	Pro-apoptotic
E2f3	Similar to E2f3 protein (LOC291105), mRNA	p53 and ATM	Repair
E2f6	E2F transcription factor 6	p53 and ATM	Repair
Tnfsf5	Tumor necrosis factor (ligand) superfamily, member 5	TNF ligand	Pro-apoptotic
Ltbr	Lymphotoxin B receptor (predicted)	TNFR	Pro-apoptotic
Rad50	RAD50 homolog (S. cerevisiae)	p53 and ATM	Repair
Tnfrsf26	Tumor necrosis factor receptor superfamily, member 26 (predicted)	TNFR	Other related function
Tnfrsf10b	Similar to TRAIL receptor2 KILLER/DR5 homologue (LOC364420), mRNA	TNFR	Pro-apoptotic
Tnfrsf12a	Tumor necrosis factor receptor superfamily, member 12a	TNFR	Pro-apoptotic
Tnfrsf4	Tumor necrosis factor receptor superfamily, member 4	TNFR	Anti-apoptotic
Tnfsf15	Tumor necrosis factor (ligand) superfamily, member 15	TNF ligand	Pro-apoptotic
Tnfsf9	Tumor necrosis factor (ligand) superfamily, member 9	TNF ligand	Other related function
Tnfaip3	TNFAIP3 interacting protein 2 (predicted)	Other related genes	Other related function
Traf2	Tnf receptor-associated factor 2 (predicted)	TRAF	Anti-apoptotic

Table 4: Ca - K-means analysis orchidectomy with testosterone maintenance

Group 1 Total number of genes: 37

Gene symbol	Common gene name	Family	Role in apoptosis
Bad	Bcl2-associated death promoter	Bcl2	Pro-apoptotic
Baiap2	Brain-specific angiogenesis inhibitor 1-associated protein 2	Other related genes	Other related function
Bak1	BCL2-antagonist/killer 1	Bcl2	Pro-apoptotic
Bax	Bcl2-associated X protein	Bcl2	Pro-apoptotic
Bcl10	B-cell CLL/lymphoma 10	CARD family	Pro-apoptotic
Becn1	Beclin 1 (coiled-coil, myosin-like BCL2-interacting protein)	Bcl2	Anti-apoptotic
Bid3	BH3 interacting (with BCL2 family) domain, apoptosis agonist	Bcl2	Pro-apoptotic
Bnip3	BCL2/adenovirus E1B 19 kDa-interacting protein 3	Bcl2	Pro-apoptotic
Bnip3l	BCL2/adenovirus E1B 19 kDa-interacting protein 3-like	Bcl2	Pro-apoptotic
Casp4	Caspase 11	Caspase	Pro-apoptotic
Cflar	CASP8 and FADD-like apoptosis regulator	Death Effector domain	Anti-apoptotic
Dlk	Similar to Death-associated protein kinase 3	Death domain	Pro-apoptotic
E2f5	E2F transcription factor 5	p53 and ATM	Repair
Ltb	Lymphotoxin B	TNF ligand	Pro-apoptotic
Ltbr	Lymphotoxin B receptor (predicted)	TNFR	Pro-apoptotic
Myd88	Myeloid differentiation primary response gene 88	Death domain	Pro-apoptotic
Ngfrap1	Nerve growth factor receptor associated protein 1	TNFR	Pro-apoptotic
Rad50	RAD50 homolog (S. cerevisiae)	p53 and ATM	Repair
Rad52	Similar to Rad52 protein	p53 and ATM	Repair
Chek2	Protein kinase Chk2	p53 and ATM	Repair
Tnfaip2	Similar to [Mouse primary response gene B94 mRNA, 3end.], gene product	Other related genes	Other related function
Tnfrsf1a	Tumor necrosis factor receptor superfamily, member 1a	TNFR	Pro-apoptotic
Tnfrsf1b	Tumor necrosis factor receptor superfamily, member 1b	TNFR	Anti-apoptotic
Tnfrsf8	Tumor necrosis factor receptor superfamily, member 8	TNFR	Pro-apoptotic
Tnfsf10	Tumor necrosis factor (ligand) superfamily, member 10	TNF ligand	Pro-apoptotic
Tnfsf12	Tumor necrosis factor ligand superfamily member 12	TNF ligand	Pro-apoptotic
CD70	Similar to CD70 protein (CD27 ligand) (LOC301132), mRNA	TNF ligand	Pro-apoptotic
Tp53	Tumor protein p53	p53 and ATM	Repair
Zranb1	Zinc finger, RAN-binding domain containing 1 (predicted)	TRAF	Other related function
Tradd	TNFRSF1A-associated via death domain	TRAF	Other related function
Traf4	Similar to TNF receptor associated factor 4 (LOC303285), mRNA	TRAF	Anti-apoptotic
Traip	TRAF-interacting protein (predicted)	TRAF	Pro-apoptotic
Ube1c	Ubiquitin-activating enzyme E1C	Other related genes	Other related function
Ube1x	Similar to ubiquitin-protein ligase (EC 6.3.2.19) E1 - mouse	Other related genes	Other related function
Ube2d2	Ubiquitin-conjugating enzyme E2D 2	Other related genes	Other related function
Ube2d3	Ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast)	Other related genes	Other related function
Ube2i	Ubiquitin-conjugating enzyme E2I	Other related genes	Other related function

Group 2	**Total number of genes: 32**		
Gene symbol	**Common gene name**	**Family**	**Role in apoptosis**
Apaf1	Apoptotic peptidase activating factor 1	CARD family	Pro-apoptotic
Pycard	Apoptosis-associated speck-like protein containing a CARD	CARD domain	Pro-apoptotic
Bcl2	B-cell leukemia/lymphoma 2	Bcl2	Anti-apoptotic
Bcl2a1	B-cell leukemia/lymphoma 2 related protein A1	Bcl2	Anti-apoptotic
Biklk	Bcl2-interacting killer-like	Bcl2	Pro-apoptotic
Birc3	Inhibitor of apoptosis protein 1	IAP	Anti-apoptotic
Birc5	Baculoviral IAP repeat-containing 5	IAP	Anti-apoptotic
Bnip1	BCL2/adenovirus E1B 19kDa-interacting protein 1	Bcl2	Anti-apoptotic
Bok	Bcl-2-related ovarian killer protein	Bcl2	Pro-apoptotic
Casp2	Caspase 2	Caspase	Pro-apoptotic
Casp6	Caspase 6	Caspase	Pro-apoptotic
Casp8	Caspase 8	Caspase	Pro-apoptotic
Casp8ap2	Midasin homolog (yeast) (predicted)	Death Effector domain	Pro-apoptotic
Casp9	Caspase 9	Caspase	Pro-apoptotic
Chek1	Checkpoint kinase 1 homolog (S. pombe)	p53 and ATM	Repair
Cidea	Cell death-inducing DNA fragmentation factor, alpha subunit-like effector A (predicted)	CIDE domain	Pro-apoptotic
Cntnap1	Contactin associated protein 1	Other related genes	Other related function
Dap3	Death associated protein 3 (predicted)	Death domain	Pro-apoptotic
Dffa	DNA fragmentation factor, alpha subunit	CIDE domain	Pro-apoptotic
Fadd	Fas (TNFRSF6)-associated via death domain	Death Effector domain	Pro-apoptotic
Gadd45a	Growth arrest and DNA-damage-inducible 45 alpha	p53 and ATM	Repair
Card9	Caspase recruitment domain protein 9	CARD	Pro-apoptotic
Tnfsf5	Tumor necrosis factor (ligand) superfamily, member 5	TNF ligand	Pro-apoptotic
Mcl1	Myeloid cell leukemia sequence 1	Bcl2	Anti-apoptotic
Rad1	Similar to Rad1p (LOC294800), mRNA	p53 and ATM	Repair
Rad23a	Similar to UV excision repair protein RAD23 homolog A (MHR23A)	p53 and ATM	Repair
Ripk2	Similar to receptor-interacting protein 2 (LOC362491), mRNA	Death domain	Pro-apoptotic
Rrad	Ras-related associated with diabetes	Other related genes	Other related function
Tank	TRAF family member-associated Nf-kappa B activator	TRAF	Pro-apoptotic
Tnfrsf11b	Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	TNFR	Anti-apoptotic
Tnfsf13	Tumor necrosis factor ligand superfamily, member 13	TNF ligand	Pro-apoptotic
Ube2n	Ubiquitin-conjugating enzyme E2N (homologous to yeast UBC13)	Other related genes	Other related function

Unclassified	**Total number of genes: 22**		
Gene symbol	**Common gene name**	**Family**	**Role in apoptosis**
Atm	Ataxia telangiectasia mutated homolog (human)	p53 and ATM	Repair
Bcl2l1	Bcl2-like 1	Bcl2	Anti-apoptotic
Bcl2l10	Bcl2-like 10	Bcl2	Anti-apoptotic

Unclassified	**Total number of genes: 22**		
Gene symbol	**Common gene name**	**Family**	**Role in apoptosis**
Bcl2l11	BCL2-like 11 (apoptosis facilitator)	Bcl2	Pro-apoptotic
Bcl2l2	Bcl2-like 2	Bcl2	Anti-apoptotic
Birc1b	Baculoviral IAP repeat-containing 1b	IAP	Anti-apoptotic
Bmf	Bcl-2 modifying factor	Bcl2	Pro-apoptotic
Casp12	Caspase 12	Caspase	Pro-apoptotic
Casp7	Caspase 7	Caspase	Pro-apoptotic
Cradd	CASP2 and RIPK1 domain containing adaptor with death domain (predicted)	Death domain	Pro-apoptotic
Dffb	DNA fragmentation factor, beta subunit	CIDE domain	Pro-apoptotic
E2f3	Similar to E2f3 protein (LOC291105), mRNA	p53 and ATM	Repair
E2f6	E2F transcription factor 6	p53 and ATM	Repair
Rfng	Radical fringe gene homolog (Drosophila)	Other related genes	Other related function
Tnfrsf26	Tumor necrosis factor receptor superfamily, member 26 (predicted)	TNFR	Other related function
Tnfrsf10b	Similar to TRAIL receptor2 KILLER/DR5 homologue (LOC364420), mRNA	TNFR	Pro-apoptotic
Tnfrsf12a	Tumor necrosis factor receptor superfamily, member 12a	TNFR	Pro-apoptotic
Tnfrsf4	Tumor necrosis factor receptor superfamily, member 4	TNFR	Anti-apoptotic
Tnfsf15	Tumor necrosis factor (ligand) superfamily, member 15	TNF ligand	Pro-apoptotic
Tnfsf9	Tumor necrosis factor (ligand) superfamily, member 9	TNF ligand	Other related function
Tnfaip3	TNFAIP3 interacting protein 2 (predicted)	Other related genes	Other related function
Traf2	Tnf receptor-associated factor 2 (predicted)	TRAF	Anti-apoptotic

Table 5: Co - K-means analysis orchidectomy without testosterone maintenance

Group 1	**Total number of genes: 21**		
Gene symbol	**Common gene name**	**Family**	**Role in apoptosis**
Apaf1	Apoptotic peptidase activating factor 1	CARD family	Pro-apoptotic
Atm	Ataxia telangiectasia mutated homolog (human)	p53 and ATM	Repair
Bid3	BH3 interacting (with BCL2 family) domain, apoptosis agonist	Bcl2	Pro-apoptotic
Biklk	Bcl2-interacting killer-like	Bcl2	Pro-apoptotic
Bnip1	BCL2/adenovirus E1B 19kDa-interacting protein 1	Bcl2	Anti-apoptotic
Bok	Bcl-2-related ovarian killer protein	Bcl2	Pro-apoptotic
Casp8	Caspase 8	Caspase	Pro-apoptotic
Casp8ap2	Midasin homolog (yeast) (predicted)	Death Effector domain	Pro-apoptotic
Casp9	Caspase 9	Caspase	Pro-apoptotic
Cflar	CASP8 and FADD-like apoptosis regulator	Death Effector domain	Anti-apoptotic
E2f5	E2F transcription factor 5	p53 and ATM	Repair
Fadd	Fas (TNFRSF6)-associated via death domain	Death Effector domain	Pro-apoptotic
Gadd45a	Growth arrest and DNA-damage-inducible 45 alpha	p53 and ATM	Repair
Myd88	Myeloid differentiation primary response gene 88	Death domain	Pro-apoptotic
Rad50	RAD50 homolog (S. cerevisiae)	p53 and ATM	Repair
Tnfrsf1b	Tumor necrosis factor receptor superfamily, member 1b	TNFR	Anti-apoptotic
Tnfsf10	Tumor necrosis factor (ligand) superfamily, member 10	TNF ligand	Pro-apoptotic
Zranb1	Zinc finger, RAN-binding domain containing 1 (predicted)	TRAF	Other related function
Ube1x	Similar to ubiquitin-protein ligase (EC 6.3.2.19) E1 - mouse	Other related genes	Other related function
Ube2d3	Ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast)	Other related genes	Other related function
Ube2n	Ubiquitin-conjugating enzyme E2N (homologous to yeast UBC13)	Other related genes	Other related function

Group 2	**Total number of genes: 26**		
Gene symbol	**Common gene name**	**Family**	**Role in apoptosis**
Baiap2	Brain-specific angiogenesis inhibitor 1-associated protein 2	Other related genes	Other related function
Bak1	BCL2-antagonist/killer 1	Bcl2	Pro-apoptotic
Bcl2a1	B-cell leukemia/lymphoma 2 related protein A1	Bcl2	Anti-apoptotic
Becn1	Beclin 1 (coiled-coil, myosin-like BCL2-interacting protein)	Bcl2	Anti-apoptotic
Birc3	Inhibitor of apoptosis protein 1	IAP	Anti-apoptotic
Casp4	Caspase 11	Caspase	Pro-apoptotic
Casp2	Caspase 2	Caspase	Pro-apoptotic
Casp6	Caspase 6	Caspase	Pro-apoptotic
Dlk	Similar to Death-associated protein kinase 3	Death domain	Pro-apoptotic
Mcl1	Myeloid cell leukemia sequence 1	Bcl2	Anti-apoptotic
Rad1	Similar to Rad1p (LOC294800), mRNA	p53 and ATM	Repair
Rad52	Similar to Rad52 protein	p53 and ATM	Repair
Ripk2	Similar to receptor-interacting protein 2 (LOC362491), mRNA	Death domain	Pro-apoptotic
Tank	TRAF family member-associated Nf-kappa B activator	TRAF	Pro-apoptotic

Group 2	**Total number of genes: 26**		
Gene symbol	**Common gene name**	**Family**	**Role in apoptosis**
Tnfaip2	Similar to [Mouse primary response gene B94 mRNA, 3end.], gene product	Other related genes	Other related function
Tnfrsf26	Tumor necrosis factor receptor superfamily, member 26 (predicted)	TNFR	Other related function
Tnfrsf11b	Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	TNFR	Anti-apoptotic
Tnfrsf12a	Tumor necrosis factor receptor superfamily, member 12a	TNFR	Pro-apoptotic
Tnfrsf4	Tumor necrosis factor receptor superfamily, member 4	TNFR	Anti-apoptotic
Tnfrsf8	Tumor necrosis factor receptor superfamily, member 8	TNFR	Pro-apoptotic
Tnfsf15	Tumor necrosis factor (ligand) superfamily, member 15	TNF ligand	Pro-apoptotic
CD70	Similar to CD70 protein (CD27 ligand) (LOC301132), mRNA	TNF ligand	Pro-apoptotic
Tnfsf9	Tumor necrosis factor (ligand) superfamily, member 9	TNF ligand	Other related function
Tnfaip3	TNFAIP3 interacting protein 2 (predicted)	Other related genes	Other related function
Traf4	Similar to TNF receptor associated factor 4 (LOC303285), mRNA	TRAF	Anti-apoptotic
Ube1c	Ubiquitin-activating enzyme E1C	Other related genes	Other related function

Group 3	Total number of genes: 30		
Gene symbol	**Common gene name**	**Family**	**Role in apoptosis**
Pycard	Apoptosis-associated speck-like protein containing a CARD	CARD domain	Pro-apoptotic
Bad	Bcl2-associated death promoter	Bcl2	Pro-apoptotic
Bax	Bcl2-associated X protein	Bcl2	Pro-apoptotic
Bcl10	B-cell CLL/lymphoma 10	CARD domain	Pro-apoptotic
Bcl2	B-cell leukemia/lymphoma 2	Bcl2	Anti-apoptotic
Bcl2l10	Bcl2-like 10	Bcl2	Anti-apoptotic
Birc1b	Baculoviral IAP repeat-containing 1b	IAP	Anti-apoptotic
Birc5	Baculoviral IAP repeat-containing 5	IAP	Anti-apoptotic
Bnip3	BCL2/adenovirus E1B 19 kDa-interacting protein 3	Bcl2	Pro-apoptotic
Bnip3l	BCL2/adenovirus E1B 19 kDa-interacting protein 3-like	Bcl2	Pro-apoptotic
Chek1	Checkpoint kinase 1 homolog (S. pombe)	p53 and ATM	Repair
Cidea	Cell death-inducing DNA fragmentation factor, alpha subunit-like effector A (predicted)	CIDE domain	Pro-apoptotic
Cntnap1	Contactin associated protein 1	Other related genes	Other related function
Dap3	Death associated protein 3 (predicted)	Death domain	Pro-apoptotic
Card9	Caspase recruitment domain protein 9	TNF ligand	Pro-apoptotic
Tnfsf5	Tumor necrosis factor (ligand) superfamily, member 5	CARD domain	Pro-apoptotic
Ltb	Lymphotoxin B	TNF ligand	Pro-apoptotic
Ngfrap1	Nerve growth factor receptor associated protein 1	TNFR	Pro-apoptotic
Rad23a	Similar to UV excision repair protein RAD23 homolog A (MHR23A)	p53 and ATM	Repair
Chek2	Protein kinase Chk2	p53 and ATM	Repair
Rrad	Ras-related associated with diabetes	Other related genes	Other related function
Tnfrsf1a	Tumor necrosis factor receptor superfamily, member 1a	TNFR	Pro-apoptotic
Tnfsf12	Tumor necrosis factor ligand superfamily member 12	TNF ligand	Pro-apoptotic

Group 3	Total number of genes: 30		
Gene symbol	**Common gene name**	**Family**	**Role in apoptosis**
Tnfsf13	Tumor necrosis factor ligand superfamily, member 13	TNF ligand	Pro-apoptotic
Tp53	Tumor protein p53	p53 and ATM	Repair
Tradd	TNFRSF1A-associated via death domain	TRAF	Other related function
Traf2	Tnf receptor-associated factor 2 (predicted)	TRAF	Anti-apoptotic
Traip	TRAF-interacting protein (predicted)	TRAF	Pro-apoptotic
Ube2d2	Ubiquitin-conjugating enzyme E2D 2	Other related genes	Other related function
Ube2i	Ubiquitin-conjugating enzyme E2I	Other related genes	Other related function

Unclassified	**Total number of genes: 14**		
Gene symbol	**Common gene name**	**Family**	**Role in apoptosis**
Bcl2l1	Bcl2-like 1	Bcl2	Anti-apoptotic
Bcl2l11	BCL2-like 11 (apoptosis facilitator)	Bcl2	Pro-apoptotic
Bcl2l2	Bcl2-like 2	Bcl2	Anti-apoptotic
Bmf	Bcl-2 modifying factor	Bcl2	Pro-apoptotic
Casp12	Caspase 12	Caspase	Pro-apoptotic
Casp7	Caspase 7	Caspase	Pro-apoptotic
Cradd	CASP2 and RIPK1 domain containing adaptor with death domain (predicted)	Death domain	Pro-apoptotic
Dffa	DNA fragmentation factor, alpha subunit	CIDE domain	Pro-apoptotic
Dffb	DNA fragmentation factor, beta subunit	CIDE domain	Pro-apoptotic
E2f3	Similar to E2f3 protein (LOC291105), mRNA	p53 and ATM	Repair
E2f6	E2F transcription factor 6	p53 and ATM	Repair
Ltbr	Lymphotoxin B receptor (predicted)	TNFR	Pro-apoptotic
Rfng	Radical fringe gene homolog (Drosophila)	Other related genes	Other related function
Tnfrsf10b	Similar to TRAIL receptor2 KILLER/DR5 homologue (LOC364420), mRNA	TNFR	Pro-apoptotic

Table 6: Co - K-means analysis orchidectomy with testosterone maintenance

Group 1	**Total number of genes: 25**		
Gene symbol	**Common gene name**	**Family**	**Role in apoptosis**
Pycard	Apoptosis-associated speck-like protein containing a CARD	CARD domain	Pro-apoptotic
Bcl2a1	B-cell leukemia/lymphoma 2 related protein A1	Bcl2	Anti-apoptotic
Birc3	Inhibitor of apoptosis protein 1	IAP	Anti-apoptotic
Casp2	Caspase 2	Caspase	Pro-apoptotic
Casp8	Caspase 8	Caspase	Pro-apoptotic
Casp8ap2	Midasin homolog (yeast) (predicted)	Death effector domain	Pro-apoptotic
Chek1	Checkpoint kinase 1 homolog (S. pombe)	p53 and ATM	Repair
Cidea	Cell death-inducing DNA fragmentation factor, alpha subunit-like effector A (predicted)	CIDE domain	Pro-apoptotic
Cntnap1	Contactin associated protein 1	Other related genes	Other related function
Dap3	Death associated protein 3 (predicted)	Death domain	Pro-apoptotic
Card9	Caspase recruitment domain protein 9	CARD domain	Pro-apoptotic
Ltb	Lymphotoxin B	TNF ligand	Pro-apoptotic
Ngfrap1	Nerve growth factor receptor associated protein 1	TNFR	Pro-apoptotic
Rad1	Similar to Rad1p (LOC294800), mRNA	p53 and ATM	Repair
Rad23a	Similar to UV excision repair protein RAD23 homolog A (MHR23A)	p53 and ATM	Repair
Rad50	RAD50 homolog (S. cerevisiae)	p53 and ATM	Repair
Chek2	Protein kinase Chk2	p53 and ATM	Repair
Rrad	Ras-related associated with diabetes	Other related genes	Other related function
Tank	TRAF family member-associated Nf-kappa B activator	TRAF	Pro-apoptotic
Tnfrsf8	Tumor necrosis factor receptor superfamily, member 8	TNFR	Pro-apoptotic
Tnfsf12	Tumor necrosis factor ligand superfamily member 12	TNF ligand	Pro-apoptotic
Tnfsf13	Tumor necrosis factor ligand superfamily, member 13	TNF ligand	Pro-apoptotic
Zranb1	Zinc finger, RAN-binding domain containing 1 (predicted)	TRAF	Other related function
Tradd	TNFRSF1A-associated via death domain	TRAF	Other related function
Ube2i	Ubiquitin-conjugating enzyme E2I	Other related genes	Other related function

Group 2	**Total number of genes: 17**		
Gene symbol	**Common gene name**	**Family**	**Role in apoptosis**
Atm	Ataxia telangiectasia mutated homolog (human)	p53 and ATM	Repair
Baiap2	Brain-specific angiogenesis inhibitor 1-associated protein 2	Other related genes	Other related function
Bak1	BCL2-antagonist/killer 1	Bcl2	Pro-apoptotic
Bcl10	B-cell CLL/lymphoma 10	CARD family	Pro-apoptotic
Becn1	Beclin 1 (coiled-coil, myosin-like BCL2-interacting protein)	Bcl2	Anti-apoptotic
Bid3	BH3 interacting (with BCL2 family) domain, apoptosis agonist	Bcl2	Pro-apoptotic
Casp6	Caspase 6	Caspase	Pro-apoptotic
Dlk	Similar to Death-associated protein kinase 3	Death domain	Pro-apoptotic

Group 2	Total number of genes: 17		
Gene symbol	**Common gene name**	**Family**	**Role in apoptosis**
E2f5	E2F transcription factor 5	p53 and ATM	Repair
Rad52	Similar to Rad52 protein	p53 and ATM	Repair
Tnfaip2	Similar to [Mouse primary response gene B94 mRNA, 3end.], gene product	Other related genes	Other related function
Tnfrsf11b	Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	TNFR	Anti-apoptotic
Tnfrsf1a	Tumor necrosis factor receptor superfamily, member 1a	TNFR	Pro-apoptotic
Tnfsf10	Tumor necrosis factor (ligand) superfamily, member 10	TNF ligand	Pro-apoptotic
Tp53	Tumor protein p53	p53 and ATM	Repair
Ube1x	Similar to ubiquitin-protein ligase (EC 6.3.2.19) E1 - mouse	Other related genes	Other related function
Ube2d3	Ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast)	Other related genes	Other related function

Group 3	Total number of genes: 32		
Gene symbol	**Common gene name**	**Family**	**Role in apoptosis**
Apaf1	Apoptotic peptidase activating factor 1	CARD family	Pro-apoptotic
Bad	Bcl2-associated death promoter	Bcl2	Pro-apoptotic
Bax	Bcl2-associated X protein	Bcl2	Pro-apoptotic
Bcl2	B-cell leukemia/lymphoma 2	Bcl2	Anti-apoptotic
Bcl2l10	Bcl2-like 10	Bcl2	Anti-apoptotic
Biklk	Bcl2-interacting killer-like	Bcl2	Pro-apoptotic
Birc1b	Baculoviral IAP repeat-containing 1b	IAP	Anti-apoptotic
Bnip1	BCL2/adenovirus E1B 19kDa-interacting protein 1	Bcl2	Anti-apoptotic
Bnip3	BCL2/adenovirus E1B 19 kDa-interacting protein 3	Bcl2	Pro-apoptotic
Bnip3l	BCL2/adenovirus E1B 19 kDa-interacting protein 3-like	Bcl2	Pro-apoptotic
Bok	Bcl-2-related ovarian killer protein	Bcl2	Pro-apoptotic
Casp4	Caspase 11	Caspase	Pro-apoptotic
Casp9	Caspase 9	Caspase	Pro-apoptotic
Cflar	CASP8 and FADD-like apoptosis regulator	Death effector domain	Anti-apoptotic
Fadd	Fas (TNFRSF6)-associated via death domain	Death effector domain	Pro-apoptotic
Gadd45a	Growth arrest and DNA-damage-inducible 45 alpha	p53 and ATM	Repair
Mcl1	Myeloid cell leukemia sequence 1	Bcl2	Anti-apoptotic
Myd88	Myeloid differentiation primary response gene 88	Death domain	Pro-apoptotic
Ripk2	Similar to receptor-interacting protein 2 (LOC362491), mRNA	Death domain	Pro-apoptotic
Tnfrsf26	Tumor necrosis factor receptor superfamily, member 26 (predicted)	TNFR	Other related function
Tnfrsf1b	Tumor necrosis factor receptor superfamily, member 1b	TNFR	Anti-apoptotic
Tnfrsf4	Tumor necrosis factor receptor superfamily, member 4	TNFR	Anti-apoptotic
Tnfsf15	Tumor necrosis factor (ligand) superfamily, member 15	TNF ligand	Pro-apoptotic
CD70	Similar to CD70 protein (CD27 ligand) (LOC301132), mRNA	TNF ligand	Pro-apoptotic
Tnfsf9	Tumor necrosis factor (ligand) superfamily, member 9	TNF ligand	Other related function
Tnfaip3	TNFAIP3 interacting protein 2 (predicted)	Other related genes	Other related function
Traf2	Tnf receptor-associated factor 2 (predicted)	TRAF	Anti-apoptotic
Traf4	Similar to TNF receptor associated factor 4 (LOC303285), mRNA	TRAF	Anti-apoptotic

Group 3	Total number of genes: 32		
Gene symbol	**Common gene name**	**Family**	**Role in apoptosis**
Traip	TRAF-interacting protein (predicted)	TRAF	Pro-apoptotic
Ube1c	Ubiquitin-activating enzyme E1C	Other related genes	Other related function
Ube2d2	Ubiquitin-conjugating enzyme E2D 2	Other related genes	Other related function
Ube2n	Ubiquitin-conjugating enzyme E2N (homologous to yeast UBC13)	Other related genes	Other related function

Unclassified	Total number of genes: 17		
Gene symbol	**Common gene name**	**Family**	**Role in apoptosis**
Bcl2l1	Bcl2-like 1	Bcl2	Anti-apoptotic
Bcl2l11	BCL2-like 11 (apoptosis facilitator)	Bcl2	Pro-apoptotic
Bcl2l2	Bcl2-like 2	Bcl2	Anti-apoptotic
Birc5	Baculoviral IAP repeat-containing 5	IAP	Anti-apoptotic
Bmf	Bcl-2 modifying factor	Bcl2	Pro-apoptotic
Casp12	Caspase 12	Caspase	Pro-apoptotic
Casp7	Caspase 7	Caspase	Pro-apoptotic
Cradd	CASP2 and RIPK1 domain containing adaptor with death domain (predicted)	Death domain	Pro-apoptotic
Dffa	DNA fragmentation factor, alpha subunit	CIDE domain	Pro-apoptotic
Dffb	DNA fragmentation factor, beta subunit	CIDE domain	Pro-apoptotic
E2f3	Similar to E2f3 protein (LOC291105), mRNA	p53 and ATM	Repair
E2f6	E2F transcription factor 6	p53 and ATM	Repair
Tnfsf5	Tumor necrosis factor (ligand) superfamily, member 5	TNFR	Pro-apoptotic
Ltbr	Lymphotoxin B receptor (predicted)	TNFR	Pro-apoptotic
Rfng	Radical fringe gene homolog (Drosophila)	Other related genes	Other related function
Tnfrsf10b	Similar to TRAIL receptor2 KILLER/DR5 homologue (LOC364420), mRNA	TNFR	Pro-apoptotic
Tnfrsf12a	Tumor necrosis factor receptor superfamily, member 12a	TNFR	Pro-apoptotic

Table 7: Cd - K-means analysis orchidectomy without testosterone maintenance

Group 1	**Total number of genes: 18**		
Gene symbol	**Common gene name**	**Family**	**Role in apoptosis**
Atm	Ataxia telangiectasia mutated homolog (human)	p53 and ATM	Repair
Bak1	BCL2-antagonist/killer 1	Bcl2	Pro-apoptotic
Bax	Bcl2-associated X protein	Bcl2	Pro-apoptotic
Becn1	Beclin 1 (coiled-coil, myosin-like BCL2-interacting protein)	Bcl2	Anti-apoptotic
Biklk	Bcl2-interacting killer-like	Bcl2	Pro-apoptotic
Casp8	Caspase 8	Caspase	Pro-apoptotic
Casp8ap2	Midasin homolog (yeast) (predicted)	Death Effector domain	Pro-apoptotic
Casp9	Caspase 9	Caspase	Pro-apoptotic
Cflar	CASP8 and FADD-like apoptosis regulator	Death Effector domain	Anti-apoptotic
Card9	Caspase recruitment domain protein 9	CARD	Pro-apoptotic
Myd88	Myeloid differentiation primary response gene 88	Death domain	Pro-apoptotic
Tnfaip3	TNFAIP3 interacting protein 2 (predicted)	Other related genes	Other related function
Zranb1	Zinc finger, RAN-binding domain containing 1 (predicted)	TRAF	Other related function
Traf2	Tnf receptor-associated factor 2 (predicted)	TRAF	Anti-apoptotic
Traf4	Similar to TNF receptor associated factor 4 (LOC303285), mRNA	TRAF	Anti-apoptotic
Ube1c	Ubiquitin-activating enzyme E1C	Other related genes	Other related function
Ube2d2	Ubiquitin-conjugating enzyme E2D 2	Other related genes	Other related function
Ube2d3	Ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast)	Other related genes	Other related function

Group 2	**Total number of genes: 10**		
Gene symbol	**Common gene name**	**Family**	**Role in apoptosis**
Birc3	Inhibitor of apoptosis protein 1	IAP	Anti-apoptotic
Casp2	Caspase 2	Caspase	Pro-apoptotic
Casp6	Caspase 6	Caspase	Pro-apoptotic
Dlk	Similar to Death-associated protein kinase 3	Death domain	Pro-apoptotic
E2f5	E2F transcription factor 5	p53 and ATM	Repair
Rad1	Similar to Rad1p (LOC294800), mRNA	p53 and ATM	Repair
Tnfaip2	Similar to [Mouse primary response gene B94 mRNA, 3end.], gene product	Other related genes	Other related function

Group 2	Total number of genes: 10		
Gene symbol	**Common gene name**	**Family**	**Role in apoptosis**
Tnfrsf11b	Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	TNFR	Anti-apoptotic
CD70	Similar to CD70 protein (CD27 ligand) (LOC301132), mRNA	TNF ligand	Pro-apoptotic
Ube2n	Ubiquitin-conjugating enzyme E2N (homologous to yeast UBC13)	Other related genes	Other related function

Group 3	Total number of genes: 15		
Gene symbol	**Common gene name**	**Family**	**Role in apoptosis**
Bcl2a1	B-cell leukemia/lymphoma 2 related protein A1	Bcl2	Anti-apoptotic
Chek1	Checkpoint kinase 1 homolog (S. pombe)	p53 and ATM	Repair
Cidea	Cell death-inducing DNA fragmentation factor, alpha subunit-like effector A (predicted)	CIDE domain	Pro-apoptotic
Rad50	RAD50 homolog (S. cerevisiae)	p53 and ATM	Repair
Rad52	Similar to Rad52 protein	p53 and ATM	Repair
Rfng	Radical fringe gene homolog (Drosophila)	Othe related genes	Other related function
Ripk2	Similar to receptor-interacting protein 2 (LOC362491), mRNA	Death domain	Pro-apoptotic
Rrad	Ras-related associated with diabetes	Othe related genes	Other related function
Tank	TRAF family member-associated Nf-kappa B activator	TRAF	Pro-apoptotic
Tnfrsf12a	Tumor necrosis factor receptor superfamily, member 12a	TNFR	Pro-apoptotic
Tnfrsf1b	Tumor necrosis factor receptor superfamily, member 1b	TNFR	Anti-apoptotic
Tnfrsf4	Tumor necrosis factor receptor superfamily, member 4	TNFR	Anti-apoptotic
Tnfrsf8	Tumor necrosis factor receptor superfamily, member 8	TNFR	Pro-apoptotic
Tnfsf15	Tumor necrosis factor (ligand) superfamily, member 15	TNF ligand	Pro-apoptotic
Tnfsf9	Tumor necrosis factor (ligand) superfamily, member 9	TNF ligand	Other related function

Group 4	Total number of genes: 22		
Gene symbol	**Common gene name**	**Family**	**Role in apoptosis**
Apaf1	Apoptotic peptidase activating factor 1	CARD	Pro-apoptotic
Atm	Ataxia telangiectasia mutated homolog (human)	p53 and ATM	Repair
Bad	Bcl2-associated death promoter	Bcl2	Pro-apoptotic
Baiap2	Brain-specific angiogenesis inhibitor 1-associated protein 2	Other related genes	Other related function
Bax	Bcl2-associated X protein	Bcl2	Pro-apoptotic

Group 4	**Total number of genes: 22**		
Gene symbol	**Common gene name**	**Family**	**Role in apoptosis**
Bcl10	B-cell CLL/lymphoma 10	CARD	Pro-apoptotic
Bcl2l2	Bcl2-like 2	Bcl2	Anti-apoptotic
Bnip1	BCL2/adenovirus E1B 19kDa-interacting protein 1	Bcl2	Anti-apoptotic
Bnip3	BCL2/adenovirus E1B 19 kDa-interacting protein 3	Bcl2	Pro-apoptotic
Bnip3l	BCL2/adenovirus E1B 19 kDa-interacting protein 3-like	Bcl2	Pro-apoptotic
Myd88	Myeloid differentiation primary response gene 88	Death domain	Pro-apoptotic
Ngfrap1	Nerve growth factor receptor associated protein 1	TNFR	Pro-apoptotic
Tnfrsf12a	Tumor necrosis factor receptor superfamily, member 12a	TNFR	Pro-apoptotic
Tnfrsf1a	Tumor necrosis factor receptor superfamily, member 1a	TNFR	Pro-apoptotic
Tnfsf12	Tumor necrosis factor ligand superfamily member 12	TNF ligand	Pro-apoptotic
Tp53	Tumor protein p53	p53 and ATM	Repair
Tradd	TNFRSF1A-associated via death domain	TRAF	Other related function
Traip	TRAF-interacting protein (predicted)	TRAF	Pro-apoptotic
Ube1c	Ubiquitin-activating enzyme E1C	Other related genes	Other related function
Ube1x	Similar to ubiquitin-protein ligase (EC 6.3.2.19) E1 - mouse	Other related genes	Other related function
Ube2d2	Ubiquitin-conjugating enzyme E2D 2	Other related genes	Other related function
Ube2d3	Ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast)	Other related genes	Other related function
Ube2i	Ubiquitin-conjugating enzyme E2I	Other related genes	Other related function

Unclassified	**Total number of genes: 22**		
Gene symbol	**Common gene name**	**Family**	**Role in apoptosis**
Bcl2l1	Bcl2-like 1	Bcl2	Anti-apoptotic
Bcl2l10	Bcl2-like 10	Bcl2	Anti-apoptotic
Bcl2l11	BCL2-like 11 (apoptosis facilitator)	Bcl2	Pro-apoptotic
Bcl2l2	Bcl2-like 2	Bcl2	Anti-apoptotic
Birc5	Baculoviral IAP repeat-containing 5	IAP	Anti-apoptotic
Bmf	Bcl-2 modifying factor	Bcl2	Pro-apoptotic
Bok	Bcl-2-related ovarian killer protein	Bcl2	Pro-apoptotic
Casp4	Caspase 11	Caspase	Pro-apoptotic
Casp12	Caspase 12	Caspase	Pro-apoptotic

Unclassified	**Total number of genes: 22**		
Gene symbol	**Common gene name**	**Family**	**Role in apoptosis**
Casp7	Caspase 7	Caspase	Pro-apoptotic
Cntnap1	Contactin associated protein 1	Other related genes	Other related function
Cradd	CASP2 and RIPK1 domain containing adaptor with death domain (predicted)	Death domain	Pro-apoptotic
Dffa	DNA fragmentation factor, alpha subunit	CIDE domain	Pro-apoptotic
Dffb	DNA fragmentation factor, beta subunit	CIDE domain	Pro-apoptotic
E2f3	Similar to E2f3 protein (LOC291105), mRNA	p53 and ATM	Repair
E2f6	E2F transcription factor 6	p53 and ATM	Repair
Gadd45a	Growth arrest and DNA-damage-inducible 45 alpha	p53 and ATM	Repair
Tnfsf5	Tumor necrosis factor (ligand) superfamily, member 5	TNF ligand	Pro-apoptotic
Ltbr	Lymphotoxin B receptor (predicted)	TNFR	Pro-apoptotic
Mcl1	Myeloid cell leukemia sequence 1	Bcl2	Anti-apoptotic
Tnfrsf26	Tumor necrosis factor receptor superfamily, member 26 (predicted)	TNFR	Other related function
Tnfrsf10b	Similar to TRAIL receptor2 KILLER/DR5 homologue (LOC364420), mRNA	TNFR	Pro-apoptotic

Table 8: Cd - K-means analysis orchidectomy with testosterone maintenance

Group 1	**Total number of genes: 26**		
Gene symbol	**Common gene name**	**Family**	**Role in apoptosis**
Pycard	Apoptosis-associated speck-like protein containing a CARD	CARD domain	Pro-apoptotic
Bcl2a1	B-cell leukemia/lymphoma 2 related protein A1	Bcl2	Anti-apoptotic
Bcl2l10	Bcl2-like 10	Bcl2	Anti-apoptotic
Becn1	Beclin 1 (coiled-coil, myosin-like BCL2-interacting protein)	Bcl2	Anti-apoptotic
Biklk	Bcl2-interacting killer-like	Bcl2	Pro-apoptotic
Birc1b	Baculoviral IAP repeat-containing 1b	IAP	Anti-apoptotic
Birc3	Inhibitor of apoptosis protein 1	IAP	Anti-apoptotic
Casp4	Caspase 11	Caspase	Pro-apoptotic
Casp2	Caspase 2	Caspase	Pro-apoptotic
Casp6	Caspase 6	Caspase	Pro-apoptotic
Casp8	Caspase 8	Caspase	Pro-apoptotic
Casp8ap2	Midasin homolog (yeast) (predicted)	Death effector domain	Pro-apoptotic
Casp9	Caspase 9	Caspase	Pro-apoptotic
Chek1	Checkpoint kinase 1 homolog (S. pombe)	p53 and ATM	Repair
Cidea	Cell death-inducing DNA fragmentation factor, alpha subunit-like effector A (predicted)	CIDE domain	Pro-apoptotic
Dap3	Death associated protein 3 (predicted)	Death domain	Pro-apoptotic
Dlk	Similar to Death-associated protein kinase 3	Death domain	Pro-apoptotic
E2f5	E2F transcription factor 5	p53 and ATM	Repair
Gadd45a	Growth arrest and DNA-damage-inducible 45 alpha	p53 and ATM	Repair
Card9	Caspase recruitment domain protein 9	CARD domain	Pro-apoptotic
Ltb	Lymphotoxin B	TNF ligand	Pro-apoptotic
Rad1	Similar to Rad1p (LOC294800), mRNA	p53 and ATM	Repair
Rad23a	Similar to UV excision repair protein RAD23 homolog A (MHR23A)	p53 and ATM	Repair
Chek2	Protein kinase Chk2	p53 and ATM	Repair
Tnfsf10	Tumor necrosis factor (ligand) superfamily, member 10	TNF ligand	Pro-apoptotic
Tnfsf13	Tumor necrosis factor ligand superfamily, member 13	TNF ligand	Pro-apoptotic

Group 2	**Total number of genes: 27**		
Gene symbol	**Common gene name**	**Family**	**Role in apoptosis**
Bak1	BCL2-antagonist/killer 1	Bcl2	Pro-apoptotic
Bcl2	B-cell leukemia/lymphoma 2	Bcl2	Anti-apoptotic
Bcl2l1	Bcl2-like 1	Bcl2	Anti-apoptotic
Bid3	BH3 interacting (with BCL2 family) domain, apoptosis agonist	Bcl2	Pro-apoptotic
Bok	Bcl-2-related ovarian killer protein	Bcl2	Pro-apoptotic
Cflar	CASP8 and FADD-like apoptosis regulator	Death effector domain	Anti-apoptotic
Fadd	Fas (TNFRSF6)-associated via death domain	Death effector domain	Pro-apoptotic
Mcl1	Myeloid cell leukemia sequence 1	Bcl2	Anti-apoptotic
Rad50	RAD50 homolog (S. cerevisiae)	p53 and ATM	Repair
Rad52	Similar to Rad52 protein	p53 and ATM	Repair
Rfng	Radical fringe gene homolog (Drosophila)	Other related genes	Other related function
Ripk2	Similar to receptor-interacting protein 2 (LOC362491), mRNA	Death domain	Pro-apoptotic
Rrad	Ras-related associated with diabetes	Other related genes	Other related function
Tank	TRAF family member-associated Nf-kappa B activator	TRAF	Pro-apoptotic
Tnfaip2	Similar to [Mouse primary response gene B94 mRNA, 3end.], gene product	Other related genes	Other related function
Tnfrsf11b	Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	TNFR	Anti-apoptotic
Tnfrsf1b	Tumor necrosis factor receptor superfamily, member 1b	TNFR	Anti-apoptotic
Tnfrsf4	Tumor necrosis factor receptor superfamily, member 4	TNFR	Anti-apoptotic
Tnfrsf8	Tumor necrosis factor receptor superfamily, member 8	TNFR	Pro-apoptotic
Tnfsf15	Tumor necrosis factor (ligand) superfamily, member 15	TNF ligand	Pro-apoptotic
CD70	Similar to CD70 protein (CD27 ligand) (LOC301132), mRNA	TNF ligand	Pro-apoptotic
Tnfsf9	Tumor necrosis factor (ligand) superfamily, member 9	TNF ligand	Other related function
Tnfaip3	TNFAIP3 interacting protein 2 (predicted)	Other related genes	Other related function
Zranb1	Zinc finger, RAN-binding domain containing 1 (predicted)	TRAF	Other related function
Traf2	Tnf receptor-associated factor 2 (predicted)	TRAF	Anti-apoptotic
Traf4	Similar to TNF receptor associated factor 4 (LOC303285), mRNA	TRAF	Anti-apoptotic
Ube2n	Ubiquitin-conjugating enzyme E2N (homologous to yeast UBC13)	Other related genes	Other related function

Group 3	**Total number of genes: 23**		
Gene symbol	**Common gene name**	**Family**	**Role in apoptosis**
Apaf1	Apoptotic peptidase activating factor 1	CARD family	Pro-apoptotic
Atm	Ataxia telangiectasia mutated homolog (human)	p53 and ATM	Repair
Bad	Bcl2-associated death promoter	Bcl2	Pro-apoptotic
Baiap2	Brain-specific angiogenesis inhibitor 1-associated protein 2	Other related genes	Other related function
Bax	Bcl2-associated X protein	Bcl2	Pro-apoptotic
Bcl10	B-cell CLL/lymphoma 10	CARD family	Pro-apoptotic
Bcl2l2	Bcl2-like 2	Bcl2	Anti-apoptotic
Bnip1	BCL2/adenovirus E1B 19kDa-interacting protein 1	Bcl2	Anti-apoptotic
Bnip3	BCL2/adenovirus E1B 19 kDa-interacting protein 3	Bcl2	Pro-apoptotic
Bnip3l	BCL2/adenovirus E1B 19 kDa-interacting protein 3-like	Bcl2	Pro-apoptotic
Myd88	Myeloid differentiation primary response gene 88	Death domain	Pro-apoptotic
Ngfrap1	Nerve growth factor receptor associated protein 1	TNFR	Pro-apoptotic
Tnfrsf12a	Tumor necrosis factor receptor superfamily, member 12a	TNFR	Pro-apoptotic
Tnfrsf1a	Tumor necrosis factor receptor superfamily, member 1a	TNFR	Pro-apoptotic
Tnfsf12	Tumor necrosis factor ligand superfamily member 12	TNF ligand	Pro-apoptotic
Tp53	Tumor protein p53	p53 and ATM	Repair
Tradd	TNFRSF1A-associated via death domain	TRAF	Other related function
Traip	TRAF-interacting protein (predicted)	TRAF	Pro-apoptotic
Ube1c	Ubiquitin-activating enzyme E1C	Other related genes	Other related function
Ube1x	Similar to ubiquitin-protein ligase (EC 6.3.2.19) E1 - mouse	Other related genes	Other related function
Ube2d2	Ubiquitin-conjugating enzyme E2D 2	Other related genes	Other related function
Ube2d3	Ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast)	Other related genes	Other related function
Ube2i	Ubiquitin-conjugating enzyme E2I	Other related genes	Other related function

Unclassified	**Total number of genes: 15**		
Gene symbol	**Common gene name**	**Family**	**Role in apoptosis**
Bcl2l11	BCL2-like 11 (apoptosis facilitator)	Bcl2	Pro-apoptotic
Birc5	Baculoviral IAP repeat-containing 5	IAP	Anti-apoptotic
Bmf	Bcl-2 modifying factor	Bcl2	Pro-apoptotic

Unclassified	**Total number of genes: 15**		
Gene symbol	**Common gene name**	**Family**	**Role in apoptosis**
Casp12	Caspase 12	Caspase	Pro-apoptotic
Casp7	Caspase 7	Caspase	Pro-apoptotic
Cntnap1	Contactin associated protein 1	Other related genes	Other related function
Cradd	CASP2 and RIPK1 domain containing adaptor with death domain (predicted)	Death domain	Pro-apoptotic
Dffa	DNA fragmentation factor, alpha subunit	CIDE domaine	Pro-apoptotic
Dffb	DNA fragmentation factor, beta subunit	CIDE domaine	Pro-apoptotic
E2f3	Similar to E2f3 protein (LOC291105), mRNA	p53 and ATM	Repair
E2f6	E2F transcription factor 6	p53 and ATM	Repair
Tnfsf5	Tumor necrosis factor (ligand) superfamily, member 5	TNF ligand	Pro-apoptotic
Ltbr	Lymphotoxin B receptor (predicted)	TNFR	Pro-apoptotic
Tnfrsf26	Tumor necrosis factor receptor superfamily, member 26 (predicted)	TNFR	Other related function
Tnfrsf10b	Similar to TRAIL receptor2 KILLER/DR5 homologue (LOC364420), mRNA	TNFR	Pro-apoptotic

APPENDIX 2

1. Materials and Methods

RNA extraction, Real-Time RT-PCR, and immunohistochemistry were done as described in chapter 3.

1.1. Dot blot

First-strand cDNA synthesis was carried out using 1µg total RNA, random primers (Invitrogen, Mississauga, ON), 10mM dNTP mix (Invitrogen), and SuperScript^{TM} III RT (Invitrogen). The synthesized cDNA was then used as a template for PCR amplification using primers for *Birc5* (forward 5'-CTGATTTGGCCCAGTGTTTT -3'; reverse 5'-TCCATTACCCCATGGTAGGA -3') and *18S rRNA* (forward 5'-AAACGGCTACCACATCCAAG-3'; reverse 5'-AGTCGGCATCGTTTATGGTC-3') designed using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi/) and synthesized by AlphaDNA (www.alphadna.com, Montreal, QC). The cycling conditions were as follow: 2 min at 94^0C, 40 cycles of 30 sec at 94^0C, 1 min at 56^0C, and 1 min at 72^0C, followed by 5 min at 72^0C, and 4^0C O/N. For dot blot analysis, PCR samples were prepared by adding 0.5M EDTA, 6N NaOH, and 2M NH_4OAc. Samples were loaded into a dot-blot manifold (Bio-Rad, Mississauga, ON) to be transferred onto a nitrocellulose membrane (Bio-Rad). Filter was removed and soaked for 15 sec in 6X SSC+0.1% SDS. The membrane was cross-linked under UV light for 4 min. Membranes were soaked for 2-4h at 42^0C in pre-hybridization solution [20X SSC, 50X Denhardt's, 20mg/ml tRNA (Roche Applied Science, Laval, QC), 20% SDS, and ddH_2O]. The internal oligonucleotide probe for *Birc5* (GCGCCTTCCTTACAGTCAAG) and *18S rRNA* (CGCGGTTCTATTTTGTTGGT) were designed using Primer3 software and synthesized by AlphaDNA. Fifty ng of oligonucleotide probe was labeled using γP32 (PerkinElmer, Woodbridge, ON), kinase buffer (Roche Applied Science), and T4 kinase (Roche Applied Science). It was incubated for 1-2h at 37^0C and passed trough a G-25 sephadex column. An activity of 10^4-10^5 cpm/ng was

considered good. Labeled oligonucleotide was added to the hybridization solution (20X SSC, 20% SDS, and ddH_2O) at a concentration of $6x10^6$cmp/ml.

1.2. Cloning of the 3kb-upstream promoter region of *Birc5*

Genomic DNA was extracted from rat testis using the Wizard Genomic DNA purification kit (Promega, Madison, WI) following the manufacturer's instructions. Concentration and quality of extracted DNA were assessed using a nanodrop 2000 spectrophotometer (Thermo Scientific, Mississauga, ON). Different sets of primers covering no ARE or all 5 AREs in the 3kb-upstream promoter of *Birc5* were designed using Primer3 software and synthesized by AlphaDNA. PCR amplification was done using 50ng to 500ng of genomic DNA , 10mM dNTP mix (Invitrogen) and Taq DNA polymerase (Invitrogen) using the following cycling conditions: 2min at 94^0C, 40 cycles of 1min at 94^0C, 1min at 55^0C, 3min at 72^0C, then 10min at 72^0C. Efficiency of amplification was checked by running the amplified products on 1% agarose gels. Products were cleaned using the Wizard SV Gel and PCR Clean-Up System (Promega) following the manufacturer's instructions. PCR inserts were cloned into vectors using the pBlue-TOPO reporter kit (Invitrogen) following the manufacturer's instructions. Competent TOP10 cells were then transformed with vectors and plated onto LB plates O/N. When colonies formed, 10 of them were selected and amplified into LB medium. Vectors were extracted from cells using the Qiaprep Spin Miniprep (Qiagen Inc.) following the manufacturer's instructions. Potential positive clones were identified by restriction analysis using BanHI, BsaAI, and SspI (New England BioLabs Inc., Pickering, ON).

2. Results

2.1. *Birc5* was highly expressed in the epididymis

We assessed the presence of *Birc5* in different rat tissues qualitatively by dot blot and quantitatively by qRT-PCR (fig. 1). We found that *Birc5* was expressed in all tissues examined with very low expression observed in the coagulating gland, heart, kidney, liver, dorsal prostate, lateral prostate, ventral

prostate, and vas deferens. The testis had the highest expression of *Birc5*, followed by the different epididymal regions and seminal vesicles. This suggested that BIRC5 may play a role in the epididymis.

2.2. BIRC5 localized in the cyptoplasm of principal cells

We determined the immunolocalization of BIRC5 in the different regions of the epididymis (fig. 2). In all regions, BIRC5 was localized to the cytoplasm of most cell types, except the clear cells of the Cd (fig. 2E). This data pointed to a role of BIRC5 as an anti-apoptotic protein in the epididymis.

2.3. Effects of orchidectomy with or without testosterone replacement on *Birc5* mRNA expression in the ventral prostate and seminal vesicles

We assessed the effects of orchidectomy with or without testosterone replacement on *Birc5* mRNA expression in the ventral prostate (fig. 3A) and seminal vesicles (fig. 3B). After orchidectomy, *Birc5* mRNA was significantly ($p<0.05$) increased at 3 and 7 days in the ventral prostate (fig. 3A), whereas it was significantly ($p<0.05$) decreased in the seminal vesicles (fig. 3B). Testosterone (T) replacement could not prevent the increase in *Birc5* mRNA in the ventral prostate, although it decreased its extent (fig. 3A). In the seminal vesicles, T replacement significantly ($p<0.05$) increased *Birc5* mRNA at only 3 days (fig. 3B).

2.4. Cloning the 3kb-upstream promoter region of rat *Birc5*

Using the pBlue-TOPO reporter kit, we could not clone the proper inserts for the promoter regions covering no ARE and 5AREs of the rat *Birc5* gene. To optimize, we tried different amounts of starting genomic DNA, different sets of primers, different PCR conditions including varying $MgCl_2$ concentrations, and different insert to vector molar ratio for transformation.

3. Discussion

It is widely accepted that BIRC5 is absent in terminally differentiated tissues, but highly expressed in most known cancers. This idea is derived from a study by Ambrosini et al. (2) that could not detect *Birc5* expression in a wide variety of adult tissues, but could in many cancers and lymphomas. It was later shown that *Birc5* was expressed in thymocytes (2), $CD34^+$ bone-marrow-derived stem cells (3), basal colonic epithelial cells (4), placenta (2), ovaries (5), and testis (6), all mitotically-active cells and tissues. Here, we report low expression of *Birc5* in most adult rat tissues examined with high expression in testis and the terminally-differentiated epididymis, using dot blot and qRT-PCR. Previously, heart, liver, kidney were shown to be negative for *Birc5* using northern blot (2). The fact that we could detect *Birc5* in those tissues, whereas others could not, is due to the higher sensitivity of the two techniques we used as compared to northern blot.

BIRC5 not only acts as an anti-apoptotic protein, but also as a regulator of mitosis by associating with the inner centromere protein (INCENP) and Aurora B thereby targeting the complex to kinetochores. It also corrects misaligned chromosomes, works to properly form the central spindle, and completes cytokinesis (7;8). In fact, function of BIRC5 in apoptosis or cell division has been associated with specific subcellular compartment; anti-apoptotic BIRC5 localizes to the cytoplasm, whereas mitotic BIRC5 resides in the nucleus (9). We have shown that in the epididymis, BIRC5 localizes to the cytoplasm of epithelial cells indicating a role as an anti-apoptotic protein.

The ventral prostate and seminal vesicles are androgen-sensitive tissues used as biomarkers of serum androgen concentrations (10). It is therefore interesting to assess their response to orchidectomy with or without T replacement. After orchidectomy, *Birc5* mRNA expression was increased in the ventral prostate, but decreased in the seminal vesicles; T replacement increased *Birc5* expression in both ventral prostate and seminal vesicles. The pattern observed in the ventral prostate was similar to the one observed for the epididymis. It is noteworthy that orchidectomy had opposite effects in the ventral

prostate and seminal vesicles, whereas T replacement caused a similar trend in both tissues. These results highlight differences in transcriptional regulation in two androgen-dependent tissues.

Changes in *Birc5* mRNA expression after orchidectomy with or without testosterone replacement suggested that *Birc5* could be regulated at the transcriptional level by androgens. In fact, we found 5 putative AREs in the upstream promoter region of *Birc5*. However, our attempts at cloning the regions covering no ARE and 5AREs using the pBlue-TOPO reporter kit were unsuccessful due to the difficulty to amplify the proper inserts. This problem was caused by the difficulty at designing primers with the right length, %GC content, and 3' sequence to amplify the desired regions; these 3 parameters are essential for proper amplification (11). An alternative method to clone the desired promoter regions would be to use genomic library screening. In addition, pLuc vectors containing up to 6270bp upstream of the human *Birc5* promoter are available and could be used to carry out reporter assays (11).

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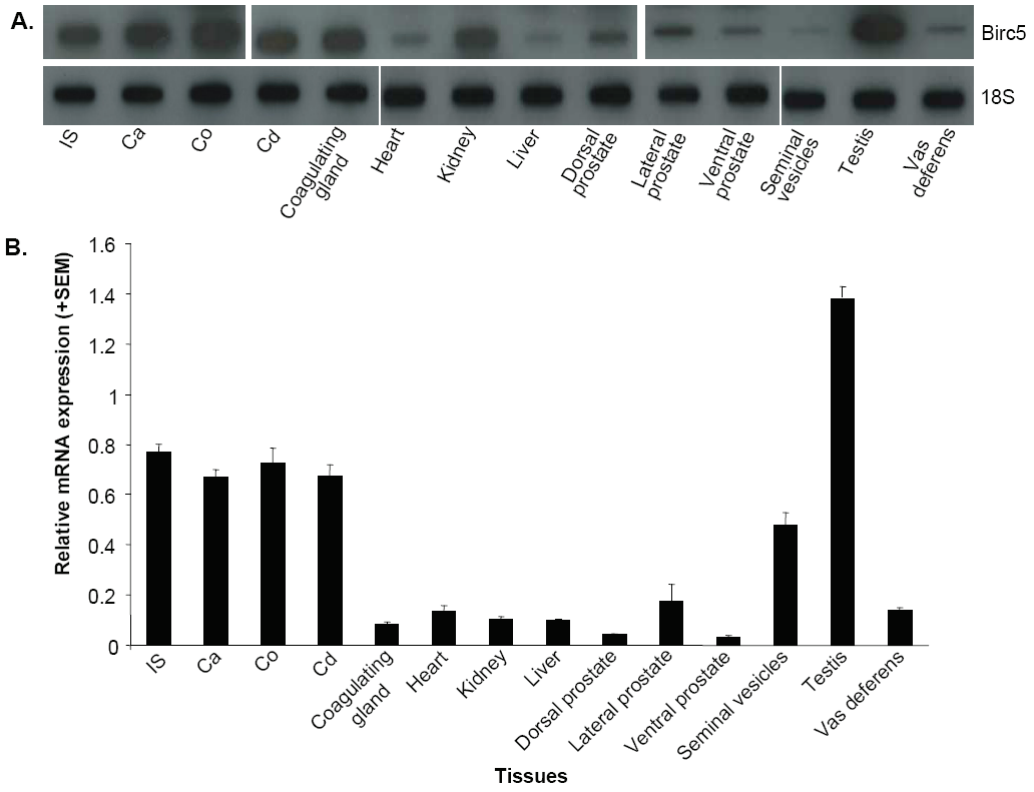

Figure 1: Identification of *Birc5* in different rat tissues. Presence of *Birc5* in IS, Ca, Co, Cd, coagulating gland, heart, kidney, liver, dorsal prostate, lateral prostate, ventral prostate, seminal vesicles, testis, and vas deferens was assessed by dot blot (A) and qRT-PCR (B). To confirm equal loading of RNA in the dot blot experiment, membranes were probed with an 18S probe. *Birc5* mRNA expression for the qRT-PCR experiment was normalized to *Ppia* (*cyclophilin A*) expression. Data are presented as mean ± SEM (n=3/group).

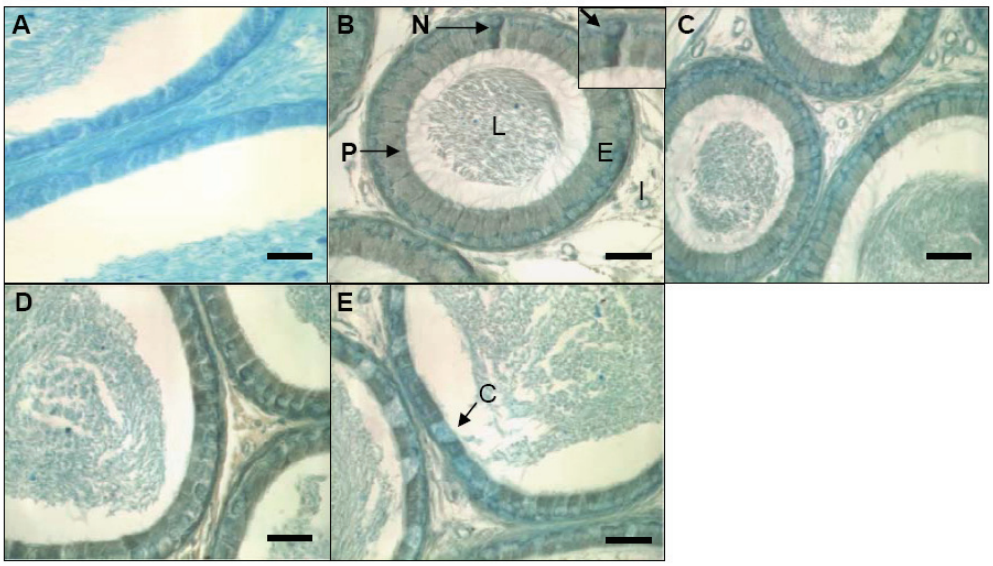

Figure 2: BIRC5 immunolocalization in the different regions of the epididymis. Epididymides of control animals (n=5) were fixed by Bouin's fixation, stained with an anti-BIRC5 antibody, and counterstained with methylene blue. Immunolocalization was determined in the IS (B), Ca (C), Co (D), and Cd (E). (A) shows a slide incubated with only secondary antibody. The insert in (B) shows a higher magnification of the labelled narrow cell. E: epithelium; L: lumen; I: interstitium; P: principal cells; N: narrow cells; C: clear cells. The bar represents 2µm.

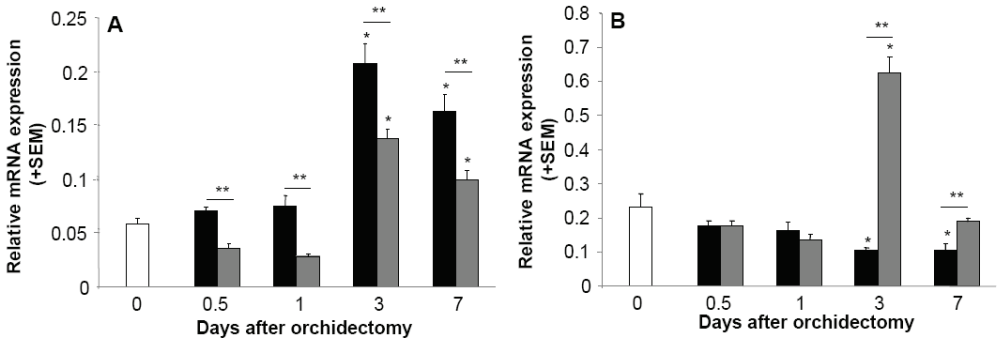

Figure 3: Effects of orchidectomy with or without testosterone replacement on *Birc5* mRNA expression in the ventral prostate and seminal vesicles. Rats were orchidectomized with empty (black bars) or testosterone-filled (grey bars) implants and sacrificed 0.5, 1, 3, and 7 days after surgery. Changes in *Birc5* mRNA expression were assayed by qRT-PCR in the ventral prostate (A) and seminal vesicles (B). *Birc5* expression was normalized to *Ppia* (*cyclophilin A*) expression. Day 0 corresponds to sham-operated animals (white bars). Data are presented as mean ±SEM (n=5/group). Significant effects ($p<0.05$) of treatment on expression are depicted by (**) and significant changes as compared to sham-operated are depicted by (*).

APPENDIX 3

1. Materials and Methods

RNA extraction, Real-Time PCR, cell viability, and IGF1 ELISA were done as described in chapter 4.

Primers for *Mcl1* were forward 5'-TTCTTTCGGTGCCTTTGTG-3' and reverse 5'-CATCCCAGCCTCTTTGTTTG-3'.

1.1. Cell culture

The mouse proximal caput epididymis PC-1 cell line (passage #15) and the mouse distal caput epididymis DC-3 cell line (passage #10) (kindly provided by Dr. M.-C. Orgebin-Crist, Department of Obstetrics and Gynecology, Vanderbilt University School of Medicine, Nashville, TN) were grown as described in chapter 4. PC-1 cells passage #15 were treated as described in chapter 4; media and cells for RNA extraction were collected after 3h, 6h, 12h, 24h, and 48h of treatment. The experiment was repeated 3 times; values were the means of the 3 individual experiments.

1.2. One-step PCR

Presence of *Tnfrsf11b* (QT00177170, Qiagen Inc., Mississauga, ON) and *Birc5* (QT00113379, Qiagen Inc.) in the PC-1 cells was determined by one-step PCR under the following cycling conditions: 50^0C for 30min, 95^0C for 15min, 40 cycles of 1min at 94^0C, 1min at 55^0C, 1min at 72^0C, followed by 10min at 72^0C, and 4^0C O/N. Amplified PCR samples were run on 1% agarose gels.

1.3. Two-steps RT-PCR

Two-steps RT-PCR was done to determine if *Tnfrsf11b* was expressed in the DC-3 cells. First, RNA was reverse transcribed into cDNA using 1µg total RNA, random primers (Invitrogen Canada Inc., Burlington, ON), 10mM dNTP mix (Invitrogen Canada Inc.), and SuperScript^{TM} II RT (Invitrogen Canada Inc.) under the following cycling conditions: 65^0C for 2min, 4^0C for 2min, 25^0C for 5min, 50^0C for 60min, 70^0C for 15min, then samples were kept at 4^0C. The synthesized cDNA was then used as a template for PCR amplification using primers for

Tnfrsf11b (QT00177170, Qiagen Inc.). The cycling conditions were as follow: 2 min at 94^0C, 40 cycles of 30 sec at 94^0C, 1 min at 56^0C, and 1 min at 72^0C, followed by 5 min at 72^0C, and 4^0C O/N. Amplified PCR samples were run on a 1% agarose gel.

1.4. Immunofluorescence

PC-1 cells were fixed with 1% formalin in PBS and slides were incubated overnight at 4°C with a primary rabbit antibody against BIRC5 (1:1500, #2808, Cell Signaling Technology, Danvers, MA). Slides were then probed with a FITC-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) and mounted using DAPI-containing Vectashield (#H-1200, Vector Laboratories Inc., Burlington, CA).

2. Results and Discussion

2.1. *Birc5* was expressed in both PC-1 and DC-3 cells and *Tnfrsf11b* only in DC-3 cells

Presence of *Birc5* and *Tnfrsf11b* transcripts was assessed in the PC-1 and DC-3 cell lines before any experiment was conducted. PC-1 cells expressed *Birc5* transcripts (fig. 1A), but not *Tnfrsf11b* (data not shown); the amplified fragment was expected to be below 250bp. On the other hand, DC-3 cells were shown to express *Birc5* (chapter 4) and *Tnfrsf11b* (fig. 1B); the amplified fragment was expected to be between 100bp and 200bp. This suggested that PC-1 and DC-3 cells were good model systems to study *Birc5*, but only DC-3 cells could be used to study *Tnfrsf11b*.

2.2. BIRC5 localized to both cytoplasm and nucleus in the PC-1 cells

BIRC5 function as a protein involved in cell division and/or cell survival is associated with its cellular localization. In fact, when BIRC5 is localized in the cytoplasm, it acts as an anti-apoptotic protein, whereas in the nucleus, it is involved in cell division (1). To assess the potential roles of BIRC5 in the PC-1 cells, we determined its cellular localization (fig. 2). We found that BIRC5 was

localized in both the nucleus and cytoplasm of PC-1 cells (fig. 2) as well as in the midbody of dividing cells (fig. 2D-F). This suggested that BIRC5 was involved in cell division and potentially cell survival in the PC-1 cells.

2.3. Androgen treatment, withdrawal and/or blockade had no effect on *Birc5*, *Igf1*, and *Igf1r* mRNA expression over time in the PC-1 cell line

Changes in mRNA expression for *Birc5* (fig. 3A), *Igf1* (fig. 3B), and *Igf1r* (fig. 3C) were assessed for passage #15 PC-1 cells. Over time, there was no change in *Birc5* (fig. 3A), *Igf1* (fig. 3B), and *Igf1r* (fig. 3C) mRNA expression among the different treatment groups.

2.4. Androgen treatment, withdrawal and/or blockade had no effect on IGF1 concentration

Although, *Igf1* mRNA expression did not change over time in the PC-1 cells, it was still possible that concentration of IGF1 in the media would increase over time after androgen withdrawal and/or blockade. Concentration of IGF1 in the media over time did not change after androgen withdrawal and/or blockade (fig. 4).

2.5. Passage number did not affect viability of PC-1 cells after androgen treatment, withdrawal and/or blockade

In order to assess if the passage number could have an effect on the response of the PC-1 cells to androgen withdrawal and/or blockade, we determined the cell viability of passage #20 PC-1 cells. Viability of passage #20 PC-1 cells was not affected by the different treatment conditions (fig. 5) and they responded similarly to passage #10 PC-1 cells (chapter 4). This suggested that at least to passage #20, viability of PC-1 cells after androgen withdrawal and/or blockade was similar to earlier passages PC-1 cells.

2.6. Androgen treatment, withdrawal and/or blockade had no effect on *Mcl1* mRNA expression

We assessed changes in mRNA expression for *Mcl1* in the PC-1 and DC-3 cells after androgen withdrawal and/or blockade (fig. 6). Mcl-1 is a Bcl2 family member that prevents apoptosis by binding and inactivating pro-apoptotic Bcl2 members (2). *Mcl1* has also been shown to be differentially regulated after orchidectomy in the epididymis (unpublished data) (3). We found that treatments had no effect on *Mcl1* expression in both PC-1 and DC-3 cells (fig. 6).

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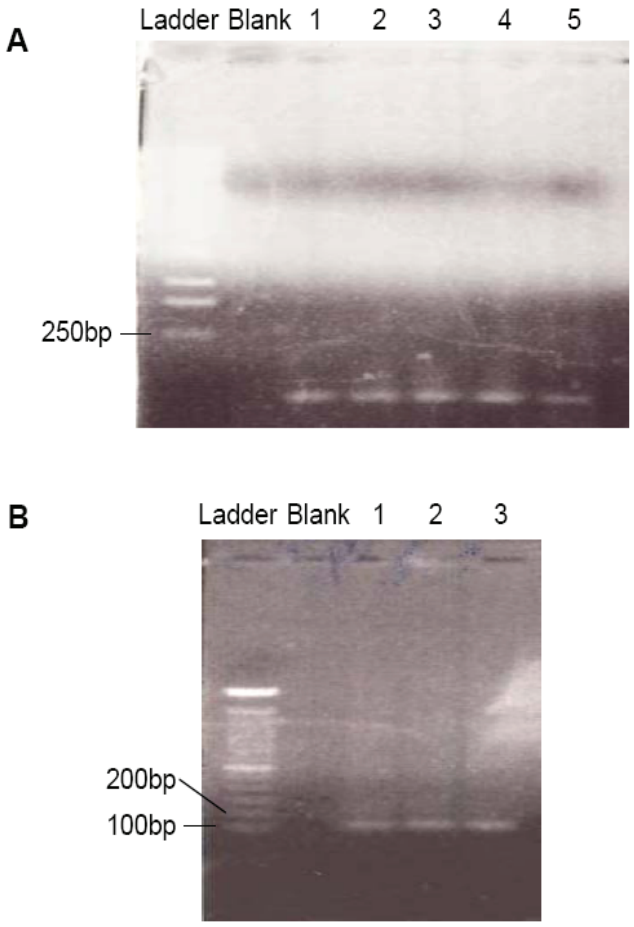

Figure 1: Presence of *Birc5* and *Tnfrsf11b* in the PC-1 and DC-3 cell lines. PC-1 (A) and DC-3 (B) cells were cultured in FBS-containing medium with DHT. Cells were collected for RNA extraction (n=3-5). Presence of *Birc5* and *Tnfrsf11b* in the PC-1 cells was determined by one-step PCR, whereas presence of *Tnfrsf11b* in the DC-3 cells was determined by two-step PCR.

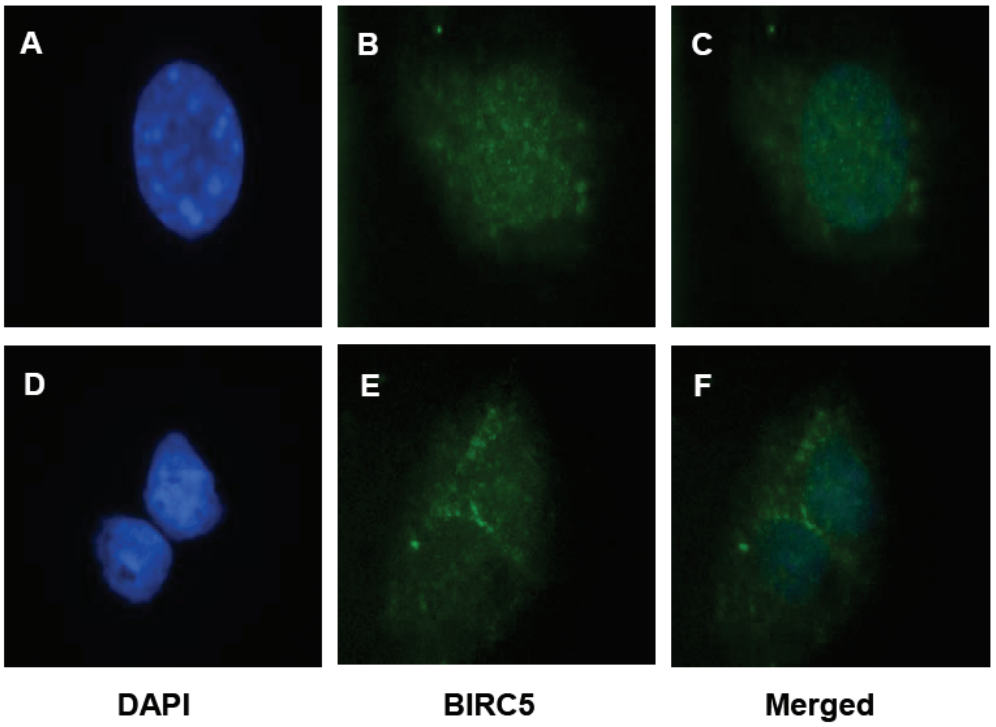

Figure 2: Localization of BIRC5 in the PC-1 cell line. PC-1 cells were fixed with 1% formalin and slides were probed with an antibody against BIRC5 followed by a FITC-conjugated secondary antibody.

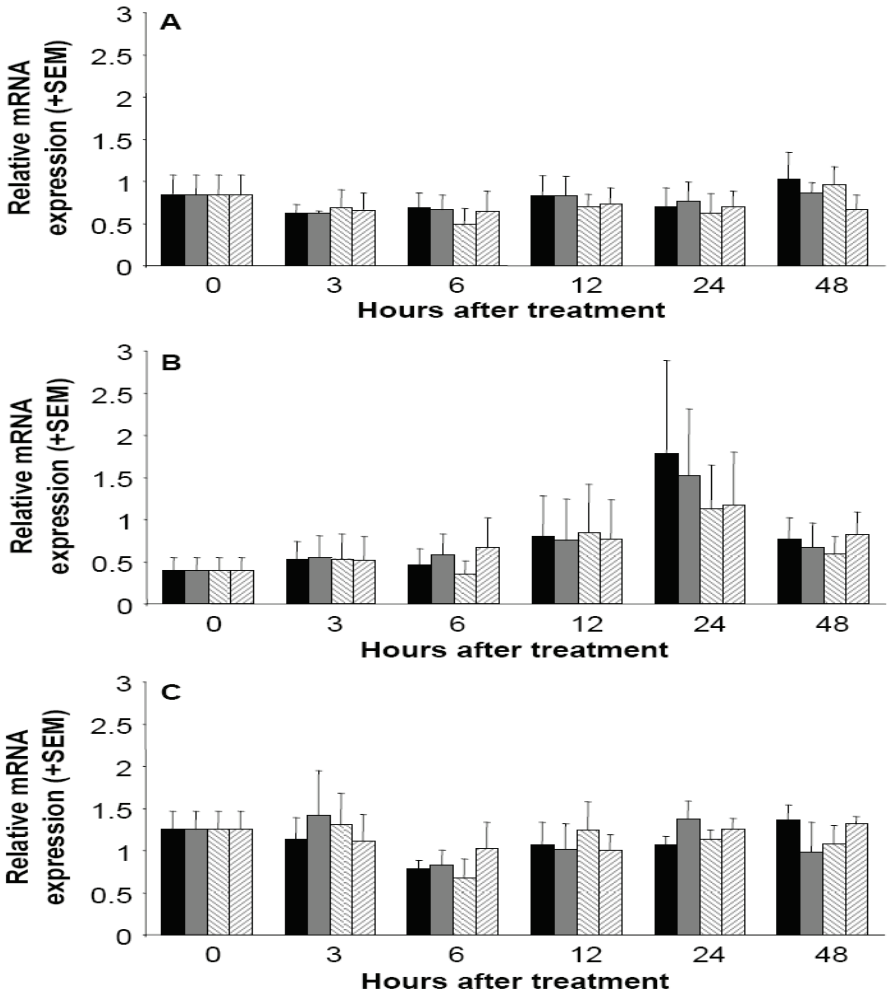

Figure 3: Effects of androgen treatment, withdrawal and/or blockade on *Birc5*, *Igf1*, and *Igf1r* mRNA expression in the PC-1 cell line. PC-1 cells were cultured (n=3/group) in CF-FBS in the absence (-) of DHT without (black bars) or with (left-sided stripped bars) hydroxyflutamide and in the presence (+) of DHT without (grey bars) or with (right-sided stripped bars) hydroxyflutamide for 3h, 6h, 12h, 24h, and 48h. Changes in expression for *Birc5* (A), *Igf1* (B), and *Igf1r* (C) were assessed by qRT-PCR and normalized to *Ppia* (*cyclophilin A*) expression. Data are presented as mean +SEM.

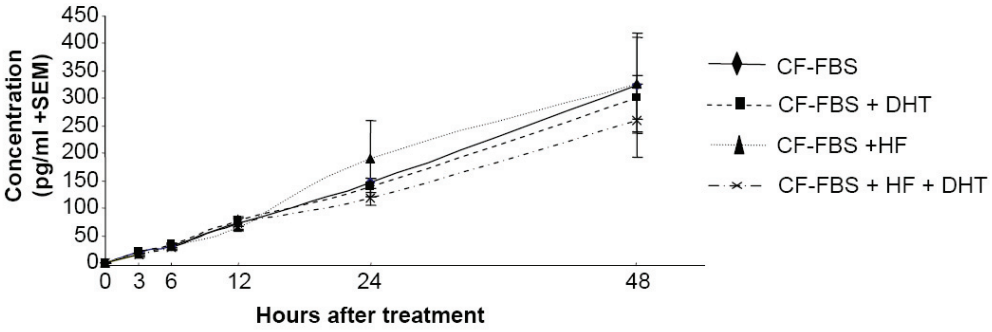

Figure 4: Effects of androgen treatment, withdrawal and/or blockade on IGF1 concentration in the PC-1 cell line. PC-1 cells were cultured (n=3/group) in CF-FBS in the absence of DHT (diamond), the presence of DHT (square), the presence of hydroxyflutamide (HF) (triangle) or the presence of DHT and HF (cross) for 3h, 6h, 12h, 24h, and 48h. IGF1 concentrations (pg/ml) in the media were measured using the IGF1 mouse quantikine ELISA assay; every measure was done in duplicate. Data are presented as mean ± SEM.

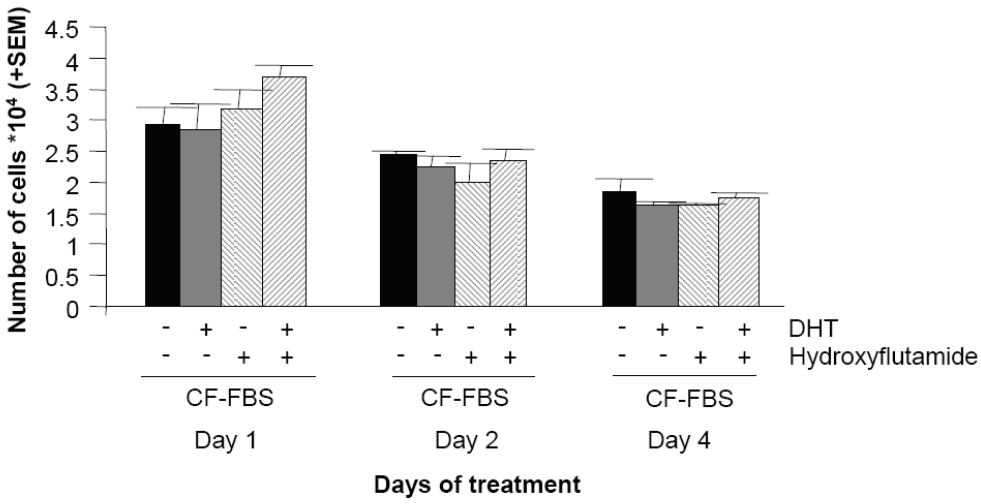

Figure 5: Effects of androgen treatment, withdrawal and/or blockade on PC-1 cell viability. PC-1 cells passage #20 were cultured (n=5/group) in CF-FBS in the absence (-) of DHT without (black bars) or with (left-sided stripped bars) hydroxyflutamide and in the presence (+) of DHT without (grey bars) or with (right-sided stripped bars) hydroxyflutamide for 1, 2, and 4 days and numbers (*10^4) of viable cells were determined by the CellTiter-Glo luminescent cell viability assay. Data are presented as mean +SEM.

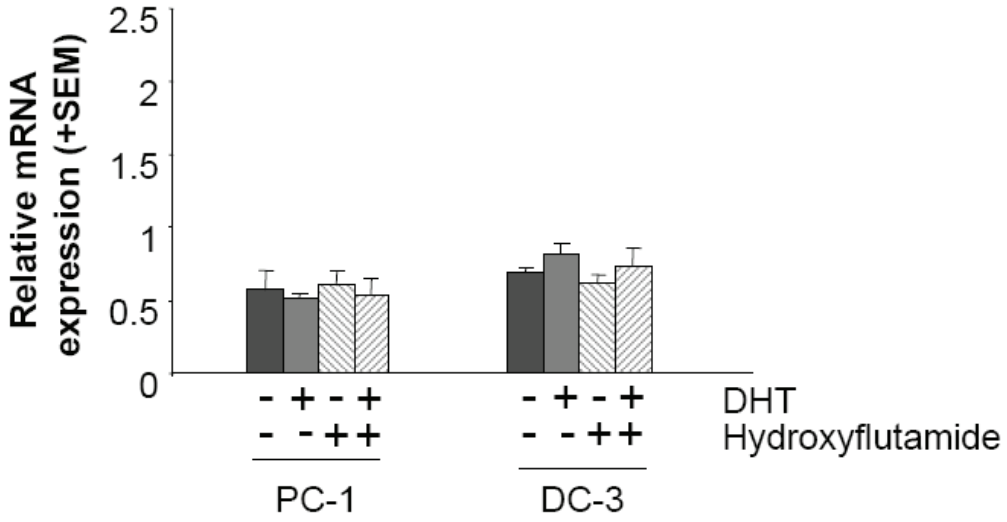

Figure 6: Effects of androgen treatment, withdrawal and/or blockade on *Mcl1* mRNA expression in the PC-1 and DC-3 cell lines. PC-1 and DC-3 cells were cultured (n=3/group) in CF-FBS in the absence (-) of DHT without (black bars) or with (left-sided stripped bars) hydroxyflutamide and in the presence (+) of DHT without (grey bars) or with (right-sided stripped bars) hydroxyflutamide for 3h, 6h, 12h, 24h, and 48h. Changes in expression for *Mcl1* were assessed by qRT-PCR and normalized to *Ppia* (*cyclophilin A*) expression. Data are presented as mean +SEM.