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REGULATION OF GAP JUNCTION ALPHA 1 BINDING PROTEINS IN THE ADULT RAT EPIDIDYMIS AND THEIR INFLUENCE ON ANDROGEN-DEPENDENT GAP JUNCTION ALPHA 1 LOCALIZATION

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Abstract

Spermatozoa acquire motility and fertility by passing through the epididymis. The epididymal epithelium is maintained by androgens and testicular factors secreted by the testes. It is thought that epithelial regulation by these testicular factors influences gap junctional intercellular communication and involves signal transduction pathways.

Little information exists regarding signal transduction pathways or on the regulation of gap junctions (GJ) in the epididymis. However, previous studies from this laboratory have shown that the targeting of the gap junction protein alpha 1 (GJA1) is reliant on testicular androgens in the initial segment of the rat epididymis (Cyr et al. 1996).

The first objective of this study was to characterize the expression and localization of signalling effectors cAMP-dependent protein kinase A catalytic subunit (PKAcat) and cellular Rous sarcoma oncogene (c-Src) in the adult, rat epididymis. Immunocytochemical and Western blot analyses demonstrated that c-Src and PKAcat were present in all four segments of the epididymis. C-Src localization was segment-specific. These data suggest that PKAcat and c-Src can mediate signalling cascades in the rat epididymis.

The second objective of this study was to determine the effect of androgens on GJA1 protein synthesis and phosphorylation in the initial segment and caput epididymidis using an orchidectomy model and Western blot analyses. This was done in order to understand the androgen-dependent targeting of GJA1. The proteins c-Src, PKAcat and tight junction protein 1 (TJP1) were also investigated, since studies have shown that they regulate GJA1 (Toyofuku et al. 1999; Giepmans et al. 2001a; Giepmans et al. 2001b; Duffy et al. 2004; Yogo et al. 2006). GJA1 expression was testicular factor-dependent in the initial segment and caput, and its phosphorylation state was testicular factor-dependent in the caput, but not the initial segment. These results indicate that GJA1 targeting is independent of its protein synthesis and phosphorylation status. Western blot analyses demonstrated that c-Src was testicular factor-dependent in the initial segment and androgen-dependent in the caput. The ratio of active to inactive c-Src was androgen-dependent in the caput, but remained constant in the initial segment. Immunocytochemical analysis indicated that c-Src cellular targeting was altered with

orchidectomy and was dependent on testicular factors. These results indicate that despite a decrease in total c-Src, c-Src is still active in orchidectomized animals and therefore capable of regulating GJA1. PKAcat expression decreased in orchidectomized animals maintained with testosterone in the caput. Orchidectomy did not influence the cellular localization of PKAcat. No differences were observed in any treatment group for TJP1. These results suggest that neither PKAcat nor TJP1 influence GJA1 intracellular targeting.

The third objective of this study was to determine if a direct association between GJA1 and c-Src, PKAcat or TJP1, played a role in GJA1's intracellular targeting. Coimmunoprecipitation analyses were done on tissue lysates from the initial segment and caput epididymidis using an orchidectomy model. In the initial segment, an association was observed between GJA1 and c-Src and GJA1 and TJP1, which was treatment-independent. In the caput, a weak association was observed between GJA1 and TJP1 in all treatment groups. There was no association between GJA1 and PKAcat in either segment. These results suggest that GJA1 is regulated in a segment-specific manner and that c-Src and TJP1 may play a role in GJ inhibition.

This study contributes several ideas to epididymal cell biology. It demonstrates that signalling pathways may contribute to segment-specific physiological differences in the epididymis. The study suggests that tight and gap junctions could form a junctional nexus in the epididymis. The data provide evidence that gap junctions are regulated in a segment-specific manner to contribute to epithelial diversity.

Student

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I dedicate this thesis to my mom, Linda Edwards, who has devoted her life to inspiring scholarship.

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List of Abbreviations

αERKO	estrogen receptor alpha knockout
ABP	androgen binding protein
ADAM	disintegrin-metalloprotease
ADP	adenosine diphosphate
AJ	adherens junction
AKAP	A kinase anchoring protein
ANGII	angiotensin II
ANOVA	analysis of variance
AP	alkaline phosphatase
AP-1	activator protein 1
AR	androgen receptor
AT1	angiotensin II receptor
ATP	adenosine triphosphate
AVP	arginine vasopressin
BEB	blood-epididymal barrier
BrdU	bromodeoxyuridine
CA	cauda epididymidis
Ca^{2+}	calcium
CAK-β	cell adhesion kinase β
cAMP	3',5' cyclic adenosine monophosphate
CAS	Crk-associated substrate
cGMP	3',5'-cyclic guanosine GMP
CIP85	connexin43-interacting protein of 85 kDa
CK1	casein kinase 1
CS	corpus epididymidis
c-Src	cellular Rous sarcoma oncogene
CT	caput epididymidis
Ctrl	sham-operated control
CYP19	cytochrome P450 19, aromatase
DHT	dihydrotestosterone
DNA	deoxyribonucleic acid
EGF/ErbB	epidermal growth factor
EGFR	epidermal growth factor receptor
Epac	exchange protein directly activated by cAMP
EphA7	erythropoietin-producing hepatoma-amplified sequence receptor A7
ERα	estrogen receptor alpha
ERβ	estrogen receptor beta
ER	estrogen receptor
ERK	extracellular signal regulated kinase
ET-1	endothelin-1
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
Flt-1	fms-like tyrosine kinase 1

Flt-4	fms-like tyrosine kinase 4
FSH	follicle-stimulating hormone
GAP	GTPase activating protein
GDP	guanine diphosphate
GEF	guanine nucleotide exchange factor
GJ	gap junction
GJA1	gap junction alpha 1
GJB1	gap junction beta 1
GJB2	gap junction beta 2
GJD3	gap junction delta 3
GJIC	gap junction intercellular communication
GJP	gap junction protein
GPCR	G-protein coupled receptor
GST	glutathione S-transferase
GTP	guanine triphosphate
H^+V-ATPase	vacuolar proton ATPase pump
H_2O_2	hydrogen peroxide
hCG	human chorionic gonadotropin
HCH	γ-hexachlorocyclohexane
HGF	hepatocyte growth factor
Hox	homeobox
HRP	horseradish peroxidase
HSP90	heat shock protein of 90 kDa
IF	immunofluoresence
IGF	insulin-like growth factor
IGF-1	insulin-like growth factor 1
IGF-1R	insulin-like growth factor 1 receptor
IGF-2	insulin-like growth factor 2
IGFBP	insulin-like growth factor binding protein
IL-1β	interleukin-1 beta
INSRR	insulin-receptor related receptor
IS	initial segment epididymidis
K^+	potassium
kDa	kilodaltons
KDR	kinase insert domain-containing receptor
LH	luteinizing hormone
LHRH	lutenizing hormone releasing hormone
MAGUK	membrane-associated guanylate kinase
MAPK	mitogen-activated protein kinase
MD	mesonephric duct
MEK	MAPK kinase/ERK activating kinase
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
NAD^+	nicotinamide adenine dinucleotide, oxidized form
Nedd4	E3 ubiquitin ligase
NGF	nerve growth factor
NOV/CCN3	nephroblastoma overexpressed gene/connective-tissue growth factor

NRTK	non-receptor tyrosine kinase
Orch + T	orchidectomy with testosterone maintenance
Orch	orchidectomy
OT	oxytocin
OTR	oxytocin receptor
P0	unphosphorylated
P1	phosphorylated on one residue
P2	phosphorylated on two residues
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
PKA RIIβ	PKA regulatory subunit beta
PKA	cAMP-dependent protein kinase A
PKAcat	PKA catalytic subunit
PKC	protein kinase C
PKI	PKA inhibitor
PTB	phosphotyrosine-binding
PTHrP	parathyroid hormone-related protein
PTU	n-2-propylthiouracil
p-Tyr-416	active form of cellular Rous sarcoma virus phosphorylated on tyrosine 416
p-Tyr-527	inactive form of cellular Rous sarcoma virus phosphorylated on tyrosine 527
RAS	renin-angiotensin system
ROS	reactive oxygen species
RTK	receptor tyrosine kinase
RT-PCR	reverse transcription polymerase chain reaction, real-time polymerase chain reaction
SEM	standard error of the mean
SH2	Src homology domain 2
SH3	Src homology domain 3
Sos	son of sevenless
Src	Rous sarcoma oncogene
StAR	steroidogenic acute regulatory protein
T	testosterone
TER	transepithelial electrical resistance
TGFβ	transforming growth factor beta
TJ	tight junction
TJP1	tight junction protein 1
TNFR-1	tumor necrosis factor receptor 1
TPA	12-*O*-tetradecanoylphorbol-13-acetate
TrkA	tyrosine kinase A
V_{1A}	arginine vasopressin receptor
VEGF	vascular endothelial growth factor
v-Src	viral Rous sarcoma oncogene

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INTRODUCTION

Male infertility is caused by several factors, including diseases (Seshagiri, 2001), poor diet (Hammoud et al., 2008) medications (Hendrick et al., 2000), environmental and occupational chemical exposures (Sharpe, 2000; Cherry et al., 2008), and genetic and molecular mutations (Seshagiri, 2001). However, the cause of infertility is unknown in over half of male infertility cases (de Kretser, 1997). Spermatozoa cannot fertilize an ovum until they have passed through the epididymis. The epididymis is a highly coiled tubule that is subdivided into the initial segment, caput, corpus and cauda, according to histological and physiological criteria (Robaire and Hermo, 1988). Each epididymal segment creates a highly specific luminal microenvironment to permit sperm acquisition of motility. The different cell types of the epididymal epithelium contribute to creating the luminal milieu, but exactly how these cells regulate the microenvironment, is unknown. Though, it is thought that gap junction intercellular communication (GJIC) plays role in coordinating the epithelium.

Gap junctions (GJ) form a pore-like structure at appositional plasma membranes to permit the passage of small molecules, including secondary messengers (Goodenough et al., 1996). Several studies indicate that abnormal pathology causes, or is caused by, aberrant localization of gap junction proteins, resulting in abnormal intercellular communication (Mesnil et al., 2005). The precise mechanism(s) that govern GJ trafficking are unknown, but studies suggest that kinases and GJ binding proteins are involved in this process.

Little information exists on the regulation of GJs in the epididymis. However, prior studies from this laboratory have shown that targeting of GJA1 is reliant on testicular androgens in the initial segment (Cyr et al., 1996). The same study reported an increase in total epididymal GJA1 protein expression and hyperphosphorylation in the absence of androgens. Segment-specific effects, however, were not determined.

In the current study, it is hypothesized that the altered trafficking of GJA1 in the initial segment is due to either: a) a segment-specific increase in GJA1 protein synthesis or phosphorylation b) androgen-dependent regulation of a GJA1 binding protein and/or c) changes in the association between GJA1 and its binding partner(s) with androgen

withdrawal. Chapter 1 of this thesis explores the current literature on epididymal and gap junctional regulation, and then focuses on the GJ protein, GJA1. Chapter 2 of this thesis is comprised of the article entitled: *Regulation of Gap Junction Alpha 1 Binding Proteins in the Adult Rat Epididymis and Their Influence on Androgen-Dependent Gap Junction Alpha 1 Localization.* This study addresses three possible mechanisms of GJA1 regulation to better understand GJ regulation in the epididymis, and investigate the mechanisms of GJA1 intracellular trafficking using a unique *in vivo* model.

GJA1 binds numerous proteins via its C-terminal domain to regulate intracellular trafficking and GJIC (Laird, 2006). The kinase Rous sarcoma virus, c-Src, disrupts GJIC by directly phosphorylating GJA1 or by activating other kinases that phosphorylate GJA1, resulting in GJ internalization. Conversely, cAMP-dependent protein kinase A catalytic subunit, PKAcat, enhances GJIC, promoting GJA1 accumulation at gap junction plaques (reviewed in Lampe and Lau, 2004). Very few signal transduction components have been identified in the epididymis and even fewer studies have identified functional signalling pathways. As such, there are no reports on the presence or regulation of c-Src or PKAcat in the epididymis, but studies suggest that they are regulated by androgens in male reproductive organs (Sadar et al., 1999; Wong et al., 2005). Therefore, the first objective of this study is to characterize c-Src and PKAcat in the rat epididymis to gain insight into epididymal signal transduction. Secondly, the study aims to determine if c-Src or PKAcat expression or cellular localization are regulated by androgens in the rat epididymis.

The scaffolding protein, tight junction protein 1 (TJP1), directly binds to GJA1 (Giepmans and Moolenaar, 1998). TJP1 may be involved in GJA1 trafficking by: i) regulating GJA1 targeting to the plasma membrane (Laing et al., 2005) or ii) influencing endocytosis of GJA1-containing GJs (Segretain et al., 2004). TJP1 is present in the epididymis and is influenced by androgens during postnatal development and aging (Levy and Robaire, 1999; DeBellefeuille et al., 2003), but its regulation in the adult epididymis is unknown. Thus, as part of the second objective of this study, the androgen-dependency of TJP1 is investigated.

Finally, to determine if GJA1 potential regulators could play a role in the androgen-dependent, segment-specific targeting of GJA1 in the rat epididymis, a direct association between GJA1 and c-Src, PKAcat and TJP1 is studied.

The results and implications from the study in Chapter 2 are discussed in Chapter 3 of this thesis. Finally, a 22-page French summary of the thesis is presented in Chapter 4.

CHAPTER 1: LITERATURE REVIEW

1.0 The Epididymis

1.1 Gross Anatomy

Spermatozoa travel from the seminiferous tubules of the testis through the rete testis to the excurrent duct system, which is comprised of the efferent ducts, epididymis and vas deferens. It is not until spermatozoa have been exposed to the epididymis that they acquire progressive motility and ability to fertilize the ovum (Orgebin-Crist, 1969). The epididymis creates a fertile ejaculate by transporting, concentrating, maturing and storing spermatozoa produced by the testes (Robaire and Hermo, 1988). Spermatozoal transport through the epididymis is accomplished by hydrostatic pressure created by testicular fluid, and by peristaltic contractions that force the distal movement of spermatozoa along the length of the epididymis (Robaire and Hermo, 1988).

The epididymis is a long, highly coiled tubule with an uncoiled length approximating 3 metres in the rat (Turner et al., 1990) and 6-7 metres in the human (Turner, 1995). In rodent models, the epididymis is subdivided into four main segments according to divisions by connective tissue septa: the initial segment, caput, corpus and cauda (Figure 1). These segments can be subdivided into 19 smaller subdivisions in the rat (Tomsig et al., 2006). Each segment creates a specific luminal microenvironment with which sperm dynamically interact to become mature (Cornwall et al., 1990). Once spermatozoa have achieved fertility, they are stored in the cauda. The cauda straightens at its most distal end to form the vas deferens. The vas deferens in turn connects to the urethra tube that culminates at the external genitalia. The ductal system is commonly divided into epithelial and luminal compartments.

A layer of smooth muscle cells, termed the myoid layer, surrounds the epithelium. Muscle contractions move spermatozoa along the length of the tubule to the cauda. The ductal system is embedded in stromal tissue containing the vascular endothelium. This provides a constant blood flow to the epididymis and permits the delivery of endocrine substances.

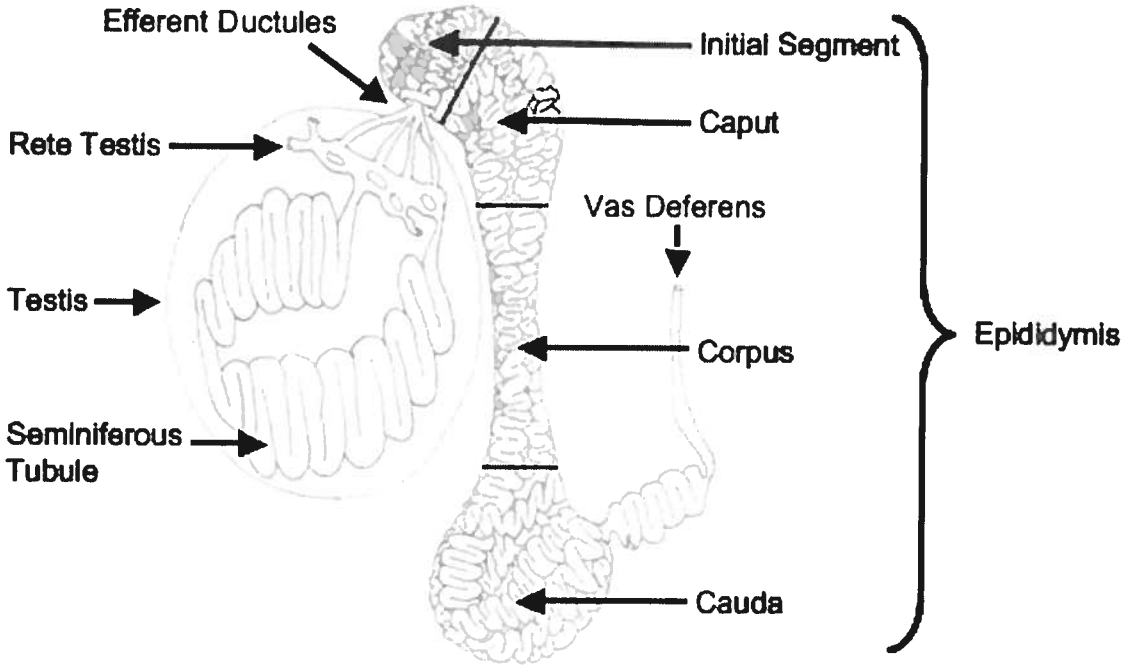

Figure 1. Schematic representation of the rat testis, efferent ductules and epididymis. Note the four segments of the epididymis: initial segment, caput, corpus and cauda. Inspired by Robaire and Hermo 1988.

1.2 Histology

1.2.1 Development and Differentiation

The caput, corpus and cauda epididymidis originate from the mesonephric (Wolffian) ductal system and share the same embryological origin as the kidney (Robaire et al., 2006). The initial segment on the other hand, may originate from the mesonephric tubules, but this is not yet apparent (Robaire et al., 2006). The mesonephric ducts are derived from the mesoderm at embryonic day 9 in the mouse (Hannema and Hughes, 2007). Mesonephric ductal formation has been investigated using knockout mice models. Important roles have been demonstrated for genes including: *Pax2, Pax8, Lim1, Emx2* and the retinoic acid receptors α, γ and their subtypes (reviewed in Hannema and Hughes, 2007).

Growth and coiling of the mesonephric ducts begins at embryonic day 15.5 to embryonic day 16.5 in the rat and is regulated by rising testosterone levels originating in the Leydig cells of the testis (Rodríguez et al., 2002; Robaire et al., 2006; Hannema and

Hughes, 2007). Testosterone exerts its effect by binding to the androgen receptor (AR) that is first expressed in mesenchymal cells and then in epithelial cells (Bentvelsen et al., 1995). Under the influence of androgens, the upper and middle portions of the mesonephric ducts differentiate into the epididymis, while the lower mesonephric ducts becomes the seminal vesicles (Higgins et al., 1989). The mesenchyme determines the organogenesis of the epithelium, as demonstrated by transplantation studies of mesenchymal and epithelial recombinants (reviewed in Cunha et al., 1980). These studies showed that seminal vesicle mesenchymal cells transplanted into the upper and middle mesonephric duct (i.e. the progenitor for the epididymis) develop into seminal vesicles and not the epididymis (Higgins et al., 1989). It has been suggested that growth factors govern mesenchymal-epithelial interactions and are involved in epithelial differentiation (Basciani et al., 2004; Hannema and Hughes, 2007).

The exact mechanism by which the mesonephric ducts further differentiates into different reproductive organs is not entirely clear. Although mesenchymal-epithelial signalling via growth factors and hormones likely influence gene expression (Rodríguez et al., 2002), the precise series of events remains unknown. Studies involving homeobox (Hox) genes indicate a role for these transcription factors in creating tissue boundaries to define the epididymis, vas deferens, seminal vesicles and other male reproductive organs (reviewed in Rao and Wilkinson, 2002). For instance, while *Hoxa9, Hoxd9, Hoxa10, Hoxd10* are expressed in the epididymis and vas deferens, *Hoxa11* is specific to the vas deferens and *Hoxa13* and *Hoxd13* are specific to the seminal vesicles (reviewed in Hannema and Hughes, 2007). *Hox* genes may also play a role in postnatal segmentation of the epididymis, as certain *Hox* genes are expressed in the adult, but not the embryonic epididymis (Rao and Wilkinson, 2002; Bomgardner et al., 2003).

After birth, epididymal tubules are coiled in the proximal segments, but coiling of the cauda does not take place until one or two days later (Robaire et al., 2006). During the undifferentiated period from postnatal day 1 to day 15 there is increased epididymal growth and the tubule begins to lengthen (Sun and Flickinger, 1979; Robaire et al., 2006). The first cell type to appear is the halo cell, which is observed on day 14 (Sun and Flickinger, 1979); the precursor to halo cells is not known, but it may be immunogenic (Sun and Flickinger, 1979; Serre and Robaire, 1999).

The period of differentiation continues from day 16 to day 44 (Sun and Flickinger, 1979). During this time, the remaining five cell types that compose the adult epithelial compartment emerge. Epididymal stem cells first differentiate into columnar and narrow cells at approximately postnatal day 16 in the rat. The columnar cell further differentiates into principal and basal cells at postnatal day 28, whereas narrow cells start to differentiate into clear cells at day 36, except in the initial segment (Robaire et al., 2006). Clear cells are not fully differentiated until day 49, hence completing epididymal differentiation (Hermo et al., 1992). The origin of apical cells in the initial segment is unknown.

Androgens, and likely other testicular factors such as hormones and growth factors, control differentiation where the end of differentiation coincides with the peak in androgen levels in the rat. The period of expansion occurs from day 45 to adult when spermatozoa appear in the epididymal lumen and the length and weight of the epididymis increases (Sun and Flickinger, 1979; Rodríguez et al., 2002).

1.2.2 Cell Types of the Epididymal Epithelium

The epididymal epithelial height decreases from the proximal to distal segments and the tubule diameter increases. The proportion of each of the six cell types within the epithelium differs depending on the segment.

Principal cells compose the largest percentage of the epithelium and have direct contact with both the luminal compartment and the basement membrane. The initial segment and caput have the highest proportion of principal cells that compose about 80% of the epithelium, while the cauda the lowest proportion at less than 69% (Robaire and Hermo, 1988). The change in height of the epithelium is in large part due to the differences in principal cell height. The role of principal cells in creating and maintaining the luminal microenvironment has been studied at the level of tight junctions. Tight junctions form between adjacent principal cells to create the blood-epididymal barrier to protect developing spermatozoa from an immune response (Cyr et al., 2007). Exactly how principal cells contribute to the luminal environment is an active area of research. Evidence suggests that the interaction between principal cell secretions and spermatozoal

remodeling is a highly regulated process controlled by a combination of hormones and testicular factors (Robaire et al., 2006).

Basal cells are also found in each segment of the epididymis with an increase in distribution along the length of the proximal epididymis with similar proportions between the corpus and cauda (Robaire and Hermo, 1988). These cells have a cytoplasmic extension enveloped between adjacent principal cells that, until recently, did not appear to contact the luminal compartment (Shum et al., 2008). This extension may contribute to the removal of degenerated sperm components in a manner similar to macrophages, or mediate intraluminal pH (Seiler et al., 2000; Shum et al., 2008). Androgens are thought to have little influence on basal cell function; however during castration, basal cells take on a dome-shaped appearance and become more compact due to tubule distention (Hermo and Papp, 1996; Seiler et al., 2000). Peptide hormones and prostaglandins have been reported to regulate basal cell function, but these studies remain controversial (Leung et al., 2004; Shum et al., 2008).

Narrow cells are present in the initial segment and intermediate zone between the distal initial segment and proximal caput and are most abundant in the intermediate zone (Adamali and Hermo, 1996). This cell's name is derived from the narrowing of its cytoplasm towards the basement membrane; the nucleus is elongated and located in the upper half of the cell. There are three proposed functions for narrow cells: regulating the luminal pH, degrading luminal proteins and protecting spermatozoa. Narrow cells may regulate the luminal pH via carbonic anhydrase XII, carbonic anhydrase II and the vacuolar proton ATPase pump (H^+V-ATPase), the latter two of which are located in small apical vesicles (Adamali and Hermo, 1996; Hermo et al., 2000; Hermo et al., 2005). There are numerous apical vesicles in narrow cells. These vesicles may form components of the endocytic pathway to remove and degrade luminal proteins (Robaire and Hermo, 1988; Adamali and Hermo, 1996; Hermo et al., 2000). The antioxidant enzyme's subunits, glutathione S-transferase (GST) Yb_1 and Yo, have been localized to narrow cells. These subunits likely play a role in conjugating electrophilic substances originating from the circulation to protect spermatozoa from harmful reactive oxygen species (ROS) (Adamali and Hermo, 1996; Andonian and Hermo, 2003).

Apical cells are goblet-shaped and make up 11% of the proximal initial segment and 1.3% of the intermediate zone (Adamali and Hermo, 1996). They do not contact the basement membrane and have a few apical vesicles and endosomes (Adamali and Hermo, 1996). While their precise role is unknown, like narrow cells, they express the Yb_1, Yf and Yo GST subunits. Thus, apical cells may also contribute to protecting spermatozoa from ROS and to the breakdown of endocytosed substances (Adamali and Hermo, 1996; Andonian and Hermo, 2003).

Clear cells are found in all segments excluding the initial segment, and are most abundant in the distal cauda (Robaire and Hermo, 1988). Their primary role is thought to be endocytic in nature, as tracers injected into the epididymal lumen are rapidly seen in endocytic, cellular organelles (Hermo et al., 1988). Clear cells also absorb the contents of the cytoplasmic droplet from spermatozoa, thus facilitating the maturational process (Robaire and Hermo, 1988). Similar to narrow cells, clear cells have been implicated in luminal acidification via the H^+V-ATPase; however, soluble adenylate cyclase plays an additional role in this cell type (Hermo et al., 2000; Pastor-Soler et al., 2003).

Halo cells are found throughout the epididymal epithelium and range from 3.8% of the epithelium in the corpus and cauda to 5% and 6.5% in the initial segment and caput, respectively (Robaire and Hermo, 1988). Halo cells have a dense nucleus with a clear cytoplasm and they are located closer to the basal part of the epithelium (Robaire et al., 2006). These cells are comprised of T lymphocytes, cytotoxic T lymphocytes and monocytes in young, adult animals (Flickinger et al., 1997; Serre and Robaire, 1999). In aging animals, eosinophils and B lymphocytes are also present (Serre and Robaire, 1998; Robaire et al., 2006).

1.3 Hormonal Regulation

1.3.1 Role of Androgens

Studies by Benoit in the 1920s demonstrated that androgens were necessary to maintain the structure and function of the epididymis (Benoit, 1926). Indeed, androgen levels in the epididymis are 10 times higher than in blood serum. Several models have been used to elucidate the role of androgens. These models include: bilateral

orchidectomy (removal of the testes), orchidectomy with testosterone maintenance, efferent duct ligation (removes luminal androgens), anti-androgen treatment, and embryonic and postnatal developmental studies. These models have demonstrated diverse roles for androgens in epididymal function.

Androgen deprivation alters epididymal size and histology. Orchidectomy results in a dramatic decrease in epididymal weight to 25% of control animals during a two-week period. There is a lower intraluminal pressure due to the absence of rete testis fluid and spermatozoa atrophy. As a result of decreased intraluminal pressure, there is a decreased tubule diameter and epithelial height, and an increased intertubular space. Other morphological changes include a thickened lamina densa of the basement membrane, reduced smooth endoplasmic reticulum content, varied mitochondria and altered principal cell morphology (reviewed in Ezer and Robaire, 2002). Many proteins and enzymes have been reported to be androgen-dependent and are described in other sections. Androgens have also been shown to regulate several cell processes such as ion and organic molecule transport and metabolic function (reviewed in Ezer and Robaire, 2002).

Androgen withdrawal also results in a wave of apoptosis that begins in the initial segment 18 hours after orchidectomy and continues to the caput, corpus and cauda at increasing time intervals. By seven days after orchidectomy, apoptotic cells are largely undetected (Fan and Robaire, 1998). Testosterone replacement reverses this wave, except in the initial segment, which also relies on luminal components (reviewed in Ezer and Robaire, 2002). Despite the ability of androgens to prevent cell death in certain epididymal segments, their influence on DNA synthesis is low in the caput and cauda of orchidectomized animals given androgen treatment for three days. This is in contrast to other androgen-dependent tissues such as the prostate and seminal vesicles (reviewed in Ezer and Robaire, 2002).

Androgens regulate the epididymis via two routes. One is by luminal androgens transported through the excurrent ducts, and the other is by androgens actively transported across the basal epithelium from the peritubular fluid (Turner and Yamamoto, 1991). Androgens originate in the testis, adrenal glands and peripheral tissues such as the skin, prostate and epididymis. Leydig cells located in the intertubular space of the testis synthesize androstenedione, testosterone and small quantities of 5α-reduced hormones,

such as dihydrotestosterone (DHT), from their cholesterol precursor via a series of enzymatic conversions (Figure 2). Cholesterol is derived from the blood plasma pool of low-density lipoprotein, or synthesized *de novo* from acetate and cholesterol esters. The steroidogenic acute regulatory protein (StAR) mediates the rate-limiting step of testosterone formation, which is the transfer of cholesterol from the outer to the inner mitochondrial membrane. Lutenizing hormone augments both acute and steady state testosterone synthesis via StAR and other enzymes involved in steroid synthesis (reviewed in Griffin and Wilson, 2003).

Testosterone and small amounts of other androgens are released from Leydig cells into the systemic circulation via endothelial vessels and distributed to target organs and tissues, including the epididymis. Interstitial testicular androgens diffuse into Sertoli cells that comprise the seminiferous epithelium, but in a restricted manner (Turner, 1991; Turner and Yamamoto, 1991). Seminiferous tubule intraluminal androgens are transported through the excurrent ductal system bound to androgen binding protein (ABP) (Turner, 1991). ABP is secreted from Sertoli cells in a follicle-stimulating hormone (FSH) and testosterone-dependent manner (Turner, 1991). The androgen-ABP complex travels to the epididymis and is internalized by the principal cells in the initial segment and caput via a receptor-mediated mechanism. Once inside the cell, testosterone is released from ABP and converted by 5α-reductase to DHT. This conversion provides the epididymis with its major source of DHT, a more active androgen with a higher affinity for the androgen receptor (reviewed in Ezer and Robaire, 2002).

There are two forms of the 5α-reductase enzyme that arise from two separate gene products, type 1 and type 2. The enzymatic activity and mRNA expression of 5α-reductase type 1 is highest in the initial segment, thereby leading to increased levels of DHT compared to the testis (Ezer and Robaire, 2002). A lumicrine factor from the testis regulates 5α-reductase type 1 in the initial segment, whereas it is controlled by circulating androgens in the other segments (Viger and Robaire, 1991; Robaire and Viger, 1995). The mRNA expression of 5α-reductase type 2 on the other hand, is highest in the caput, but its enzymatic activity is not likely as efficient as type 1 (reviewed in Ezer and Robaire, 2002).

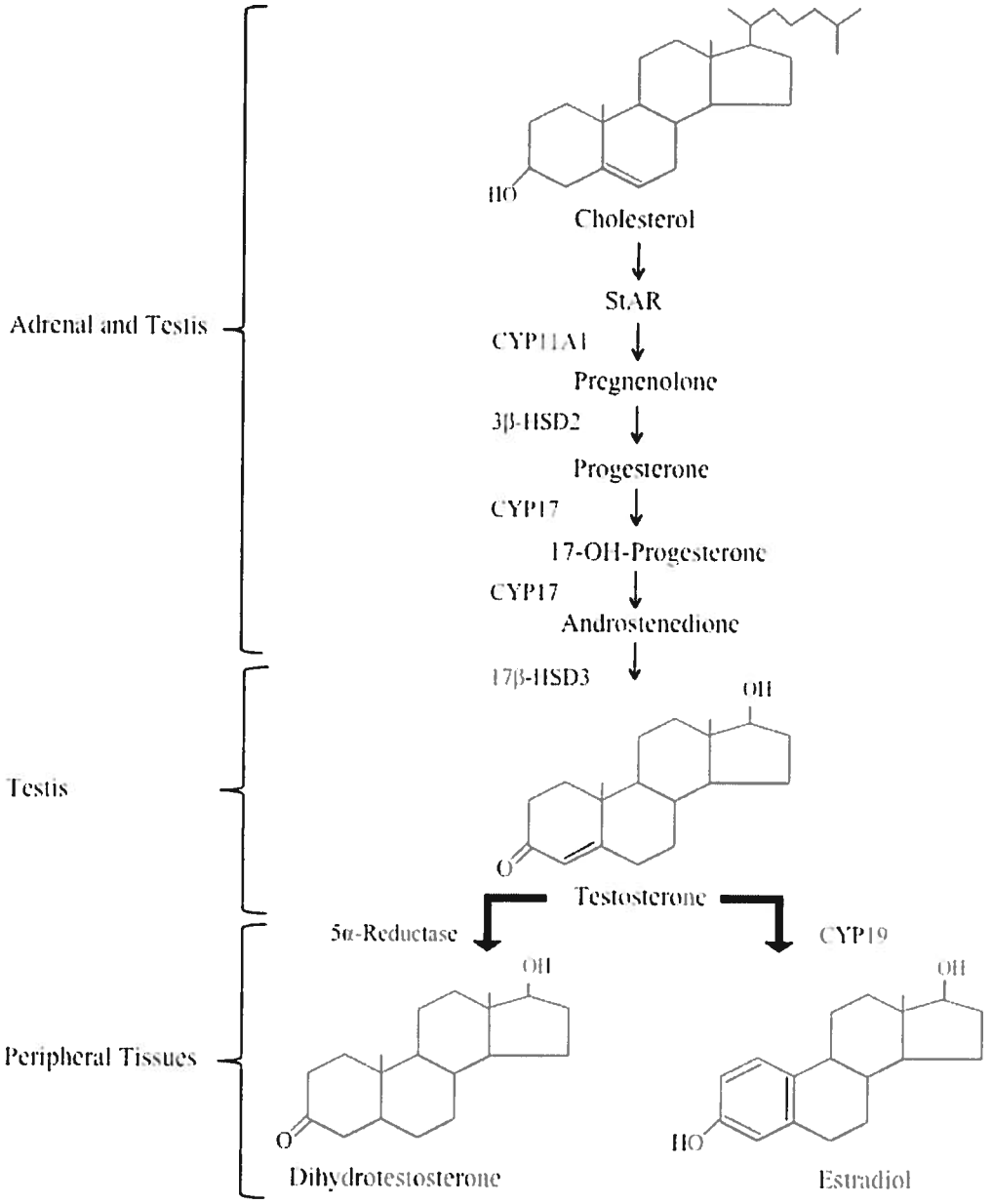

Figure 2. General pathway of testosterone synthesis and its conversion to dihydrotestosterone (DHT) and to estradiol in peripheral tissues. Cholesterol is derived from plasma or synthesized *de novo* and transported to the inner mitochondrial membrane via steroidogenic acute regulatory protein (StAR). The enzymatic reactions are carried out by cytochrome P450 (CYP) isozymes and by β-hydroxysteroid dehydrogenases (HSD) to synthesize testosterone. 5α-Reductase and CYP19 form 5α-reduced metabolites such as DHT and estradiols, respectively, predominantly in peripheral tissues. Adapted from Griffin and Wilson 2003.

Once in the cytosol androgens, mostly in the form of 5α-reduced metabolites of testosterone, bind to the androgen receptor (AR). Agonist binding results in AR dimerization and its translocation to the nucleus. In the nucleus, the androgen receptor acts as a cis-regulatory transcription factor through a DNA binding domain, resulting in gene transcription (Rodriguez et al., 2001). The androgen receptor is distinctly localized to the nuclei of epithelial cells, and is not found at the plasma membrane (Goyal et al., 1998; Zhu et al., 2000). Thus, unlike the testis and prostate, the AR cannot act via a non-genomic mechanism in the epididymis (Kampa et al., 2002; Cheng et al., 2007).

Studies suggest that androgens alone do not regulate androgen receptor expression in the rat. Androgen receptor immunolocalization is unchanged in the rat epididymis after two weeks of orchidectomy (Paris et al., 1994). Additionally, rats treated with a lutenizing hormone releasing hormone (LHRH) antagonist have decreased AR expression in the epithelial compartment of the caput and cauda by 30 and 45%, respectively (Zhu et al., 2000). In the goat epididymis on the other hand, circulating androgens are sufficient to regulate the androgen receptor indicating species variations (Goyal et al., 1998).

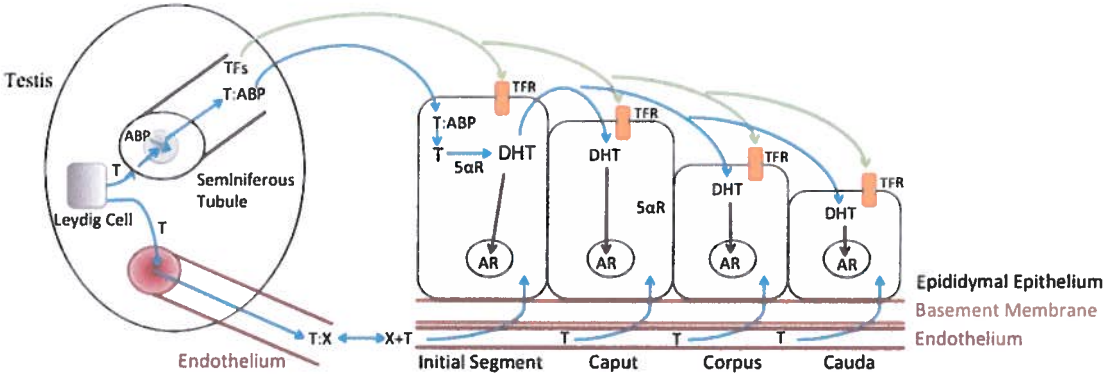

Figure 3. Regulation of the epididymal epithelium by circulating androgens, luminal androgens and testicular factors. Androgens, mainly testosterone (T), produced by Leydig cells enter the seminiferous tubules of the testis and bind to androgen binding protein (ABP) in the lumen. Testosterone bound to ABP (T:ABP) is transported through the excurrent ducts to the epididymis where T:ABP is internalized by principal cells in the initial segment and caput. T is converted by 5α-reductase (5αR) to more active metabolites, such as dihydrotestosterone (DHT). DHT then regulates distal epididymal segments. T from Leydig cells also enters the systemic circulation and is distributed to the epididymis and other target organs. Testicular factors (TFs), including hormones and growth factors, are transported through the excurrent ducts to the epididymal lumen and bind to their cognate receptor on the apical plasma membrane [testicular factor receptor (TFR)]. The epididymis itself is a source of growth factors and may mediate paracrine and lumicrine signalling (not shown). X: blood carrier protein. T:X: Testosterone bound to blood carrier protein. AR: androgen receptor. Adapted from Robaire and Viger, 1995.

1.3.2 Role of Estradiol

In the male, estradiol is produced in peripheral tissues, but also at several sites in the reproductive tract including Sertoli cells, germ cells and minor quantities in Leydig cells. Cytochrome P450 19 (CYP19) catalyzes the conversion of testosterone to estradiol. In spermatozoa, CYP19 is expressed in the cytoplasmic droplet located on the flagellum and is the most abundant source of estradiol in the male reproductive tract (Hess et al., 2002). The epididymal epithelium does not express CYP19 and therefore, cannot be a source of estradiol (Schleicher et al., 1989).

Estradiol exerts its effects by binding to the estrogen receptor (ER) that has two different subtypes in mammals, ERα and ERβ (Menuet et al., 2001). The distribution of ERα throughout the male reproductive tract has been somewhat controversial due to differences in reported immunolocalization between and within species. With respect to the rat epididymis, ERα has been reported to be either absent, expressed only in the initial segment or to be strongly expressed in the initial segment, but weakly expressed in the caput, corpus and cauda (reviewed in Hess et al., 2002). Despite inconsistencies in ERα localization in the epididymis, the presence of ERα in efferent duct tubules is rather constant. ERα's role in efferent duct tubules has been studied extensively using ERα knockout mice (αERKO) and anti-estrogens (Hess, 2003).

ERα promotes fluid reabsorption in the efferent ducts to concentrate spermatozoa transported from the rete testis. The epididymides of the αERKO mice have subtle abnormalities compared to wildtype controls (Hess et al., 2000). Apical, narrow and clear cells have morphological changes and Hess et al. (2002) has pointed out that the function of these cell types parallels that of the efferent ductules in ion transport and endocytosis. αERKO mice are infertile, while ERβ knockout mice appear to be normal (Hess et al., 2002).

ERβ is ubiquitously distributed in the epididymis. ERβ shows strong immunoreactivity in the efferent ductules, a weaker reaction in the initial segment and a stronger reaction in the distal regions in all the epithelial cell types except halo cells, and in the stromal compartment (Saunders et al., 2001; Zhou et al., 2002; Hess, 2003). Thus,

there is a possibility for functional binding of estrogen to at least one of its receptor subtypes throughout the epididymis (Hess et al., 2002).

To further elucidate the role of estrogens in the epididymis studies have examined the effects of estrogen treatment with or without orchidectomy, and used anti-estrogens on the adult epididymis. These effects are varied and include decreased epididymal weight, decreased fertility, decreased spermatozoal transit time and altered ion concentrations (reviewed in Hess et al., 2002). Unfortunately, these studies do not determine if estrogen effects are direct or indirect with respect to the epididymis and could be related to interference with gonadotropin release (Hess et al., 2002). Moreover, the regressive effects of orchidectomy on the epididymis are reversed with testosterone maintenance in the caput, corpus and cauda, suggesting that estradiol from spermatozoa plays a more minor role in these segments (Ezer and Robaire, 2002; Hess et al., 2002). In the initial segment, however, testosterone maintenance does not completely reverse regressive changes. It is therefore possible that, along with other testicular factors, an estradiol-mediated paracrine interaction between spermatozoa and the initial segment is more prominent.

1.3.3 Role of Retinoids

Retinoids are a pleiotropic class of compounds related to vitamin A. Many of the enzymes and carrier proteins required for retinoid biosynthesis, transport and signalling have been identified in the epididymis, though the details of this complex system will not be described here (for a review see Orgebin-Crist et al., 2002). In general, vitamin A deficiency arrests spermatogenesis in the testis, and the epididymal epithelium phenotype is squamous metaplasia (Robaire et al., 2006). The retinoic acid receptor α knockout genotype produces the most severe epididymal phenotype, indicating an important role for this receptor in epididymal function (Costa et al., 1997). Several studies suggest that testosterone and testicular factors mediate retinoid signaling via retinoid carrier molecules, lipocalins (Zwain et al., 1992; Lareyre et al., 2001; Robaire et al., 2006). Thus, there may exist complex overlap between androgen and retinoid regulation in the epididymis.

1.3.4 Oxytocin and Peptide Hormones

Several hormones mediate spermatozoal transport through the epididymis including: oxytocin (OT), endothelin-1 (ET-1), arginine vasopressin (AVP) and estradiol. OT and ET-1 receptors are located in the human and rabbit epididymal epithelium and smooth muscle layers (Vignozzi et al., 2008). OT is produced locally in the epididymal epithelium and in the hypothalamus, while ET-1 is synthesized by the epithelium (Vignozzi et al., 2008). OT and ET-1 synergistically cause smooth muscle contractions leading to semen expulsion (reviewed in Thackare et al., 2006; Vignozzi et al., 2008). While the exact role for estrogens in regulating contractions is unclear, OT receptor (OTR) mRNA and protein expression are upregulated in the rabbit epididymis by estrogens, but not testosterone (Filippi et al., 2002). In the rat, OTRs have not been reported. While some studies suggest OT does mediate rat epididymal contractions (Studdard et al., 2002), others suggest that AVP is more important (Jaakkola and Talo, 1981). It may be that OT acts via the AVP receptor, V_{1A} (Gupta et al., 2008). OT also plays a role in the production of DHT by stimulating 5α-reductase in the initial segment, although the mechanism is unknown (Thackare et al., 2006).

Peptide hormones angiotensin II (ANGII), AVP, bradykinin and ET-1 have been reported to promote anion secretions across the epididymal epithelium by stimulating prostaglandin formation (Lai et al., 1994; Wong et al., 1999; Cheuk et al., 2002; Leung et al., 2004). In this manner, these peptide hormones may contribute to regulating the luminal microenvironment.

1.4 Testicular Factors

Sertoli cells secrete signalling molecules into the lumen of the seminiferous epithelium that are transported by the efferent ducts to the epididymis. In this way, the testis regulates the epididymis via the luminal compartment, giving rise to the term lumicrine signalling. The luminal fluid contains water, ions, small organic molecules, proteins, glycoproteins and spermatozoa, but its precise makeup is unknown (Robaire and Hermo, 1988). Hence, the term testicular factor is used to indicate a component originating from the testicular compartment and not the circulation. Testicular factors

include: binding proteins, hormones, growth factors, mitogens and spermatozoa. Androgens, androgen binding protein and estrogens have been discussed in previous sections.

Growth factors promote cellular proliferation and differentiation by binding to and activating receptor tyrosine kinases, or serine/threonine kinases, activating signal transduction pathways. The testes are a source of growth factors and if the epididymis expresses the growth factor receptor, the testes can influence the epididymis via a lumicrine pathway (reviewed in Tomsig and Turner, 2006). It is important to note that the epididymis is itself a source of growth factors, as described below, and may employ paracrine, autocrine or juxtacrine modes of regulation.

Insulin-like growth factor (IGF) is structurally related to proinsulin and its bioavailability is mediated by IGF binding proteins (IGFBP) (Laron, 2001). IGF-1 and IGF-2 regulate cellular proliferation, differentiation and apoptosis (Laron, 2001; Subramanian et al., 2008). IGF-1 has been localized to both the stromal and epithelial compartments in the rat epididymis (Leheup and Grignon, 1993). The IGF-1 receptor (IGF-1R) is expressed in all four epididymal segments, but levels are higher in the corpus and cauda (Henderson et al., 2006; Robaire et al., 2007). IGF is important in testicular and epididymal development, steroidogenesis and fertility, as demonstrated in *Igf-1* knockout mice (Baker et al., 1996; Robaire et al., 2007). Many androgen-regulated genes in a mouse epididymal cell line have been linked to IGF-1 (reviewed in Robaire et al., 2007), but its precise secondary messengers are unknown in the epididymis.

Epidermal growth factor (EGF)/ErbB receptor (EGFR) functions' include proliferation, migration, differentiation and survival (Ohtsu et al., 2006). In the mouse epididymis, the *Egf* gene expression profile follows a segment-specific pattern and increases from the proximal to distal segments; however, *Egfr* gene expression is constant throughout the epididymis (Tomsig and Turner, 2006). In the rat, *Egf* and *Egfr* expression were demonstrated in the initial segment (Tomsig et al., 2006). EGFR is localized to the basolateral and apical plasma membranes of epididymal principal cells in the monkey and the cytoplasm of principal cells in mice, but EGFR localization is unknown in the rat (reviewed in Tomsig and Turner, 2006; and Robaire et al., 2007).

The fibroblast growth factor (FGF) family has more than 20 members with four different receptor types (FGFR 1-4) to regulate cell growth and motility, differentiation, chemotaxis and apoptosis (Cotton et al., 2008). FGF2, 4 and 8 have been reported in the rat rete testis fluid and *Fgfr-1 IIIcα* and *IIIcβ* mRNA in the principal cells of the initial segment (Kirby et al., 2003). *Fgf1, 2* and *9, Fgfr1-3 IIIb* and *IIIc* mRNA and FGFR1-4 protein are expressed in rat initial segment tissue, but the compartment is unknown (Kirby et al., 2003; Tomsig et al., 2006). *Fgf10* and *Fgfr2* mRNA are present throughout the rat epididymis (Henderson et al., 2006). *Fgf 7* and *12* and *Fgfr1-4* have been demonstrated throughout the mouse epididymis by microarray analysis (Tomsig and Turner, 2006).

The transforming growth factor-β (TGF-β) family is comprised of three members, TGF-β1, TGF-β2 and TGF-β3. This growth factor regulates growth, differentiation, apoptosis and wound healing and is capable of transforming normal cells into tumorous ones (Shi and Massague, 2003; Tomsig and Turner, 2006). TGF-β1 is the most prominent member in the rat and mouse epididymides. It is expressed in all four segments shown by microarray, RT-PCR and immunocytochemistry (Desai et al., 1998; Henderson et al., 2006; Tomsig and Turner, 2006). *Tgf-β3* mRNA is expressed throughout the rat epididymis, but its protein is only detected in the corpus by immunohistochemistry (Desai et al., 1998). *Tgf-β2* mRNA is not expressed under normal conditions, but upon castration, it is upregulated in the caput and corpus (Desai and Kondaiah, 2000). Similarly, castration upregulates *Tgf-β3* mRNA in the caput and cauda in a testosterone-dependent manner (Desai and Kondaiah, 2000). *Tgf-β1* receptor is expressed in all for segments of the rat epididymis shown by RT-PCR (Henderson et al., 2006). In the marmoset monkey, TGF-β receptor type 2 is immunolocalized to principal cells with a gradient in immunoreaction that is strong in the caput and becomes weaker more distally (Bomgardner et al., 1999). The cellular localization of TGF-β receptors remains to be established in the rat epididymis.

Vascular endothelial growth factor (VEGF) promotes angiogenesis, endothelial permeability and is anti-apoptotic (Epstein, 2007; Shibuya, 2008; Vestweber, 2008). VEGF consists of at least seven members (A,B,C,D,E, F and placenta growth factor) and has three receptors: VEGFR1-3, also known as Flt-1, KDR and Flt-4, respectively (Ferrara et al., 2003; Epstein, 2007). *Vegfb* and *c* and *Vegfr 1* and *2* have been

demonstrated in the rat initial segment and *Vegfa* and *Vegfr2* in all four segments of the rat epididymis by RT-PCR (Henderson et al., 2006; Tomsig et al., 2006). Microarray analysis established *Vegf a-c* expression throughout the mouse epididymis (Tomsig and Turner, 2006). *Vegfa* is the most abundant, particularly in the proximal epididymis (Tomsig and Turner, 2006). Immunolocalzation studies in the human epididymis report that FLT-1 is present in lymphatic vessels, but not in the epididymal epithelium, while KDR is present in some basal cells of the corpus (Ergun et al., 1998). Reports of VEGFR localization in the rat have yet to emerge.

The role for nerve growth factor (NGF) has more traditionally been associated with neuron development and differentiation, but is now believed to regulate non-neuronal cells (Li et al., 2005). *Ngf* mRNA is present in mouse and rat epididymides (Ayer-LeLievre et al., 1988). NGF is localized throughout the rat epididymal epithelium and smooth muscle cells (Ayer-LeLievre et al., 1988; Li et al., 2005). The high affinity NGF receptor, TRKA, and the low affinity NGF receptor, p75, are present throughout the epithelium of the rat epididymis (Li et al., 2005).

Platelet derived growth factor (PDGF) has four family members (A, B, C and D) that bind to their receptor PDGFR α or β to elicit responses in connective tissue, and to promote differentiation, wound healing and blood vessel tonus (Heldin and Westermark, 1999). *Pdgfa* and *b* and *Pdgfr* α and β mRNA and protein are expressed in the rat and mouse epididymal epithelium (Basciani et al., 2004). PDGFA and B and PDGFR α and β are localized to principal cells throughout the rat and mouse epididymis, but are absent from the myoid layer and the clear cells of the rat cauda (Basciani et al., 2004). PDGFR α staining disappears in adult rats older than sixty days, whereas PDFGR β staining is prominent in the epithelium (Basciani et al., 2004). This suggests that the β isoform is the receptor responsible for mediating signalling cascades in adult rats.

Hepatocyte growth factor (HGF) may be involved in sperm motility maintenance via interaction with its receptor, c-met, which is localized on spermatozoa (Catizone et al., 2002). Whether or not HGF-c-met binding to spermatozoa influences the epididymal epithelium remains unknown. Previous studies, however, have demonstrated that spermatozoa can release proteins that are reabsorbed by clear cells (for instance Hermo et al., 1988; Turner, 1991; Perry et al., 1994).

The extent to which these growth factors influence epididymal function is for the most part, unknown. Although all segments of the epididymis are regulated by testicular factors, the initial segment is the most reliant on testicular inputs (Robaire et al., 2006 and references within). Yet, despite constant exposure to growth factors, cells in the initial segment do not proliferate. It has therefore been proposed that cells may cycle between "on" and "off" states in terms of their ability to elicit a response to growth factors. In this way, there would be sufficient stimulation to control signalling cascades, but insufficient stimulation for cells to enter the cell cycle (Robaire et al., 2006). The extent to which growth factor and hormone effectors overlap further adds to the complexity of this system. Deciphering testicular factor regulated processes from androgen regulated ones can be studied using an orchidectomy model with testosterone maintenance.

1.5 Orchidectomy as a Model to Study Androgen Regulation

Orchidectomy and orchidectomy with testosterone maintenance is a model used to distinguish testicular factor regulation from androgen regulation by eliminating testicular factor inputs from the epididymal lumen. Several groups have investigated changes in epididymal gene expression with the orchidectomy model by microarray analysis (for instance Ezer and Robaire, 2003; Chauvin and Griswold, 2004), while others have used 5α-reductase inhibitors (Henderson et al., 2004; Henderson et al., 2006). These studies have illustrated the complexity of androgen-regulated processes in the epididymis, but the question remains as to how these transcriptional changes relate to translational ones.

Although many studies have investigated changes in protein expression or localization caused by orchidectomy (for instance Gregory et al., 2001; DeBellefeuille et al., 2003; Primiani et al., 2007), a global picture has yet to emerge, due to technical limitations. Elucidating changes in protein expression and activity, especially those involved in signal transduction, will help decipher androgen-dependent mechanisms in the epididymis.

1.6 Signal Transduction

Secondary messengers allow the cell to translate environmental signals into biological and physiological effects. There are numerous pathways that converge to create a secondary message, but the focus of the following discussion will be on receptor and non-receptor tyrosine kinases and G-protein coupled receptors and their downstream targets in the epididymis.

Receptor tyrosine kinases (RTK), and their serine/threonine counterparts, are a group of single-pass, transmembrane glycoprotein receptors that contain a cytoplasmic, C-terminal, tyrosine kinase domain. As described earlier, growth factor receptors are RTKs that are activated by an agonist binding to the extracellular domain; agonists include, but are not limited to, their cognate growth factor. Ligand binding to the receptor induces receptor dimerization and transactivation of the kinase domain, whereby the kinase domain from one receptor phosphorylates tyrosine residues in the activation loop of the adjacent receptor. This autophosphorylation provides docking sites for various domains such as the Rous sarcoma virus (Src) homology 2 domain (SH2) and the phosphotyrosine-binding (PTB) domain found within cytoplasmic adaptor proteins (for instance Grb2 and Shc) (reviewed in Margolis and Skolnik, 1994; and Shoelson, 1997). The adaptor protein recruits and binds guanine nucleotide exchange factor (GEF) proteins such as son of sevenless (Sos) via a Src homology 3 domain (SH3). The adaptor provides a link between the RTK and the proto-oncogene Ras (reviewed in Margolis and Skolnik, 1994). Ras is membrane-associated, monomeric G-protein that is active when bound by guanine triphospate (GTP) and inactive when bound by guanine diphosphate (GDP). The exchange of RasGDP for RasGTP is regulated by GEFs to activate Ras signalling, whereas GTPase activating proteins (GAPs) inactivate Ras by hydrolyzing RasGTP to RasGDP (reviewed in Lundquist, 2006).

Activation of Ras leads to the MAPK cascade that is comprised of a series of sequential serine/threonine phosphorylations of target proteins. This cascade is illustrated in Figure 4 (Margolis and Skolnik, 1994). MAPKs can phosphorylate numerous membrane, cytoplasmic and nuclear associated proteins to mediate a cellular response. In

the nucleus, these phosphorylations activate transcription factors. The MAPK cascade can also be activated by G-protein coupled receptors (reviewed in Hur and Kim, 2002).

Heterotrimeric G-protein coupled receptors (GPCRs) are receptors with seven transmembrane helices that bind a variety of ligands ranging from nucleotides, Ca^{2+}, eicosanoids and peptide and protein hormones (Siderovski and Willard, 2005). The transmembrane receptor is coupled to a trimeric G-protein consisting of G_α, G_β and G_γ subunits. G_β and G_γ are usually referred to as $G_{\beta\gamma}$ due to their tight association within the cell. A GPCR-stimulating hormone can activate G_α (GTP-bound) allowing it to dissociate from $G_{\beta\gamma}$ and activate downstream effectors such as adenylate cyclase, ion channels, non-receptor tyrosine kinases, phospholipase C and phosphatidylinositol-3-kinase (reviewed in Hur and Kim, 2002). There are four major classes of G_α subunits that contribute to the diversity of GPCR signalling. One class, G_s, binds to and activates adenylate cyclase that

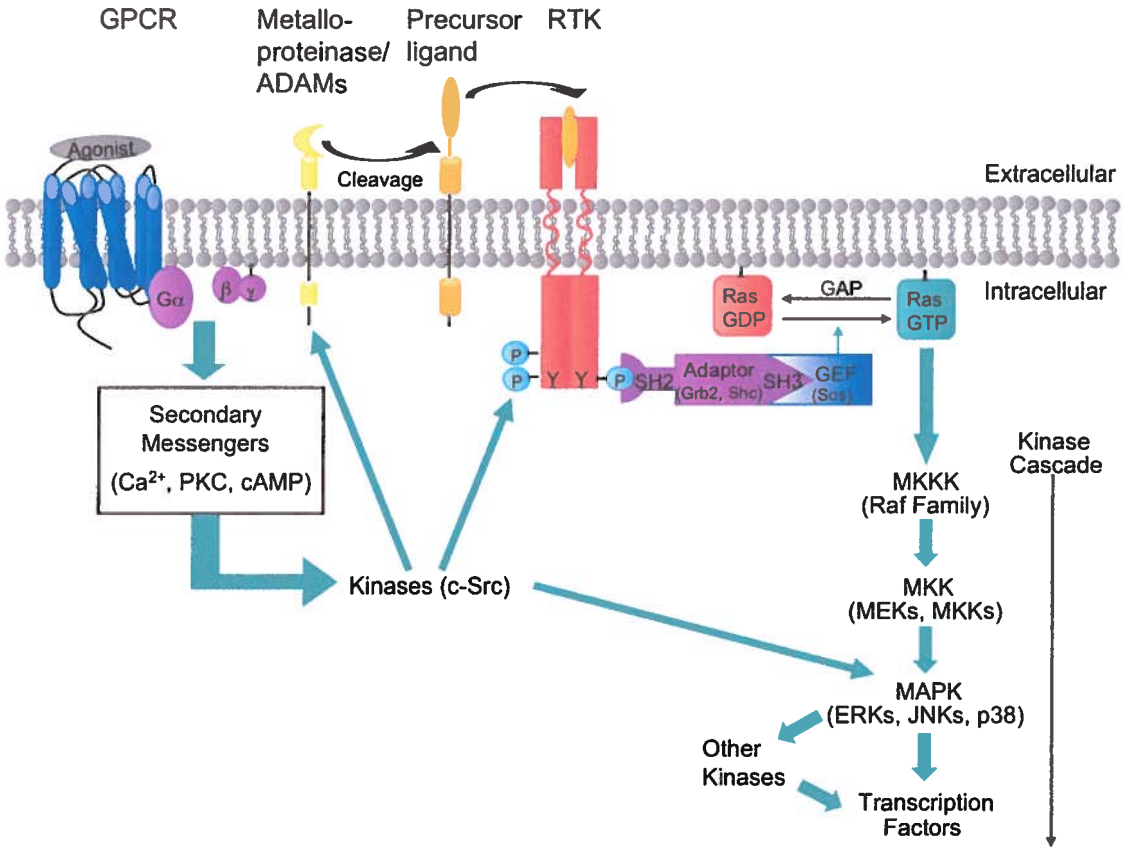

Figure 4. G-protein coupled receptor (GPCR) and receptor tyrosine kinase (RTK) signalling pathways. Agonist binding to the GPCR leads to Gα and Gβγ dissociation and activation of downstream secondary messengers. GPCR signalling effectors can cross-regulate metalloproteinases, disintegrin-metalloproteases (ADAMs) and RTKs. Metalloproteinases and ADAMs proteolytically cleave precursor RTK agonists to the active ligand. RTK ligand binding results in receptor dimerization, autophosphorylation and recruitment of adaptor proteins such as Grb2 and Shc. Adaptors then recruit guanine nucleotide exchange factor proteins (GEF) to activate the Ras G-protein. Active Ras promotes the kinase cascade resulting in sequential phosphorylation of downstream kinases and gene transcription. The kinase cascade is inactivated by GTPase activating proteins (GAP). Inspired by Gschwind et al., 2001; Ohtsu et al., 2006; Egan and Weinberg, 1993.

converts adenosine triphosphate (ATP) to 3',5'-cyclic adenosine monophosphate (cAMP). Cyclic AMP activates cAMP-dependent protein kinase A (PKA), a serine/threonine kinase whose function will be discussed in further detail later. In addition to G_{α} cascades, $G_{\beta\gamma}$ subunits activate similar and diverse signal transduction pathways including components of the phosphoinositide signalling system, such as protein kinase C (PKC) (reviewed in Hur and Kim, 2002; and Siderovski and Willard, 2005). Non-receptor tyrosine kinases (NRTK) contain a kinase domain capable of autophosphorylation, but they do not have extracellular ligand-binding or transmembrane domains. Rather, NRTKs are usually associated with a tyrosine-phosphorylated receptor via its SH2 or SH3 domain and can be cytosolic or membrane-anchored (Sandilands and Frame, 2008). The proto-oncogene cellular Src (c-Src) is comprised of a family of at least nine NRTKs and will be described in detail later. NRTKs can provide a link between RTK and GPCR signal transduction.

Signal transduction pathways are highly complex, as many components of a given pathway converge with other pathways. An example of this is transactivation. Transactivation involves membrane-anchored proteins called disintegrin-metalloproteases (ADAMs), which are activated by secondary messengers generated by GPCR signal transduction. Once activated, ADAMs can proteolytically cleave a precursor ligand to its active form so that it can then bind its cognate RT (reviewed in Gschwind et al., 2001; and Ohtsu et al., 2006). While the exact sequence of events involved in ADAM activation is unclear, c-Src is involved in the process and is an ADAM-interacting protein (reviewed in Seals and Courtneidge, 2003). Matrix metalloproteinases (MMPs) may also be involved in transactivation (reviewed in Ohtsu et al., 2006). While other components are involved in cross signalling between GPCRs and RTKs, the details will not be described here.

Very little is known about secondary messengers in the epididymis. While microarray studies have provided some information regarding the components involved, few studies have demonstrated the presence of a functional signal transduction pathway (Tomsig et al., 2006; Pastor-Soler et al., 2008). Though, several studies have alluded to their presence (Lan et al., 1998; Rodriguez et al., 2001; Cotton et al., 2008).

Other non-growth factor RTKs reported in the epididymis include the insulin-receptor related receptor α (INSRR) and erythropoietin-producing hepatoma-amplified sequence (Eph) receptor A7 (EphA7 also known as Ehk3) (Henderson et al., 2004; Henderson et al., 2006). *Insrr* and *Epha7* were detected in the caput by microarray analysis (Henderson et al., 2004; Henderson et al., 2006). The orphan RTK, c-Ros, is the most studied RTK in the epididymis. C-Ros is necessary for fertility, as demonstrated by knockout mice that lack an initial segment (Yeung et al., 1998). Cooper *et al.* (2003) proposed that the infertility was due to an abnormal luminal fluid, as many transporters were upregulated or downregulated in the knockout versus the wildtype mice. C-Ros has also been reported in the human epididymis (Legare and Sullivan, 2004). *C-Ros* mRNA and protein were reported to be expressed along the length of the human epididymis, excluding the proximal caput, and the protein was localized to basal and principal cells (Legare and Sullivan, 2004).

Adaptor proteins have been identified by microarray analysis in the initial segment of the rat epididymis. Turner and colleagues (2007) studied changes in gene expression in the four subsegments of the rat initial segment following efferent duct ligation. The adaptors *Grb2* and *Shc1* were present in all four segments, whereas *Shc2* and *Shc3* were undetected. In a separate microarray study, the Crk-associated substrate (CAS) adaptor was found to increase in the initial segment of rats treated with the 5α-reductase inhibitor PNU157706 for 28 days (Henderson et al., 2004).

Few reports have demonstrated the presence of GEFs in the epididymis. *Sos 1* and *2* were present on the microarray reported by Turner *et al.* (2007) in the four segments investigated. The GEF exchange proteins EPAC 1 and 2 were immunoprecipitated from rat tissue lysates from the cauda/vas deferens (Pastor-Soler et al., 2008).

GAPs, or small GTPases, from the Ras superfamily have several subfamilies including Ras/Rap/Ral, Rho, Rab, Arf/Sar and Ran (Lundquist, 2006). An *H-ras* knockin study demonstrated that constitutive overexpression of the oncogene *in vivo* caused distension of the epididymis, which also developed thick connective tissue containing numerous inflammatory cells (Gilbert et al., 1997). The males were also infertile, potentially due to an epididymal inflammatory response and failure of spermatozoal maturation (Gilbert et al., 1997). Pan-Ras, K-Ras and N-Ras, but not H-Ras, were

detected in hamster spermatozoa from the caput and cauda by Western blot analysis (NagDas et al., 2002). Various members of Ras/Rap/Ral and Rho familes were present on the microarray from Turner *et al.* (2007). Ral A was reported in the corpus of rats by microarray analysis and Rab12 in the caput from the same study (Henderson et al., 2006). In a microarray study comparing control adult rats to those given a DHT inhibitor, Henderson *et al.* (2004) reported *p120Gap* in the initial segment, *Rab13* in the corpus and *Rab4b* in the cauda, all of which decreased with DHT inhibition relative to controls. Recently, a new GAP designated as MacGap was detected in the caput of the human epididymis by Western blot analysis, but was not observed in the corpus or cauda (Li et al., 2008). By immunocytochemistry, MacGap was localized in the cytoplasm at the base of the epithelium in the distal caput with a weaker signal in the proximal corpus (Li et al., 2008).

Various kinases have been reported in the epididymis, but few have examined them at the protein level. Tomsig *et al.* (2006) demonstrated the presence of signal transduction pathways in segments 1 and 2 of the rat initial segment. In this study, microperfusion of the growth factors EGF, FGF2 and VEGFA activated MAPK1 and MAPK3 detected by Western blot using phosphospecific antibodies (Tomsig et al., 2006). Using microarray analysis, *Mek2* was reported in the initial segment and caput of control, but not orchidectomized rats (Ezer and Robaire, 2003 supplemental data). In the microarray analysis by Henderson *et al.* (2004), *Jak1* tyrosine kinase and phosphatidylinositol 4-kinase decreased in the corpus and cauda, respectively, with DHT inhibition. In a separate study by the same authors, casein kinase II beta subunit was reported to increase with DHT inhibition (Henderson et al., 2006). Members of the Raf family have been reported in the mouse epididymis by Northern blot and *in situ* hybridization. *A-raf* and *C-raf 1* were found throughout the epididymis, with *A-raf* levels being highest in the proximal caput and androgen-dependent (Huleihel et al., 1986; Winer et al., 1993; Winer and Wolgemuth, 1995). B-*raf*, however, was not detected (Winer et al., 1993). A-Raf was reported in the adult mouse epididymis and localized to epithelial cells (Luckett et al., 2000).

Only one study has reported the presence of a non-receptor tyrosine kinase, although members of the c-Src family were detected on the microarray from Turner *et al.*

(2007). Mitaka *et al.* (1997) reported that cell adhesion kinase β (CAK-β), a member of the focal adhesion kinase family, is expressed in the rat epididymis. The authors report *Cak-β* mRNA in the cytoplasm by *in situ* hybridization, whereas the protein was localized to microvilli.

G-protein coupled receptors often bind hormones such as vasopressin, angiotensin II, endothelin-1 and bradykinin described in previous sections (Inagami and Eguchi, 2000). Additional GPCRs reported in the rat epididymis by microarray analysis include tumor necrosis factor receptor 1 (TNFR-1) and vasopressin V2 receptor (Ezer and Robaire, 2003; Henderson et al., 2006). *Tnfr-1* was expressed throughout the epididymis, while vasopressin V2 receptor was only in the initial segment (Ezer and Robaire, 2003; Henderson et al., 2006). The GPCR subunit $G_{\alpha i2}$ that is involved in phospholipase signalling was reported in the corpus (Henderson et al., 2006).

GPCR activation leads to adenylate cyclase stimulation and production of cAMP. The soluble adenylate cyclase mRNA and protein were reported in the rat cauda and immunolocalized to clear cells, the myoid layer and caudal spermatozoa, but not principal cells (Pastor-Soler et al., 2003). The same group used a PKA activator to demonstrate that PKA could induce H^+ V-ATPase apical accumulation in clear cells of the rat cauda, which was prevented by pretreament with the PKA inhibitor, PKI (Pastor-Soler et al., 2008). *Pki* α was also reported in the rat cauda by microarray and was decreased after DHT inhibition (Henderson et al., 2004). The same authors found that the PKA regulatory subunit, RIIβ, was present in the rat caput and was decreased after high doses of a DHT inhibitor (Henderson et al., 2004).

ADAMs and MMPs have been reported in the epididymis, suggesting transactivation of GPCRs and RTKs may occur. *Adam 7* mRNA is present in the caput, corpus and cauda of the mouse epididymis and is androgen and testicular factor-dependent (Cornwall and Hsia, 1997). *Adam 28* was detected by microarray in the mouse initial segment and regulated by a testicular factor (Sipila et al., 2006). Several *Adam* genes were also detected in the four segments of the initial segment microarray by Turner *et al.* (2007). *Mmp-11* was found to increase seven days after orchidectomy in the rat caput, corpus and cauda by microarray, whereas *Mmp-14* was found to increase in the corpus (Ezer and Robaire, 2003). *Mmp-7* mRNA is highly expressed in the mouse

efferent ducts and initial segment and less so in the cauda epididymidis (Wilson et al., 1995).

While many components of the RTK and GPCR signal transduction pathways have been documented at the level of gene expression, few of the studies discussed used functional assays to confirm their results. In order to understand epididymal regulation, it is essential to begin elucidating the signal transduction pathways that control the epithelium. One aspect of epithelial regulation involves highly coordinated intercellular communication via gap junctions.

2.0 Gap Junctions

2.1 Composition of Gap Junctions

Gap junctions (GJ) form an aqueous, transcellular pore between two apposing plasma membranes that allow intercellular communication by the passage of small molecules (<1 kDa), including secondary messengers (Goodenough et al., 1996). Each GJ is comprised of two connexon hemichannels, one contributed from each cell, which are formed by the oligomerization of six transmembrane proteins called gap junction proteins (GJP) (previously known as connexins). To date, 20 GJPs have been identified in the mouse and 21 in the human, of which 19 are orthologous pairs (Sohl and Willecke, 2003). Gap junction proteins are named according to their subgroup: alpha (A), beta (B), gamma (C), delta (D) or epsilon (E) and their order of discovery (1,2,3 etc.) (Sohl and Willecke, 2003). The GJP's subgroup is determined by its degree of sequence homology with other members of the subgroup, and the length of its cytoplasmic domain (Sohl and Willecke, 2003). For instance, GJA1 was the first gap junction protein identified from the alpha subgroup, whereas GJB2 was the second gap junction protein identified from the beta subgroup. GJB1 and GJB2 are more homologous to one another than to GJA1. Species gene and protein nomenclature follow currently accepted guidelines. A second nomenclature exists for gap junction proteins, where the predicted molecular weight in

Table 1. Comparative Protein Nomenclature for Gap Junction Proteins/Connexins

Current Nomenclature	GJA1	GJA4	GJA5	GJA6[a]	GJB1	GJB2	GJB4	GJB5	GJC1	GJD3[b]
Old Nomenclature	Cx43	Cx37	Cx40	Cx33	Cx32	Cx26	Cx30.3	Cx31.1	Cx45	Cx31.9

[a] Mouse GJP for which there is no human ortholog
[b] GJD3 is mouse Cx30.2

kDa follows the abbreviation Cx, for connexin. This nomenclature will not be used, but Table 1 compares the two nomenclatures for GJPs discussed in this review.

GJPs are four-pass, transmembrane proteins containing two extracellular loops, one cytoplasmic loop and cytoplasmic N and C-termini (Figure 5). Generally, sequence conservation is found among the transmembrane domains, whereas sequence diversity is evident in the cytoplasmic loop and C-terminus (Mese et al., 2007).

Different combinations of GJPs and connexons confer specific biochemical and physical properties to the GJ. Several combinations are possible (Figure 5) (Mese et al., 2007). The type of connexon and GJs that are formed are limited by the GJP expressed within a given cell type and tissue, and the compatibility of those GJPs to oligomerize (Mese et al., 2007). GJs may be found within the plasma membrane among several or thousands of other GJs, where the latter array is referred to as a GJ plaque.

2.2 Intracellular Trafficking of Gap Junctions

2.2.1 Transport to the Plasma Membrane

Gap junctions are dynamic structures with half-lives of 1-5 hours; therefore, they are constantly being synthesized and degraded (Fallon and Goodenough, 1981; Laird et al., 1991). GJP synthesis and correct folding occurs in the endoplasmic reticulum. GJP oligomerization can occur in the endoplasmic reticulum or *trans*-Golgi, depending on the GJP (Koval, 2006), but is perhaps a progressive event that is completed in the *trans*-Golgi (Laird, 2006). Transport between the endoplasmic reticulum and Golgi takes place in the endoplasmic reticulum-Golgi intermediate compartment and may involve GTPases (Thomas et al., 2005; Laird, 2006; Das Sarma et al., 2008). Most GJPs pass through the

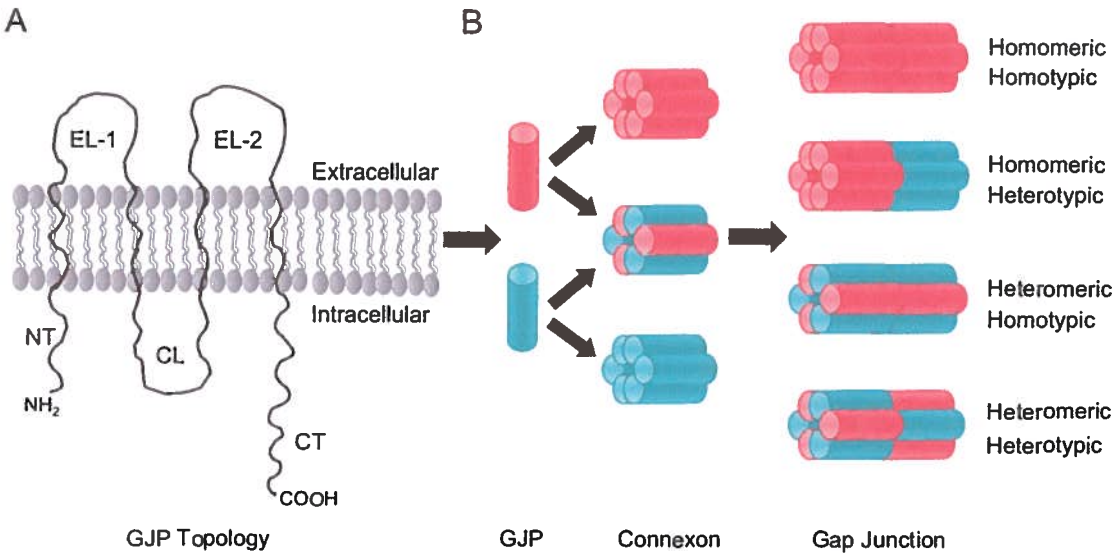

Figure 5. Schematic representation of gap junction proteins (GJP) and channels. (A) GJPs have four transmembrane domains, two extracellular loops (EL), one cytoplasmic loop (CL) and intracellular N and C-termini (NT and CT, respectively). (B) Six GJPs can selectively oligomerize to form homomeric and heteromeric connexons. Two connexons from adjacent cells dock at the plasma membrane to form homotypic and heterotypic gap junctions. Connexons can thereby create diverse gap junction channels by altering the content and spatial arrangement of their GJP subunits. Adapted from Mese et al. 2007.

Golgi apparatus, but GJB2 can reach the cell surface independent of the Golgi apparatus (reviewed in Laird, 2006).

Transport of the connexon to the plasma membrane is not entirely understood, but numerous types of intracellular vesicles appear to be involved (Jordan et al., 1999; Thomas et al., 2005). Depending on the GJP, intact microtubules may or may not be required (Thomas et al., 2005). Studies have demonstrated that certain GJPs can directly bind to caveolin-1 and 2 and partition to lipid raft domains (Schubert et al., 2002; Langlois et al., 2008). Caveolins may also assist in the exocytic transport of connexons. In support of this, newly synthesized GJA1 associates with caveolin 1 and 2 in the Golgi (Langlois et al., 2008). Once inserted into the plasma membrane, connexons may communicate to the extracellular space as a hemichannel (Ebihara, 2003; Goodenough and Paul, 2003), or diffuse laterally to regions of cell-to-cell contact (Lauf et al., 2002). Connexons from adjacent cells dock via the cysteine residues in each extracellular loop (Foote et al., 1998; Hofer and Dermietzel, 1998) to form a GJ, provided adherens junctions are present (Jongen et al., 1991; Meyer et al., 1992). Studies using green fluorescent protein and tetracysteine-tagged GJPs with fluorescent recovery after photobleaching in living cells demonstrated that newer GJs are transported to the outer

edges of the GJ plaque (Gaietta et al., 2002; Lauf et al., 2002). Older channels are located at the centre of the plaque and are removed from this site when degraded (Gaietta et al., 2002; Lauf et al., 2002).

2.2.2 Internalization and Degradation

Gap junction internalization may involve several different, and possibly converging, mechanisms. One mechanism is the formation of annular junctions termed "connexosomes" when specifically containing GJs (Laird, 2006). Annular junctions are double-membrane vesicles that form from the internalization of a large span of membrane from the cell surface of two adjacent cells. Annular junctions facilitate the removal of GJ plaques in a manner similar to pinocytosis or endocytosis (Jordan et al., 2001). GJ internalization may also occur via clathrin-mediated endocytosis. While this is traditionally thought to be a separate method of GJ internalization (Laird, 2006), recent studies suggest that it may be related to annular junctions. In support of this, clathrin is localized to GJA1-containing annular junctions (Nickel et al., 2008), and clathrin and other components of the endocytic machinery are required for GJ internalization into double-membrane vesicles (Piehl et al., 2007; Gumpert et al., 2008). A convergent mechanism is further evidenced by studies demonstrating that annular junctions degrade by budding into smaller vesicles (Leithe et al., 2006; Piehl et al., 2007). It was suggested that the smaller vesicles represented a hybrid between annular junctions and early endosomes (Leithe et al., 2006). GJPs have been localized to early endosomes (Mograbi et al., 2003; Segretain et al., 2004), but more studies are required to determine if clathrin-associated annular junctions represent a GJP-specific or a global mechanism of GJ internalization. While annular junctions and clathrin-coated vesicles have been demonstrated in the epididymis (Cyr et al., 1995; Pelletier, 1995a; Hermo and Smith, 1998), they have not been directly associated with GJ internalization.

The degradation pathway for GJs is controversial and may involve both lysosomal and proteosomal pathways (reviewed in Laird, 2006). Lysosomes appear more likely to be involved in the degradation of gap junctional components, while the proteasome may influence the stability of GJs and aid in their internalization (Laing et al., 1997; Musil et

al., 2000; Qin et al., 2003; Leithe and Rivedal, 2007). GJA1 is ubiquitinated by the E3 ubiquitin ligase, Nedd4, in a phosphorylation-modulated process (Leykauf et al., 2006). It has been suggested, although not extensively proven, that GJA1 is ubiquinated via multiple monoubiquitins and not a polyubiquitin chain (for example Laing and Beyer, 1995; versus Leithe and Rivedal, 2004). Multiple monoubiquitins are associated with nonproteasomal processes and polyubiquitin chains with proteasomal degradation (Leithe and Rivedal, 2007). Leithe *et al.* (2007) have proposed a model whereby monoubiqutinations of the GJ plaque aids in its internalization into connexosomes and subsequent degradation via the lysosomal pathway. It remains to be established if this is indeed the case and if so, how the clathrin-mediated endocytic machinery is incorporated into this process, or if the two mechanisms are mutually exclusive.

2.3 Role of Gap Junctions

2.3.1 Cellular Communication

Gap junctions link the cytoplasm between two cells to allow the exchange of ions (K^+ and Ca^{2+}), secondary messengers (cAMP, cGMP and inositol 1,4,5-triphosphate (IP_3)), small metabolites (glucose) and amino acids (glutamate) to permit GJ intercellular communication (GJIC) (reviewed in Mese et al., 2007). Hemichannels link the cytoplasm with the extracellular space by releasing factors such as ATP, glutamate and NAD^+ (Goodenough and Paul, 2003). The importance of cellular communication has been demonstrated by targeted disruptions of GJ genes in mice. These studies have indicated diverse functions for GJPs such as cardiac development (GJA1), male fertility (GJA1), female fertility (GJA4) and neuron function (GJB1) (reviewed in Simon and Goodenough, 1998; Sridharan et al., 2007).

Each GJ has a selective ionic and metabolic permeability governed by the composition of the connexon hemichannel and GJ channel (Martinez et al., 2002; Desplantez et al., 2004). Different tissues can express one or many types of GJPs to create GJIC variability. For example, cardiomyocytes express GJA1, GJA5, GJC1 and GJD3 (Beyer et al., 1995; White et al., 2002). In these excitable cells, GJIC coordinates intercellular current flow and the propagation of action potentials throughout the heart

(Saffitz et al., 2000). The epididymal epithelium on the other hand, is comprised of nonexcitable cells, which express GJA1, GJB1, GJB2, *Gjb4* and *Gjb5* (Cyr et al., 1996; Dufresne et al., 2003). In these cells GJIC may mediate coordinated responses through metabolic coupling (Mese et al., 2007). For example, GJ channels composed of GJA1 have an eight fold higher permeability to AMP and ADP, and a 300 fold higher permeability to ATP compared to GJB1 channels (Goldberg et al., 2002). In contrast, GJB1 has a 12 fold higher permeability to adenosine than GJA1, suggesting that the energy status of a cell can be controlled by the type of GJP expressed (Goldberg et al., 2002). Each GJP can create numerous combinations of GJ channels that may selectively transfer signals to produce striking complexity and physiological diversity.

2.3.2 Carcinogenic Process

A loss of gap junctional communication is common in cancerous cells. This has lead to the idea of GJPs as tumor suppressors (reviewed in Mesnil et al., 2005). Whether or not the loss of GJPs is causative in tumor development or a consequence of it, is unclear. In support of a causative role, higher tumor rates are observed in GJP-knockout models (for instance Naus et al., 1997; Temme et al., 1997). On the other hand, numerous tumorous tissues have aberrant GJP localization and decreased expression. For example, in Sertoli cells infiltrated with carcinomas in situ, there is a decrease in GJA1 expression and GJB2 becomes cytoplasmic (Brehm et al., 2002). In a human testicular seminoma cell line, GJB1 is localized in the *trans*-Golgi network, but after overexpression is targeted to the membrane (Roger et al., 2004). In prostate cancer cell lines, the major cause of deficient communication has been attributed to the impaired trafficking of gap junction proteins (Govindarajan et al., 2002). Thus, defects in GJP trafficking play a role in the carcinogenic process; however, it is unclear how these defects arise. Furthermore, the deficiencies in GJP trafficking are dependent on numerous factors including: the type of GJP, tissue, type of tumor and stage of cancer progression (Mesnil et al., 2005).

2.3.3 Barrier Function

2.3.3.1 Formation of the Bood-Epididymal Barrier by Tight Junctions

Apical tight junctions (TJ) between principal cells of the epididymal epithelium create a selective seal that forms the blood-epididymal barrier (BEB). The BEB protects maturing spermatozoa from the immune system. The fence function of TJs separates the basolateral and apical regions of the cell to generate and maintain cell polarity, while their barrier function controls the movement of solutes, ions and water through the paracellular space (reviewed in Cyr et al., 2007). The formation of the BEB is progressive along the epididymis and is completed by postnatal day 21 in the rat, as determined by lanthanum nitrate perfusion (Agarwal and Hoffer, 1989). The length of the TJ varies along the epididymis (Cyr et al., 1995). In the initial segment, TJs are much longer and contain few desmosomes, whereas in the other regions the length of the TJ is reduced, but there are numerous desmosomes (Cyr et al., 1995).

Tight junctions are comprised of several components (reviewed in Cyr et al., 2007). Two components are the integral transmembrane proteins occludin and claudins that connect adjacent cells via extracellular loops. There are at least 20 claudin family members with variable tissue distributions (Morita et al., 1999; Furuse and Tsukita, 2006). Three other components are the membrane-associated guanylate kinase (MAGUK) proteins tight junction proteins (TJP) (also referred to as zonula occludens), TJP1, TJP2 and TJP3 (Cyr et al., 2007). The TJP members were traditionally thought to associate only with TJs, but studies have indicated that their role is more variable. TJPs are scaffolding proteins that link membrane proteins to the cytoskeleton (Gonzalez-Mariscal et al., 2000). These membrane proteins include, but are not limited to, components of adherens and gap junctions.

2.3.3.2 Barrier Function of Gap Junction Proteins

Gap junction proteins appear to aid in the barrier and fence functions of tight junctions in certain cell types. For instance, Go *et al.* (2006) treated *GJB2* transfected human airway epithelial cells with a TJ inhibitor and found that there was no change in

transepithelial electrical resistance (TER). Conversely, untransfected cells had a significant decrease in TER (Go et al., 2006). The same authors showed *GJB2* transfected cells retained a BODIPY-sphingomylin probe that diffused across the TJs in untransfectd cells. While the mechanism was not elucidated, GJIC inhibitors did not prevent the increased barrier function, suggesting that GJIC-independent factors were involved (Go et al., 2006). Similar studies in a *Gjb1*-deficient immortalized mouse hepatocyte cells were done; however in this case, the fence and barrier functions were GJIC-dependent (Kojima et al., 2002).

In a study employing a porcine blood-brain barrier endothelial primary culture, GJA1 and GJA5 colocalized and coimmunoprecipitated with junctional proteins claudin-5, occludin and TJP1 (Nagasawa et al., 2006). While GJ blockers inhibited the barrier function of TJs, GJA1, GJA5, claudin-5, occludin and TJP1 expressions were unchanged (Nagasawa et al., 2006). Thus, it has been hypothesized that the close proximity of gap and tight junctions allow GJ-permeable molecules to act directly on the TJ, thereby aiding their barrier function (Nagasawa et al., 2006; Kojima et al., 2007). This has contributed to the idea of a junctional nexus.

2.4 Formation of a Junction Nexus

The gradual discovery that tight, adherens and gap junctions share common scaffolding proteins and close spatial proximity has lead to the re-emergence of the idea of a junctional nexus, initially described in the late seventies (for example Gros et al., 1978). The following section will discuss the association of GJPs with tight and adherens junctional components in various models, focusing on the components that have also been identified in the rat epididymis.

2.4.1 Gap Junction Proteins and Tight Junction Components

In a rat lung endothelial cell line induced to express claudin-1, it was found that GJA1 and claudin-1, and GJA1 and TJP1 colocalized by immunofluorescence (IF) and coimmunoprecipitation (Nagasawa et al., 2006). GJA1 associates with TJP2 in normal rat kidney epithelial cells demonstrated by IF, coimmunoprecipitation and GST-pull down assay with the C-terminus of GJA1 (Singh et al., 2005). In the same study, GJA1 and

TJP1 colocalized by IF in mouse heart tissue (Singh et al., 2005). Interestingly, TJP2-GJA1 colocalization was cell cycle-independent, whereas TJP1-GJA1 colocalization was significantly greater in G_0 cells versus S-phase cells (Singh et al., 2005). Numerous other reports have identified a direct interaction between GJA1 and TJP1 and will be described in more detail in a following section.

In primary rat hepatocytes, GJB1 colocalizes with claudin-1 at the most apical region of the membrane and coimmunoprecipitates with occludin, claudin-1 and TJP1 (Kojima et al., 2001). Immortalized mouse hepatocytes lacking endogenous *Gjb1* were transfected with *Gjb1* to investigate the impact on TJ strands and TER (Kojima et al., 1999). It was found that GJB1 colocalized with occludin by IF and coimmunoprecipitation and increased the number of TJ strands relative to parental cells (Kojima et al., 1999). Similarly, when *Gjb1* was knocked down with short interfering RNA, a downregulation of claudin-1 was also observed (Kojima et al., 2007). This suggests a positive feedback of GJB1 and claudin-1 expression. GJB1 also colocalizes with TJP2 in cultured rat hepatocytes (Kojima et al., 2001).

GJB2 interactions have not been widely reported, but one study showed that GJB2 interacted with a 27 amino acid peptide of occludin in a human intestinal cell line (Nusrat et al., 2000).

The significance of the interactions between GJPs and TJ components are not entirely clear, but they suggest that gap and tight junctions are intimately arranged in the cell. The associations, however, may be indirect with respect to the transmembrane proteins (Laird, 2006). In the adult rat epididymis, TJ components are localized to the same regions of the cell as GJPs. For instance, claudin-1 is found between basal and principal cells and at the lateral plasma membrane of adjacent principal cells (Gregory et al., 2001). GJA1, GJB1 and GJB2 are also localized between basal and principal cells (Cyr et al., 1996; Dufresne et al., 2003) and GJB1 and GJB2 at the lateral plasma membrane of adjacent principal cells (Dufresne et al., 2003). TJP1 and GJB1 are both localized at the apical region of adjacent principal cells (Levy and Robaire, 1999; DeBellefeuille et al., 2003; Dufresne et al., 2003). Direct interactions between GJPs and TJ components have not been reported in the epididymis, despite similarities in their cellular localizations.

2.4.2 Gap Junction Proteins and Adherens Junction Components

Adherens junctions (AJ) are necessary for cell adhesion. They serve both a structural function and a signalling function. Some components of AJs include: cadherins, catenins and TJP1 (reviewed in Cyr et al., 2002). GJA1 colocalizes and coimmunoprecipitates with α-catenin in neonatal rat cardiomyocytes (Wu et al., 2003) and a mouse embryonic fibroblast cell line, NIH3T3 (Wei et al., 2005). In mouse neural crest cells, GJA1 colocalizes with p120 catenin (Xu et al., 2001). GJA1 also colocalizes and coimmunoprecipitates with β-catenin in neonatal rat cardiomyocytes (Ai et al., 2000), in NIH3T3 cells (Wei et al., 2005) and colocalizes in mouse neural crest cells (Xu et al., 2001). The significance of such an interaction may be related to the Wnt signalling pathway, of which β-catenin is a downstream effector (Ai et al., 2000). Alternatively, the interaction between GJA1 and β-catenin may reflect the differentiation stage of the cell types used, where β-catenin has been shown to associate with tight junctional proteins early in development, but less so in adulthood (DeBellefeuille et al., 2003). Whether or not this is similar for GJ proteins remains to be established.

GJB2 colocalized with E-cadherin by IF in the mouse liver 48 hours after partial hepatectomy, when GJs are beginning to reform, and with α-catenin by electron microscopy with immunogold labeling at the same time point (Fujimoto et al., 1997). Interestingly, 60 hours after partial hepatectomy when GJs have reformed, GJB2 and α-catenin no longer colocalize (Fujimoto et al., 1997).

AJ and GJs are reciprocally regulated, where the formation of one junctional type aids in the formation of the other (Jongen et al., 1991; Meyer et al., 1992). Thus, the association of GJPs with AJ components may reflect the establishment of new junctional contacts and not steady-state ones. Similar to TJ components, AJ components are also localized to the same regions of the cell as GJPs in the adult rat epididymis. For example, β-catenin, α-catenin and p120 catenin and GJB1 are localized between basal and principal cells (DeBellefeuille et al., 2003; Dufresne et al., 2003), but their colocalization has not been reported.

2.5 Gap Junction Protein Compensation

GJPs have unique and differing properties (Wang and Veenstra, 1997; White et al., 2002; Weber et al., 2004), but it has been shown that in certain physiological situations, the loss of one GJP is functionally compensated for by the expression of another. In mouse ovarian follicles, oocytes and the surrounding granulosa cells are coupled by GJA4, whereas granulosa cells are coupled to one another by GJA1. Female mice lacking *Gja4* are sterile due to disruption of folliculogenesis and oocyte development (Simon et al., 1997). Li *et al.* (2007) tested the sufficiency of GJA4 to confer fertility by crossing transgenic mice lacking *Gja4* with transgenic mice that expressed *Gja1* in oocytes. The authors found that the *Gja1* knockin restored oocyte-granulosa coupling and fertility. Hence, despite differences in GJA1 and GJA4 properties, GJA1 could compensate for the loss of GJA4 in this model.

In the testes, GJA1 channels couple adjacent Sertoli cells, adjacent Leydig cells and germ cells with Sertoli cells (Risley et al., 1992; Tan et al., 1996; Batias et al., 2000). *Gja1* knockout models have demonstrated that GJA1 is essential for normal testicular development, spermatogenesis and male fertility (Roscoe et al., 2001; Brehm et al., 2007; Sridharan et al., 2007). To determine if another GJP could compensate for GJA1 in the testis, knockin studies were done where the coding region of *Gja1* was replaced with *Gjb1*, *Gjb2* or *Gja5* (Plum et al., 2000; Winterhager et al., 2007). These homozygous knockin mice were infertile, and lacked differentiated cells indicative of intermediate stages of spermatogenesis. The seminiferous tubules were developed, but they were mostly lined by Sertoli cells (Plum et al., 2000; Winterhager et al., 2007). This phenotype was similar to GJA1 null mice (Roscoe et al., 2001; Brehm et al., 2007; Sridharan et al., 2007). Hence, in the testes neither GJB1, GJB2 nor GJA5 could compensate for GJA1.

Additional studies have investigated GJP compensation. For instance, GJA6 can compensate for GJA1 in bone formation (Minkoff et al., 1999), but astrocytes isolated from *Gja1*-null mice cannot transfer microinjected Lucifer yellow, despite the presence of other GJPs (Naus et al., 1997; Dermietzel et al., 2000). In doubly mutant mice deficient in *Gja1* and *Gjb1*, organs that express both GJPs in the same cell type have normal prenatal development (Houghton et al., 1999). Thus, the ability of GJPs to compensate for one another remains somewhat unresolved and is likely GJP and tissue-dependent.

2.6 GJA1

GJA1 is the most ubiquitously expressed GJP, and hence, the most widely studied (reviewed in Laird, 2006). Many male reproductive tissues express GJA1 including the testis, prostate and epididymis (reviewed in Pointis et al., 2005). The regulation of GJA1 is complex and can be controlled at various points throughout its life cycle such as biosynthesis, oligomerization, targeting and degradation. GJP regulation is not entirely understood as described in previous sections, but studies suggest that hormones and their effectors play a role in the process.

2.6.1 Regulation of GJA1

2.6.1.1 Hormones Implicated in GJA1 Regulation

Several different hormones regulate GJA1. In the rat epididymis, GJA1 targeting is regulated by androgens in the initial segment (Cyr et al., 1996). In control animals, GJA1 is localized between basal and principal cells in all four segments. In orchidectomized animals sacrificed seven days after surgery, GJA1 targeting is altered in the initial segment and is also found at the apical region of the epithelium between adjacent principal cells (Cyr et al., 1996). GJA1 expression also increases in tissue lysates from the entire epididymis and GJA1 becomes hyperphosphorylated (Cyr et al., 1996). When androgen levels in orchidectomized animals are maintained with testosterone implants, GJA1 localization resembles control rats (Cyr et al., 1996).

In the stallion, cryptorchidism (failure of testicular descent) decreases GJA1 expression in the seminiferous tubules and Leydig cells, determined by IF (Hejmej and Bilinska, 2008). Concomitant decreases in serum testosterone and increases in serum estradiol were observed. Thus, an androgen-estrogen hormonal imbalance affects GJA1, but may represent altered differentiation or maturation of Leydig and Sertoli cells (Hejmej and Bilinska, 2008).

GJA1 is also influenced by thyroid hormones, demonstrated by propylthiouracil (PTU)-induced neonatal hypothyroidism in rats (St-Pierre et al., 2003). GJA1 localization was altered in the testis from 30-day-old, PTU-treated rats, where the immunoreaction

was cytoplasmic, and did not localize to the plasma membrane of Sertoli cells as in control rats (St-Pierre et al., 2003). The authors suggested that thyroid hormones play a role in GJA1 intercellular targeting in the testis. In support of this, Sertoli cell cultures treated with triiodothyronine show an increase in GJA1 expression and localization to GJ plaques via a non-genomic mechanism (Gilleron et al., 2006). In the epididymis, hypothyroidism lead to lower *Gja1* mRNA levels and undetectable protein levels in the initial segment, caput and corpus until 22 and 30 days of age, respectively (St-Pierre et al., 2003). In adults, however, GJA1 localization and mRNA expression was normal, indicating a more prominent role for thyroid hormone in development (St-Pierre et al., 2003).

Human chorionic gonadotropin (hCG) and 8-bromo-cAMP analog increase Leydig cell steroidogenesis, but downregulate *Gja1* mRNA and IF staining in cultured rat Leydig cells and in testes (You et al., 2000). Targeting to the plasma membrane may also be altered (You et al., 2000).

Retinoids also regulate GJA1. In retinoid X receptor β knockout mice, *Gja1* decreases in Sertoli cells shown by *in situ* hybridization and IF (Batias et al., 2000). Similarly, retinoic acid induced *Gja1* mRNA expression and appearance of GJA1-positive punctate spots by IF in an immortalized human osteoblastic cell line (Chiba et al., 1994). After 12 hours of exposure to retinoic acid, dye coupling was also increased by 2.2 fold (Chiba et al., 1994). Additionally, in vitamin-A-deficient rats supplemented with vitamin A, *Gja1* expression increases in parallel with the reinitiation of spermatogenesis (Luk et al., 2003).

FSH and luteinizing hormone (LH) regulate GJA1 in male and female reproductive models. In Sertoli cells cultured from immature rat testes, treatment with FSH enhances dye transfer between the cells via cAMP (Pluciennik et al., 1994). This may reflect changes in gap junction protein synthesis (Pluciennik et al., 1994). In the rat ovary, FSH and LH differentially regulate GJA1 during oocyte maturation. LH mediates the disruption of oocyte-granulosa cell contacts via growth factors (Jamnongjit and Hammes, 2005), perhaps by inhibiting *Gja1* translation (Kalma et al., 2004). FSH, on the other hand, upregulates *Gja1* mRNA in granulosa cells and increases electrical cell coupling (Sommersberg et al., 2000).

GJA1 expression is tightly regulated during pregnancy. GJIC by GJA1-containing GJs helps to coordinate smooth muscle contractions at the onset of labour (Challis et al., 2001). In the rat endometrium, ovariectomy with estradiol maintenance increases *Gja1* mRNA and protein expression (Grummer et al., 1994). In contrast, treatment with progesterone alone or in combination with estradiol is suppressive for GJA1, an effect that is reversed with progesterone withdrawal (Grummer et al., 1994). Similarly, in the rat myometrium, GJA1 expression increases throughout pregnancy and is maximal during labour and correlates with an increase in the plasma estrogen to progesterone ratio (Lye et al., 1993). Interestingly, while anti-estrogen-treated ovarictomized rats have decreased GJA1 expression in the myometrium, progesterone-treated ones have suppressed targeting of GJA1 (Hendrix et al., 1995). In the latter treatment group, GJA1 accumulates in the Golgi region (Hendrix et al., 1995). In contrast to estrogen, parathyroid hormone-related protein (PTHrP) plays a role in preventing the onset of labour. PTHrP significantly reduces the expression of *Gja1* mRNA and protein in the myometrium when injected into pregnant rats (Mitchell et al., 2003).

ANGII also regulates GJA1. Primary cultures of human saphenous vein smooth muscle cells treated with ANGII have increased GJIC compared to untreated cells, as demonstrated by scrape loading and Lucifer Yellow dye transfer (Jia et al., 2008). The same study reported that ANGII increases GJA1 protein expression in a MAPK-dependent manner via a mechanism involving the transcription factor, AP-1 (activator protein-1) (Jia et al., 2008). Likewise, in neonatal rat cardiomyocytes, administration of an ANG II receptor antagonist abolishes the six fold increase in GJA1 expression observed after application of mechanical stress (Shyu et al., 2001). Increased GJA1 immunolabeling and mRNA expression were also observed (Shyu et al., 2001).

Thus, many hormones are implicated in regulating GJA1 expression and localization in a cell type and tissue-dependent manner. Studies investigating the influence of growth factors on GJA1 regulation have added further complexity.

2.6.1.2 Growth Factors Implicated in GJA1 Regulation

Growth factors influence several aspects of GJA1 regulation in a cell-type-dependent manner (Chiba et al., 1994; Reuss and Unsicker, 1998; Reuss et al., 2000).

Gja1 mRNA and protein expression may be upregulated (Chiba et al., 1994; Rivedal et al., 1996; Nadarajah et al., 1998) or downregulated (Moorby et al., 1995; Reuss et al., 1998; Reuss et al., 2000) by growth factors. Growth factors influence GJIC, demonstrated by changes in dye transfer between microinjected cells (reviewed in Reuss and Unsicker, 1998). The discovery of new GJPs suggests that these coupling changes are not necessarily associated with GJA1, but likely involve other GJPs (for instance Dermietzel et al., 2000). While some studies report a proliferative-independent effect for growth factors on GJA1 regulation, tested by BrdU incorporation (Nadarajah et al., 1998; Reuss et al., 1998), other effects are related to changes in the proliferative state of the cell population (Gibson et al., 1994; Rivedal et al., 1996). Growth factor activation of receptor tyrosine kinases also induces changes in GJA1 phosphorylation, disrupting GJIC (Lau et al., 1992; Doble et al., 1996; Lampe and Lau, 2004). Since each growth factor has specific effects associated with a given model system, the following sections will focus on converging mechanisms downstream of hormonal and growth factor stimuli that have demonstrated direct regulation of GJA1.

2.6.1.3 Hormone and Growth Factor Effectors Implicated in GJA1 Regulation

2.6.1.3.1 Transcription Factors

Hormones and growth factors influence genomic and non-genomic mechanisms of GJA1 regulation, the former resulting in direct regulation of *Gja1* transcripts and the latter affecting posttranslational modifications and GJ trafficking. Known *Gja1* transciptional regulators are described below.

Gja1 basal promotor activity is reliant on the transcription factors Sp1, Sp3 and AP-1 in several different cell types as demonstrated by luciferase reporter assays and electrophoretic mobility shift assays (Teunissen et al., 2003). Mitchell *et al.* (2005) showed that distinct combinations of AP-1 family members differentially activated *Gja1* transcription in hamster myometrial cells. While Jun dimers were rather weak *Gja1* transcriptional activators, Fos/Jun heterodimers significantly increased promotor activity (Mitchell and Lye, 2005). AP-1 transcription factors have not been directly shown in the epididymis, but one study suggests that Fos may be present (Kuhara et al., 2005).

Sp1 and Sp3 are expressed in the nuclei of the rat epididymal epithelium (Seenundun and Robaire, 2005) and bind to the 5' Sp1 binding site of the rat claudin-1 promotor (Dufresne and Cyr, 2007). It has been suggested that the expression pattern of Sp1 and Sp3 in the initial segment and caput may relate to the testicular factor regulation of claudin-1 in the rat initial segment (Gregory et al., 2001; Dufresne and Cyr, 2007). Whether or not Sp1 and Sp3 transcription factors regulate *Gja1* transcription in the rat epididymis, and/or androgen-dependent expression has yet to be established.

In NIH3T3 cells with constitutively expressed Ras, *Gja1* mRNA and protein levels are increased by a DNA-binding protein complex containing HSP90 (heat shock protein of 90 kDa) and c-Myc (Carystinos et al., 2003). C-*myc* is expressed at low levels in the mouse epididymis and is not regulated by androgens or testicular factors (Cornwall et al., 2001), suggesting it may not play a role in regulating *Gja1* in the rat epididymis.

Gja1 does not appear to contain an androgen response element. Studies have suggested that *Gja1* contains a retinoic acid response element (Chiba et al., 1994), but retinoic acid appears to enhance *Gja1* transcription via Sp1 and Sp3 (Vine et al., 2005). Authors have proposed that *Gja1* is directly regulated via a cAMP response element (for example Huynh et al., 2001; Bailey et al., 2002), yet no studies have shown direct binding of a cAMP-dependent transcription factor to the *Gja1* promotor. *Gja1* does contain a thyroid hormone response element (Stock and Sies, 2000), but regulation by this mechanism may be more important for development. Hence, activation of Sp1 and Sp3 by hormones and growth factors may, at this time, be the most likely candidates in regulating *Gja1* in the rat epididymis.

2.6.1.3.2 Kinases

GJA1 is a phosphoprotein. Phosphorylation of the GJA1 C-terminus is linked to several stages of the GJP life cycle including: channel assembly (reviewed in Solan and Lampe, 2005), channel gating (reviewed in Moreno, 2005), GJIC (reviewed in Lampe and Lau, 2004) and GJ internalization and degradation (reviewed in Laird, 2005). Changes in GJA1 phosphorylation are also linked to development, carcinogenesis and wound healing (reviewed in King and Lampe, 2005). Phosphorylation can be detected by Western blot analysis. The non-phosphorylated form of GJA1 (P0) migrates more quickly on

polyacrylamide gels, followed by GJA1 with at least one phosphorylation (P1) and then by P2, with at least two phosphorylations (Lampe and Lau, 2004). However, phosphorylation of certain residues does not influence GJA1 migration (King and Lampe, 2005).

GJA1 phosphorylation fluctuates throughout the cell cycle checkpoints (Solan and Lampe, 2005). The serine kinase, $p34^{Cdc2}$, is active during mitosis. $P34^{Cdc2}$ phosphorylates GJA1 on serine residues 255 and 262, either directly or indirectly, leading to downregulation of GJIC and GJA1 internalization (Lampe et al., 1998; Laird, 2005).

Protein kinase C (PKC) phosphorylates GJA1 at serines 368 and 372. This is associated with a decrease in GJ conductance, permeability and GJIC, although there are exceptions in certain cell types (reviewed in Lampe and Lau, 2004). Different PKC isoforms regulate GJA1, depending on the model system. For example, in rabbit and bovine lens epithelial cells the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and the growth factor IGF-1 induce an interaction between PKCγ and GJA1 in lipid rafts (Lin et al., 2003). The association resulted in redistribution of GJA1 and diminished GJ plaques (Lin et al., 2003). In cardiomyocytes, on the other hand, FGF-2 mediates the PKCε phosphorylation and interaction with GJA1, independently of the MAPK pathway (Doble et al., 2000).

Activation of the MAPK pathway by NRTKs and RTKs is associated with GJA1 phosphorylation (Lampe and Lau, 2004; Pahujaa et al., 2007). The activation of the EGF receptor induces direct phosphorylation of GJA1 by MAPK on serine residues 255, 279 and 282, disrupting GJIC (reviewed in Lampe and Lau, 2004). VEGF-A also reduces GJIC and increases GJA1 phosphorylation in a MAPK and c-Src-dependent manner (reviewed in Lampe and Lau, 2004). The PDGF receptor, however, required both PKC and MAPK activity to disrupt GJA1 activity (reviewed in Lampe and Lau, 2004). In terms of disassembly of GJA1-containing GJs, the MAPK pathway may also mediate GJ internalization, as demonstrated by administration of the carcinogen lindane in a murine Sertoli cell line (Mograbi et al., 2003). Although MAPKs are involved in GJA1 regulation, the specific MAPK subfamily depends on the cell type. For instance, in rat liver epithelial cells, EGF induces ERK1/2, but not ERK5 to phosphorylate GJA1 and decrease GJIC (Abdelmohsen et al., 2007). Alternatively, EGF induces GJA1 uncoupling

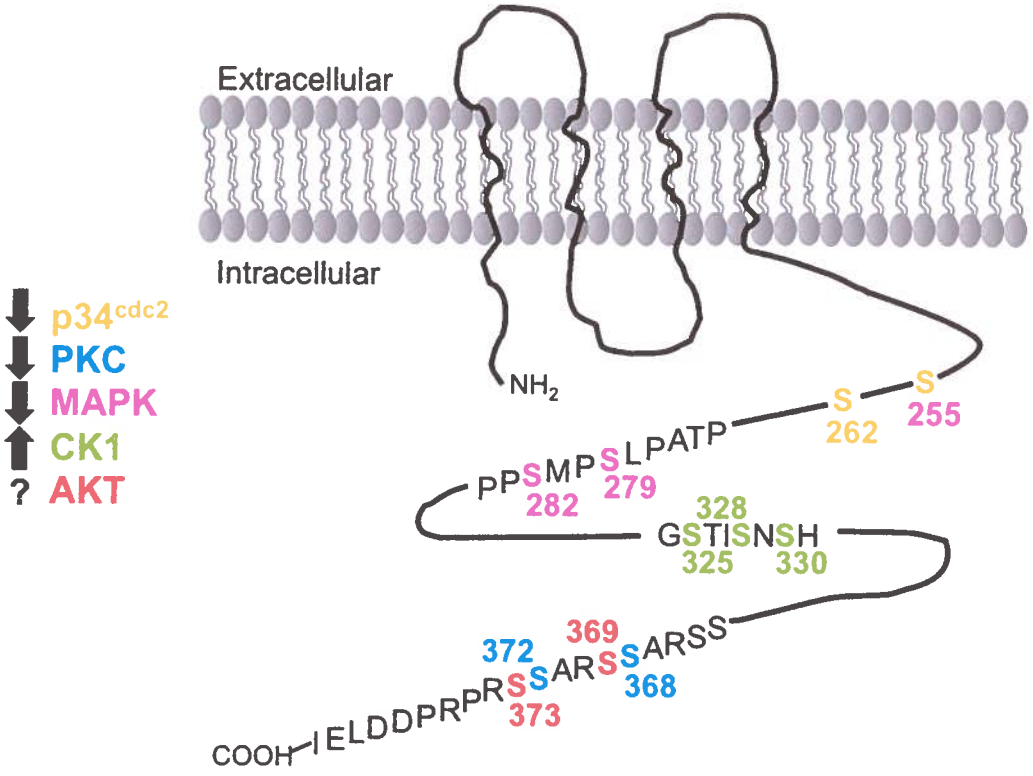

Figure 6. GJA1 residues phosphorylated by kinases. $p34^{cdc2}$ in yellow, PKC in blue, MAPK in pink, CK1 in green and AKT in red. Serine 255 is a target for $p34^{cdc2}$ and MAPK. Down arrows indicate a general inhibitory effect on GJA1, while up arrows indicate a stimulatory effect. Question mark indicates an unknown effect. Adapted from Lampe and Lau, 2004.

via the big MAPK 1 (BMK1)/ERK5 and not ERK1/2 in human embryonic kidney cells (Cameron et al., 2003). Thus, the specific MAPK associated with GJA1 regulation must be studied in a cell-type and growth factor-dependent manner.

One study has shown that casein kinase 1 (CK1) directly phosphorylates GJA1 in NRK cells (Cooper and Lampe, 2002). It was systematically suggested that the residues involved were serines 325, 328 and 330 (Cooper and Lampe, 2002). Using CK1 inhibitors, the authors also demonstrated that GJA1 localization increases at non-junctional plasma membranes and decreases Lucifer yellow dye transfer. In general, CK1 was suggested to promote GJ assembly under basal conditions (Cooper and Lampe, 2002).

Akt/protein kinase B phosphorylates GJA1 on serines 369 and 373 in Rat-1 fibroblasts (Park et al., 2006; Park et al., 2007). This creates a binding site for 14-3-3 θ

that coprecipitates with GJA1, which was suggested to play a role in trafficking (Park et al., 2007). In rat liver epithelial cells, the chemopreventive phytochemical indole-3-carbinol prevented the H_2O_2-induced inhibition of GJIC and GJA1 phosphorylation via inactivation of the Akt pathway, but not the MAPK pathway (Hwang et al., 2008).

The kinases c-Src and its viral oncogenic counterpart (v-Src) and cAMP-dependent protein kinase A (PKA) also regulate GJA1.

2.6.2 GJA1 Binding Proteins

In addition to kinases, many other GJA1 binding proteins have been identified including: TJP1, TJP2, β-catenin, drebin, α- and β-tubulin, caveolin-1 and 2, nephroblastoma overexpressed gene/connective-tissue growth factor (NOV/CCN3) and connexin43-interacting protein of 85 kDa (CIP85) (reviewed in Laird, 2006). Some of these binding proteins have been described in previous sections. The significance of the associations range from growth suppression (NOV/CCN3), to regulation of GJP transport (α and β tubulin), to regulating GJA1 turnover (CIP85) (reviewed in Laird, 2006). The GJA1 binding proteins that are reported to a) regulate intracellular trafficking and b) are regulated by androgens are described in detail.

2.6.2.1 C-Src

C-Src is a proto-oncogene most commonly known for promoting neoplastic transformation and cancerogenesis. V-Src is the mutated oncogene of c-Src that is constitutively active. C-Src belongs to a non-receptor tyrosine kinase family that consists of at least nine members (Sandilands and Frame, 2008). The biological function of the c-Src family is diverse ranging from: cell adhesion assembly and turnover, modulation of endosomal transport and signalling dynamics, proliferation, and tumor promotion (Sandilands and Frame, 2008). The family members are similar in structure with molecular masses between 52-62 kDa, with c-Src at 60 kDa (Sandilands and Frame, 2008). When c-Src is inactive, it is phosphorylated at tyrosine (Y) residue 527 that keeps the protein in a closed conformation. Upon c-Src activation, Y527 is dephosphorylated allowing c-Src to open and release the kinase domain, which will then autophosphorylate

Y416 (Figure 7). Unfolding of c-Src permits its SH2 and SH3 domains to interact with other proteins, allowing c-Src to phosphorylate its substrates.

There is strong evidence that Src downregulates GJIC through various mechanisms (Solan and Lampe, 2008). As a link between G-protein coupled receptor pathways and receptor tyrosine kinase pathways, Src can integrate signalling cascades leading to GJA1 phosphorylation on tyrosine, or serine (S) residues (reviewed in Lampe and Lau, 2004; Pahujaa et al., 2007; Solan and Lampe, 2008). V-Src enhances GJA1 phosphorylation at Y247, Y265, S262, S279/282 and S368 and reduces phosphorylation at S364/S365 (Solan and Lampe, 2008). Many studies have investigated the direct interaction of GJA1 with c-Src or v-Src.

A series of experiments utilizing coimmunoprecipitation, coimmunofluorescence and site-directed mutagenesis of GJA1 and v and c-Src in a variety of cell lines has lead to a working model for their interaction (reviewed in Lampe and Lau, 2004) (Figure 7). The current model of GJA1-v-Src interaction proposes that the SH3 domain of v-Src interacts with a proline-rich region of GJA1 at the plasma membrane to bring the v-Src kinase domain in close proximity to Y265 located on the C-terminus of GJA1. V-Src phosphorylation of Y265 creates an additional interacting site between the v-Src SH2 domain and phosphorylated Y265. Finally, v-Src phosphorylation of Y247 on the C-

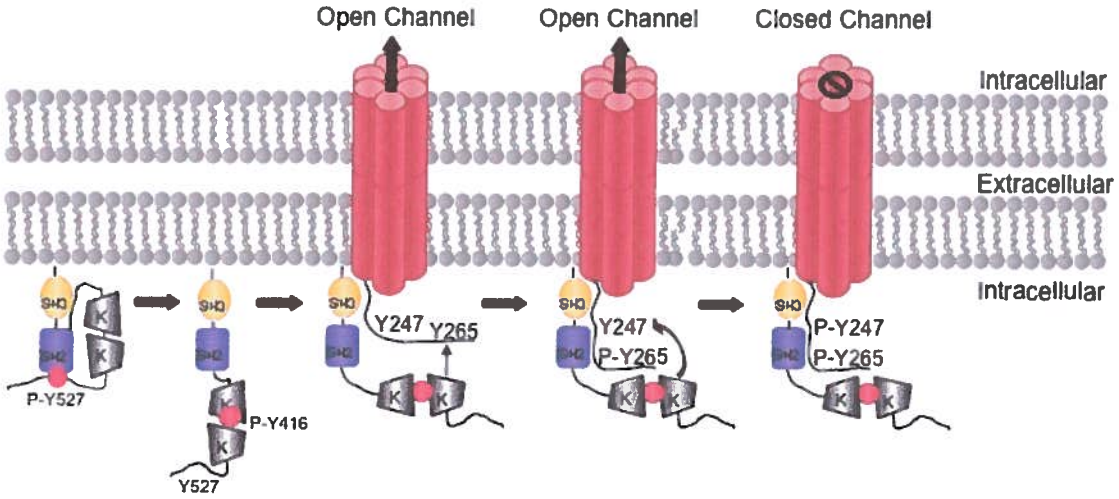

Figure 7. Model illustrating Src regulation of GJA1 gap junction channels. Inactive Src is phosphorylated at Y527 keeping the protein in a closed conformation. Dephosphorylation of Y527 releases the kinase domains, which are then free to autophosphorylate Y416, activating Src. Active Src binds to the C-terminus of GJA1 via its SH3 domain and then phosphorylates Y265 to provide an additional docking site for its SH2 domain. Finally, Src phosphorylates Y247 causing GJA1 channel closure. Adapted from Lin et al. 2001.

terminus of GJA1 leads to closure of the GJ channel (Kanemitsu et al., 1997; Lin et al., 2001). C-Src also appears to fit this model (Toyofuku et al., 1999; Giepmans et al., 2001a). The direct interaction between Src and GJA1 influences the association between GJA1 and its other binding proteins, as described later. C-Src also influences not only GJ channel closure, but the internalization of GJA1-containing GJs into annular junctions (Gilleron et al., 2008).

2.6.2.2 PKAcat

G-protein coupled receptor activation of adenylate cyclase promotes ATP cyclization to cAMP. The effector of cAMP is the cAMP-dependent protein kinase A (PKA) holoenzyme that consists of two regulatory subunits and two catalytic subunits (Hansson et al., 2000). Two cAMPs directly bind to each regulatory subunit, which releases the PKA catalytic subunits (PKAcat) that are then free to phosphorylate substrates on serine or threonine residues. PKA has two isozymes, termed I and II, and each isozyme has different isoforms. The PKAcat subunit isoforms are termed α, β and γ; thus, the catalytic subunit forms are: CIα, CIβ, CIγ and CIIα, CIIβ, and CIIγ that have a molecular mass approximating 40 kDa. Cellular signalling specificity is thought to involve A kinase anchoring proteins (AKAPs) that sequester PKA and their substrates to subcellular compartments (Skalhegg and Tasken, 2000). Up to 20 AKAPs have been identified and each AKAP associates with different, sometimes overlapping, organelles (Skalhegg and Tasken, 2000). PKAcat has also been shown to be necessary for membrane trafficking (Rodionov et al., 2003), specifically, apical, but not basolateral transport (Pimplikar and Simons, 1994).

Studies using agents to increase intracellular cAMP have demonstrated that GJA1 phosphorylation and GJIC is enhanced as a result of increased biosynthesis and/or intracellular trafficking of GJA1 to the plasma membrane (reviewed in Lampe and Lau, 2004). PKAcat directly phosphorylates serine residues 364, 365, 368, 369 and 373 in the C-terminus of GJA1 (TenBroek et al., 2001; Yogo et al., 2006); however, the importance of such phosphorylations is unclear and may i) prime GJA1 for sequential

phosphorylations by alternate kinases (Shah et al., 2002) or ii) promote GJA1-mediated gap junction channel activity (Yogo et al., 2006). At this time, it is unclear if PKAcat plays a direct or indirect role in regulating GJA1, but other than casein kinase 1, it is the only reported stimulatory kinase of GJA1 and GJIC. Furthermore, both Src and PKAcat regulate phosphorylation at S364 and S365, residues involved in regulating GJA1 trafficking to or within the plasma membrane (Solan and Lampe, 2007; Solan and Lampe, 2008).

2.6.2.3 TJP1

As described earlier, tight junction protein 1 (TJP1) is a scaffolding protein that interacts with components from tight, adherens and gap junctional components. Many studies have investigated the direct association with the C-terminus of GJA1 and the PDZ2 domain of TJP1 (for instance Giepmans and Moolenaar, 1998; Toyofuku et al., 1998). Yet, the functional relevance of this interaction is not entirely understood.

Some studies suggest that TJP1 functions to regulate the size and stability of GJA1 GJ plaques. In ROS osteosarcoma cells transfected with a dominant-negative TJP1 fusion protein, GJA1 abundance and localization to the plasma membrane was unchanged (Laing et al., 2005). GJIC, however, was interrupted and GJA1 was redistributed within the plasma membrane from lipid raft domains to gap junction plaques (Laing et al., 2005). In neonatal rat cardiomyocytes, a peptide that blocks the TJP1-GJA1 interaction was shown to increase the size of GJA1-positive GJs (Hunter et al., 2005). It was suggested that TJP1 may therefore regulate the overall size and distribution of GJ plaques by constraining the accumulation of GJA1 at the periphery of GJ plaques (Hunter et al., 2005).

Other studies suggest that TJP1 plays a role in GJA1 internalization. Using the non-genomic carcinogen γ-hexachlorocyclohexane (HCH) that induces endocytosis, Segretain *et al.* (2004) demonstrated that in untreated murine Sertoli cells GJA1 and TJP1 have a moderate colocalization at the plasma membrane. Conversely, in HCH-treated cells, the TJP1-GJA1 association was more pronounced in the cytoplasmic compartment (Segretain et al., 2004). The same group later demonstrated that the internalization process involved the formation of annular junctions containing c-Src on the outer

membrane of the vesicle and TJP1 on the inner membrane (Gilleron et al., 2008). Similarly, in porcine pulmonary artery endothelial cells, thrombin and endothelin-1 caused a rapid internalization of GJs that was dependent on TJP1 and involved clathrin-1 (Baker et al., 2008). TJP1 was also found at the centre of annular junctions colocalized with GJA1 (Baker et al., 2008).

TJP1 and c-Src appear to be competitive GJA1 binding proteins. Duffy *et al.* (2004) demonstrated in cortical astrocytes that GJA1 preferentially associated with c-Src at a lower intracellular pH, whereas TJP1 was preferred at a higher intracellular pH. Furthermore, c-Src binding to the C-terminus of GJA1 prevents and reverses the interaction between GJA1 and TJP1 (Duffy et al., 2004; Sorgen et al., 2004). The study by Gilleron *et al.* (2008), demonstrated that the internalization of GJA1 into annular junctions required activation of c-Src and disruption of the TJP1-GJA1 interaction only from the cell that contributed active c-Src.

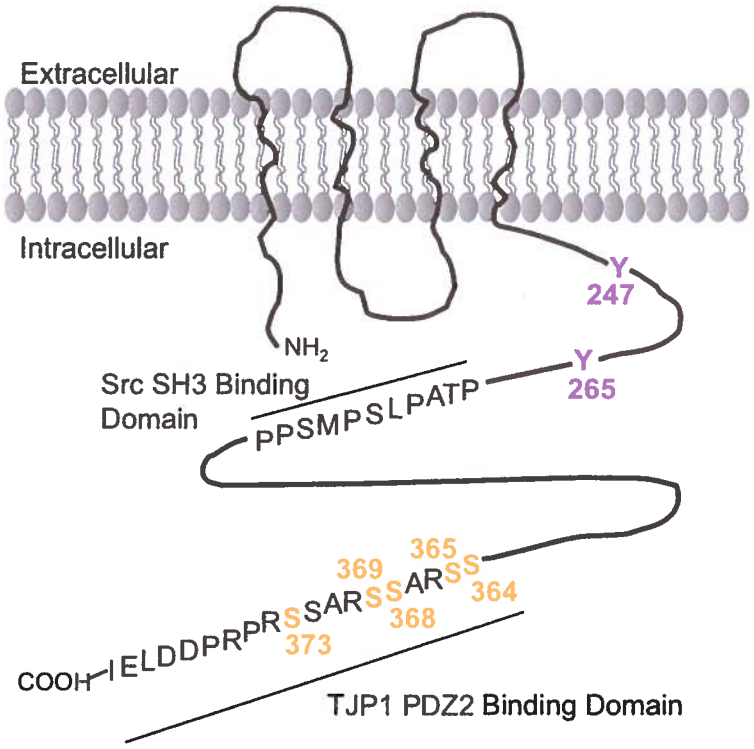

Figure 8. Schematic representation of GJA1 binding sites for Src, PKAcat and TJP1. Residues that are phosphorylated by Src are shown in purple, while those phosphorylated by PKAcat are shown in yellow. TJP1 and Src binding sites are denoted by lines adjacent to the amino acid residues. Inspired by Lampe and Lau 2004.

3.0 Objectives

In the epididymis, it is unknown what governs GJA1 trafficking to the plasma membrane of homologous or heterologous cell types, or if GJA1 regulators c-Src, PKAcat or TJP1 are involved in the process. Thus, the objective of this study was to investigate possible mechanisms of androgen-dependent, GJA1 targeting in the rat initial segment.

It is hypothesized that the segment-specific, androgen-dependent change in GJA1 intracellular localization is influenced by either the expression, or the interaction of GJA1 and its regulators.

CHAPTER 2: ARTICLE

Résumé

Les spermatozoïdes acquièrent leur motilité et leur pouvoir fécondant en traversant l'épididyme. L'épididyme est un tubule hautement enroulé qui est généralement divisé en quatre segments principaux (le segment initial (IS), la tête (CT), le corps et la queue). L'épithélium épididymaire est maintenu par des androgènes et des facteurs testiculaires qui sont sécrétés par les testicules. On pense que la régulation de l'épithélium par ces facteurs testiculaires influence la communication intercellulaire par l'intermédiaire des jonctions lacunaires (GJIC) et donc, le processus d'acquisition de la fertilité.

Dans l'épididyme de rat, cinq protéines des jonctions lacunaires (GJPs) sont exprimées, mais peu d'information existe sur leur régulation dans l'épididyme. Cependant, des études antérieures dans notre laboratoire ont démontré que la localisation de GJP alpha 1 (GJA1) est dépendante des androgènes testiculaires dans l'IS.

Des protéines de liaison de GJA1 peuvent régulent la localisation et le GJIC de GJA1. Les objectifs de cette étude ont été d'examiner la localisation et l'association directe entre GJA1 et ses régulateurs potentiels: la sous-unité catalytique de la protéine kinase dépendante de l'AMP cyclique A (PKAcat), la protéine du proto-oncogène pp60 (c-Src) et la protéine de jonction serrée 1 (TJP1) dans l'épididyme de rat adulte. Les analyses immunocytochimiques et par Western ont montré l'expression des protéines c-Src et de PKAcat le long de l'épididyme qui ont été localisées à différentes régions cellulaires. L'orchidectomie bilatérale a entraîné des niveaux de GJA1, de PKAcat et de c-Src plus faibles dans l'IS et la CT par rapport aux animaux contrôles. Le traitement avec la testostérone (T) permet de maintenir partiellement le niveau d'expression de c-Src dans la CT. Le rapport de la protéine c-Src active sur la protéine inactive a été augmenté dans la CT chez les rats orchidectomisés; ceci a été renversé avec le traitement de la T. C-Src a été retrouvé à la région apicale et aux microvillosités chez les rats orchidectomisés, et le traitement à la T a empêché l'accumulation apicale de c-Src, mais pas l'immunoréaction sur les microvillosités. La co-immunoprécipitation a indiqué une association directe entre GJA1, c-Src et TJP1 dans l'IS, mais non pas avec PKAcat. Dans

la CT, cependant, GJA1 est associé faiblement avec TJP1, mais non pas avec les autres protéines qui pourraient lier GJA1. Ces résultats ont indiqué que GJA1, c-Src et PKAcat sont régulés par les facteurs testiculaires, et que c-Src et TJP1 pourraient jouer un rôle dans l'inhibition des jonctions lacunaires dans l'IS.

REGULATION OF GAP JUNCTION ALPHA 1 BINDING PROTEINS IN THE ADULT RAT EPIDIDYMIS AND THEIR INFLUENCE ON ANDROGEN-DEPENDENT GAP JUNCTION ALPHA 1 LOCALIZATION

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Running Title: Regulation of GJA1 binding proteins

Keywords: Epididymis, Androgens, Orchidectomy, Gap Junctions, GJA1, Connexin 43, PKA, c-Src, TJP1, Coimmunoprecipitation, Western blot

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ABSTRACT

The cellular localization of gap junctional protein alpha 1 (GJA1) is androgen-dependent in the initial segment (IS) of the epididymis. GJA1 binding partners can regulate GJA1 localization and gap junctional intercellular communication (GJIC). The present objectives were to examine the localization, and direct binding of GJA1 and its potential regulators: cAMP-dependent protein kinase A catalytic subunit (PKAcat), cellular Rous sarcoma oncogene (c-Src) and tight junction protein 1 (TJP1) in the adult rat epididymis. Western blot analysis and immunocytochemistry indicated that c-Src and PKAcat were expressed throughout the epididymis and were localized between basal and principal cells. Bilateral orchidectomy resulted in a 2.4, 1.7 and 4.1 fold decrease in protein expression for GJA1, PKAcat and c-Src, respectively in the IS, and by 11, 1.4 and 6.3 fold in the caput (CT). Testosterone (T) partially reversed the effect for c-Src in the CT. The ratio of active to inactive c-Src was increased by 4.5 fold in the CT of orchidectomized rats; this was reversed with T. C-Src accumulated in the apical region and microvilli of orchidectomized animals, while T-maintenance prevented the apical accumulation, but not microvilli staining. Coimmunoprecipitation indicated a direct association between GJA1, c-Src and TJP1 in the IS, but not with PKAcat. In the CT, however, GJA1 weakly associated with TJP1, but not the other binding proteins. These results indicate that GJA1, PKAcat and c-Src are regulated by a combination of testicular factors, and that c-Src and TJP1 may contribute to segment-specific differences in GJA1 localization in the rat epididymis.

INTRODUCTION

Spermatozoa are produced by the testis, but require passage through the epididymis where they acquire motility and fertilization capabilities [1]. The epididymis is comprised of a single, highly coiled tubule that is subdivided into four main segments (the initial segment, caput, corpus and cauda) according to the histological appearance of the epithelium and its physiological function [2]. The epididymal epithelium is maintained by androgens secreted by the testis that enter the epididymis either directly via the lumen, or indirectly via the circulation [3, 4]. Along with androgens and spermatozoa, testicular factors, including hormones and growth factors, enter the epididymal lumen and regulate the epithelium via a lumicrine mechanism [5, 6].

Gap junctions (GJ) are composed of transcellular pores between neighboring cells that permit the passage of small molecules (< 1Kda) including secondary messengers [7]. Each pore is comprised of two connexons, one from each cell, which are formed by the oligomerization of six transmembrane proteins called gap junction proteins (also known as connexins). Up to 21 gap junction proteins have been identified in mammals and they are named according to their sequence homology and length of their cytoplasmic domain [8]. Five gap junction proteins have been reported in the rat epididymis and likely contribute to segment-specific differences in epididymal physiology [9]. GJA1 is differentially targeted in the rat epididymis by androgens [10]. GJA1 is localized between basal and principal cells of the epididymal epithelium. However, following orchidectomy, GJA1 also localizes to the apical region of adjacent principal cells in the initial segment [10]. In orchidectomized rats given testosterone implants, GJA1 is again localized between basal and principal cells.

GJA1, like most gap junction proteins, is a phosphoprotein. Changes in phosphorylation have been associated with altered gap junction permeability. In the epididymis, GJA1 appears hyperphosphorylated following orchidectomy in total epididymal protein extracts [10], although GJA1 hyperphosphorylation is not believed to influence its targeting. Thus, the change in GJA1 localization resulting from orchidectomy raises the question as to whether or not alternate kinase activity and/or binding to GJA1 is regulated by androgens. GJA1 is a substrate for several kinases, including cyclic AMP-dependent protein kinase A (PKA), and the cellular Rous sarcoma oncogene (c-Src) [11]. PKA is a holoenzyme comprised of two regulatory and two catalytic subunits. Binding of cAMP to the regulatory subunits releases the catalytic subunits (PKAcat) from the holoenzyme, allowing phosphorylation of serine and threonine residues of downstream substrates [12]. PKAcat has been reported to phosphorylate the C-terminal region of GJA1 on serine residues 364, 365, 368, 369 and 373, enhancing gap junctional intercellular communication (GJIC) [13, 14]. Cellular Src is a non-receptor tyrosine kinase that is active when phosphorylated at tyrosine residue 416 and inactive when phosphorylated at tyrosine 527 [15]. In the active state, c-Src can phosphorylate GJA1 at tyrosine 247 and 265, inhibiting GJIC [16, 17]. Limited information exists on signaling molecules in the epididymis and neither PKAcat nor c-Src have been reported in the epididymal epithelium.

Tight junction protein 1 (TJP1) is a scaffolding protein and member of the membrane-associated guanylate kinase homologues (MAGUK) family associated with the integral proteins of tight, adherens and gap junctions [18]. Several groups have studied the role of TJP1 in regulating GJA1-containing GJs. The association of TJP1 to GJA1 has been implicated in targeting GJs to the plasma membrane [19]. TJP1 also

provides a scaffold for the regulation of GJ channels [20], assembly of GJs [21], and regulation of endocytosis of connexons [22] by other signaling effectors. In the rat epididymis, TJP1 is localized primarily to the apical plasma membrane of principal cells where tight and adherens junctions are located [23-25]. There are no reports regarding TJP1 association with gap junction proteins in the epididymis.

The objectives of the present study were: 1. to characterize the expression and localization of PKAcat and c-Src in the rat epididymis; 2. investigate the androgen regulation of GJA1 potential regulators: c-Src, PKAcat and TJP1; and 3. determine if these potential regulators could play a role in the androgen-dependent, segment-specific targeting of GJA1 in the epididymis.

MATERIALS AND METHODS

Animals

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

Androgen regulation

To assess whether or not PKAcat, c-Src or TJP1 were regulated by testicular factors or androgens, adult Sprague Dawley rats were anesthetized with an intraperitoneal injection of ketamine/xylazine/acepromazine (50/5/2.5mg/kg). A subcutaneous injection of the analgesic buprenorphine (0.3mg/kg) was administered following the anesthetic. Rats

(n=5 per group) were either sham-operated, orchidectomized via an abdominal incision, or orchidectomized and given three subcutaneous implants of testosterone (3 x 6.2 cm), as described previously, to maintain epididymal T [23]. Testosterone-filled polydimethylsiloxane capsules were prepared according to the method outlined by Stratton et al. [26] and have well characterized steroid release rates [27]. The implants mimic epididymal testosterone levels, which are 10 times greater than blood levels. The animals were killed seven days after surgery and epididymides were either perfused with Bouin fixative as previously described [23] or dissected, subdivided into the initial segment, caput, corpus and cauda and frozen in liquid nitrogen. Paired epididymides, seminal vesicles and the ventral prostates were weighed to assess testosterone implant efficacy [28].

Immunohistochemical analysis

Immunohistochemistry was performed as described by Dubé et al., [29] without heat induced epitope retrieval. Slides were incubated with a rabbit monoclonal antibody against c-Src (13.3 ng/mL, Cell Signaling, Beverly, MA) or a rabbit polyclonal antiserum against PKAcat (1.3 μg/mL, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 1 hr at room temperature. Sections incubated for 1 hr with PBS in the absence of primary anti-sera were used as negative controls.

Western blot analysis

Individual epididymal segments (n=3 per segment) were ground with a mortar and pestle in liquid nitrogen and transferred to lysis buffer (1:3 wt/vol; 60mM Tris-HCl (pH 6.8), 2mM $CaCl_2$, 40mM octyl β-D-glucopyranoside, 1μg/mL Pepstatin A; Sigma-

Aldrich Corp. St. Louis, MO) and supplemented with a protease inhibitor cocktail (Sigma-Aldrich Corp.). The tissue was homogenized with a Polytron (PowerGen 500, Fisher Scientific, Ottawa, ON) and transferred to a microcentrifuge tube and centrifuged as previously described [23]. Protein concentrations were determined using Bradford Reagent (Sigma-Aldrich Corp.) according to the manufacturer's instructions.

A temperature gradient was done for each antibody to determine optimal heating conditions for Western blot analyses. Protein samples (50-150 μg) were diluted in 2X sample buffer and either boiled (c-Src) or warmed to 70°C, 80°C, 27°C, or 45°C for GJA1, TJP1, PKAcat and p-Tyr-416-c-Src, p-Tyr-527-c-Src for 5 min, respectively. The samples were loaded onto polyacrylamide gels (see Table 1). Proteins were separated by electrophoresis until the dye front reached the end of the gel. Proteins were then transferred onto a nitrocellulose membrane (Bio-Rad Laboratories, Mississauga, ON) at either 400mA for 1 h or 900mA for 1.5 hrs under cooling conditions. Membranes were incubated with Ponceau red (0.6% wt/vol, 1% acetic acid) for 10 min at room temperature and subsequently washed with water to assess the efficiency of sample loading and protein transfer. Blots were then blocked in either 5% powdered milk in TBS-T (20mM Tris-HCl, 150mM NaCl, pH 7.4 and 0.1-0.25% Tween 20) or 5% Bovine serum albumin in TBS-T, for phospho-specific antibodies, for 1-2 hrs at room temperature with slow rocking. Membranes were incubated overnight at 4°C with the primary antibody diluted in blocking solution according to Table 1. The following day, blots were washed 3 x 10 min in TBS-T and subsequently incubated with the secondary antibody for 1 hr at room temperature (Table 1). Blots were washed 3 x 10 min in TBS-T followed by incubation with either Lumi-light Western blotting substrate (Roche, Rockford, IL) for horseradish

peroxidase secondary antibodies or with an alkaline phosphatase (AP)-conjugate substrate kit (Bio-Rad Laboratories) for AP-conjugated secondary antibodies, according to the manufacturer's instructions. Protein levels were quantified with a Fluor Image analyzer (Bio-Rad Laboratories) and normalized to either actin or p-Tyr-527-c-Src for p-Tyr-416-c-Src. For GJA1, the major band was used to quantify expression, whereas total expression was quantified for the different isoforms of c-Src and PKAcat.

Coimmunoprecipitation

Epididymal proteins were isolated as described above and used to immunoprecipitate GJA1. ExactaCruz agarose beads (50µl, Santa Cruz Biotechnology, Santa Cruz, CA) were incubated with 2µg of GJA1 antibody (Santa Cruz Biotechnology) or 2 µg of rabbit IgG for negative controls in 500µl of PBS (pH 6.8) for 2 hrs at 4°C in a rotating tube to allow the formation of the bead-antibody complex. The complex was centrifuged in a microcentrifuge at maximum speed for 30s and washed in PBS according to the manufacturer's instructions. Tissue lysates (800 µg, n=3 per group) were precleared with ExactaCruz preclearing matrix (Santa Cruz Biotechnology) according to the manufacturer's instructions; negative controls consisted of 266 µg of tissue lysate from three biological replicates. The precleared lysate was transferred to the washed bead-antibody complex and incubated overnight at 4°C in a rotating tube. The following day the beads were recovered by centrifugation in a microcentrifuge at maximum speed for 30s. The resulting pellet was washed three times in 500 µl of PBS (pH 6.8) with centrifugation at maximum speed for 30s between washes. After the final wash, the complex was eluted from the beads by adding 40 µl of 2X sample buffer and heating to

70°C for 5 min. The samples were quick spun and 20 µl was loaded for Western blot analysis as described above. As a positive control, 100µg of tissue lysate from one animal was used.

Statistical analysis

Statistical analysis was performed on Western blot data to determine significant differences in normalized protein expression with orchidectomy or orchidectomy with testosterone maintenance as compared to controls. Normal distribution was determined using the Kolmogorov-Smirnov test and equal variance was tested using the Levene Median test. A one-way ANOVA was performed to detect statistical differences between groups and Tukey's post-hoc test (or Student-Newman-Keuls method as specifically indicated) where appropriate. All statistical tests were carried out with SigmaStat computer software (Jandel Scientific Software, San Rafael, CA). Significance was established at $P<0.05$.

RESULTS

Characterization of c-Src and PKAcat in the adult rat epididymis

To determine if c-Src or PKAcat were expressed in the adult rat epididymis, Western blot analysis was done on proteins from each of the four segments of the epididymis. Cellular Src and PKAcat were strongly expressed in each of the epididymal segments (Fig. 1). The c-Src antibody recognizes several members of the c-Src tyrosine kinase family; multiple bands on the Western blot between 50 and 60 kDa indicated that several members of this family are expressed in all four epididymal segments. Similarly, the PKAcat antibody recognizes three isoforms of the subunit. Both the alpha and beta

isoforms of PKAcat (approximately 40kDa) were detected in all four segments of the adult rat epididymis.

The cellular localization of c-Src along the epididymis indicated a segment-specific expression pattern (Fig. 2). Cellular Src was localized at the plasma membrane between basal and principal cells in all four epididymal segments (Fig. 2). In the initial segment, c-Src was also present at the apical region of narrow cells. In the caput, c-Src immunoreaction was detected in some microvilli of principal cells, along the lateral plasma membrane of some principal cells and faintly in the cytoplasm. The corpus was similar to the caput; however, there was a much stronger immunoreaction at the lateral plasma membrane between principal cells, the microvilli of principal cells and the apical plasma membrane of principal and clear cells. A strong cytoplasmic reaction was noted in the corpus, as was an immunoreaction over the myoid cells of this segment that was not observed in other segments. In the cauda epididymidis, a strong c-Src immunoreaction in the microvilli and apical plasma membrane was observed.

PKAcat immunolocalization was less variable throughout the epididymis and the immunoreaction appeared punctate, suggesting localization to intracellular vesicles or association with A kinase anchoring proteins (AKAPs) [30, 31]. This punctate reaction was observed between basal and principal cells, in nuclei, at the lateral plasma membrane between principal cells, in the cytoplasm, myoid layer, endothelium and spermatozoa of all four segments, with the cytoplasmic reaction appearing much stronger in the corpus (Fig. 3). This punctate reaction was also observed by immunofluorescence on cryosections using the same antibody (data not shown).

Assessment of testosterone implant efficacy

Paired epididymides, seminal vesicles and the ventral prostates were weighed to assess testosterone implant efficacy in orchidectomized animals with testosterone maintenance. The values were expressed as the percent of total animal body weight ± SEM (Table 2). The percent weight of prostate and seminal vesicles for orchidectomized animals was significantly different from control and orchidectomized animals with testosterone maintenance.

Regulation of epididymal GJA1, c-Src, PKAcat and TJP1

To determine if the androgen-dependent targeting of GJA1 was associated with changes in GJA1 protein synthesis, Western blot analyses of proteins from the initial segment and caput epididymidis from sham-operated control, orchidectomized, and orchidectomized with testosterone maintenance were compared (Fig. 4). GJA1 unphosphorylated (P0) protein levels were similar in the initial segment and caput for all treatment groups, with no significant differences between the two segments (Fig. 4A). For both segments, orchidectomy decreased GJA1 expression that was significant in the caput, but was not reversed by testosterone maintenance for either segment. This suggests that while GJA1 targeting is androgen-dependent, its protein expression is dependent on testicular factors. While there were no apparent changes in GJA1 phosphorylation in the initial segment, the P1 phosphorylated form was dependent on testicular factors in the caput (Fig. 4B).

Total c-Src expression significantly decreased with orchidectomy in both segments and was partially maintained in orchidectomized rats given testosterone in the caput. This suggests that c-Src expression is both androgen and testicular factor-

dependent (Fig. 5A). Although actin levels appeared to increase in orchidectomized animals, Ponceau red staining indicated that this was a reflection of protein loading and not changes in actin expression. To determine if androgens influenced the activity of c-Src, phospho-specific antibodies against the active (p-Tyr-416) and inactive (p-Tyr-527) forms of c-Src were used (Fig. 5B). No significant differences were observed in the ratio of active to inactive c-Src among the different treatment groups in the initial segment. However, in the caput, the ratio was significantly increased with orchidectomy. In orchidectomized rats given testosterone implants, the ratio of active to inactive c-Src was similar to controls. Thus, while total c-Src expression was partially androgen-dependent for both the initial segment and caput, c-Src activity was only androgen-dependent in the caput.

PKAcat levels were lower, although not significantly, in the initial segment and caput of orchidectomized rats. In orchidectomized rats given testosterone, PKAcat expression was significantly decreased in the caput (Fig. 5C).

TJP1 levels were also lower in the initial segment and caput epididymidis of orchidectomized and orchidectomized with testosterone maintenance rats; however, the effects were not statistically significant (Fig. 5D).

Regulation of c-Src and PKAcat targeting by androgens

To determine if androgens regulate the intracellular targeting of c-Src and/or PKAcat in the initial segment or caput epididymidis, immunocytochemistry was performed on sections from bilaterally orchidectomized adult rats sampled seven days after surgery (Fig. 6). In orchidectomized animals, there was an intense accumulation of c-Src immunostaining at the apical region of principal cells dispersed within a cross-

sectional tubule in both the initial segment and caput epididymidis (Fig. 6). Cellular Src was also observed in the perinuclear region of some principal cells that did not demonstrate this apical accumulation. Microvilli were immunopositive, but basal and principal cell staining was similar to control animals. In orchidectomized rats administered testosterone, the apical and perinuclear immunostaining resembled controls. Basal and principal cell staining was much less intense, but immunostaining of the microvilli persisted.

There were no apparent changes in the localization of PKAcat in either epididymal segment with orchidectomy (Fig. 6).

Association of GJA1 with c-Src, PKAcat, and TJP1

To examine if there was a direct association between GJA1 and c-Src, PKAcat or TJP1 coimmunoprecipitation was performed using protein from the initial segment and caput epididymidis of sham-operated control, orchidectomized and orchidectomized rats given testosterone implants (Fig. 7). In the initial segment, a direct association was observed between GJA1 and c-Src and GJA1 and TJP1, but not GJA1 and PKAcat, for all three treatment groups (n=1 for controls, n=2 for orchidectomized animals and n=3 for orchidectomized animals with testosterone maintenance) (Fig. 7A). In the caput, a GJA1-c-Src interaction was not observed (Fig. 7B). An interaction between TJP1 and GJA1 was observed in the caput, although this appeared to be much weaker than in the initial segment (n=3 for all treatment groups) (Fig. 7B). There was no association between GJA1 and PKAcat. No differences were observed between treatment groups.

DISCUSSION

There is little information on the regulation of gap junctional proteins in the epididymis. Previous studies from our laboratory have revealed that the cellular localization of GJA1 in epididymal principal cells is regulated, at least in part, by testicular androgens [10]. While the intracellular targeting of GJA1 may be regulated by numerous factors, studies have reported that c-Src, PKAcat and TJP1 directly bind to GJA1 in other tissues [14, 16, 32-34] suggesting that these may be involved in the localization of GJA1. PKAcat and c-Src have been reported by microarray analysis in the rat initial segment [35], but their protein expression and regulation are unknown in the epididymis.

Both immunohistochemistry and Western blot analyses indicated that PKAcat was present throughout the epididymis. PKAcat immunostaining was most intense in the corpus, and western blot analyses indicated multiple PKAcat isoforms, suggesting that PKAcat may be associated with different signaling functions or pathways in the epididymis. PKAcat is a downstream effector of receptor-mediated processes [36], but has also been shown to act as a transcriptional regulator [37], and environmental mediator [38]. The punctate immunoreaction throughout the epididymis suggests that PKAcat may associate with other proteins, such as A kinase anchoring protein (AKAPs) [30]. AKAPs confer signaling specificity to the PKA holoenzyme by sequestering it to different subcellular compartments [31]. AKAPs are present in spermatozoa [30, 39, 40], and AKAP transcripts have been detected in murine epididymides using microarray analyses [35].

PKAcat levels were significantly decreased in the caput with orchidectomy plus testosterone maintenance, whereas no effect was observed in the initial segment. The

decrease in PKAcat may reflect differences in cAMP response, since cAMP has been shown to upregulate PKA expression [41]. It has been reported that epididymal epithelial cells isolated from orchidectomized rats have decreased response to cAMP [42].

Multiple c-Src tyrosine family kinase members were also detected by Western blot in all four segments of the epididymis. This suggests that c-Src may mediate signaling cascades in all epididymal regions, although analyses of active c-Src in the initial segment and caput (Fig. 5B) suggest that the activation of c-Src is segment-specific. Immunocytochemical data indicate that c-Src is localized between basal and principal cells throughout the epididymis and at the lateral plasma membrane of adjacent principal cells in the caput and corpus, as well as in narrow cells of the initial segment, and in microvilli and apical plasma membrane of principal cells in the cauda. Differences in c-Src localization along the length of the epithelium support the notion that the regulation of cellular signaling pathways in each epididymal segment is unique and may provide specific contributions to spermatozoal maturation [3, 43].

Total c-Src expression significantly decreased with orchidectomy in both the initial segment and caput. Testosterone maintenance partially maintained c-Src expression, but only in the caput. This is in contrast to the testis, where Wong et al. (2005) reported that androgen suppression resulted in upregulated c-Src expression. Phospho-specific antibodies indicated that the ratio of active to inactive c-Src remained constant in the initial segment, in all treatment groups, but in the caput this ratio was significantly increased. These results indicate that despite a decrease in total c-Src expression, orchidectomy does not change c-Src kinase activity in the initial segment. These data therefore suggest that changes in GJA1 targeting are not due to alternate c-Src kinase activity in the initial segment.

The presence of c-Src and PKAcat between basal and principal cells, where GJA1 has been reported [10], suggested that they may directly regulate GJA1-containing gap junctions. This has been demonstrated in other tissues and species [14, 16, 32, 34, 44].

The immunolocalization of PKAcat was unaltered by orchidectomy, suggesting that it is unlikely to be involved in regulating the localization of GJA1. Conversely, in orchidectomized rats, c-Src was targeted to the apical region of some principal cells, to the perinuclear region of cells where apical staining was absent, and to microvilli, but basal and principal cell staining was similar to that observed in controls. Inactive c-Src is normally found at the perinuclear region of cells associated with endosomal compartments, while active c-Src is found at the periphery associated with focal adhesions [45]. Since c-Src is active in orchidectomy and at the apical region of some principal cells (this study) and associates with GJA1 in the active state [16], it is plausible that a direct association between c-Src and GJA1 influenced GJA1 localization.

TJP1 levels were unchanged by orchidectomy or by orchidectomy with testosterone maintenance in both the initial segment and caput epididymidis. TJP1 has been reported to be an important component of both adherens and tight junctions in the epididymis [23, 25]. In the aging Brown Norway rat, decreased levels of TJP1 are associated with a loss in blood-epididymal barrier function [25].

While GJA1 expression and phosphorylation were reported to increase with orchidectomy using total epididymal protein, the segment-specific effect of androgens was not established [10]. In this study, Western blots were done on the initial segment to determine if orchidectomy influenced the levels of GJA1. The caput was used as a control, since GJA1 targeting is not altered in this segment. The results indicated that there was a significant decrease in GJA1 protein expression with orchidectomy in the

caput and orchidectomy with testosterone maintenance in the caput and initial segment. The results suggest that testicular factors are important in regulating GJA1 expression. The testicular factor-dependency of the GJA1 P1 form in the caput also supports this. Several growth factors regulate GJA1 in other tissues [46] and these may play a role in regulating gap junctions in the epididymis [5]. GJA1 phosphorylation was unchanged in the initial segment, indicating that direct changes in phosphorylation were not influencing GJA1 targeting.

Coimmunoprecipitation studies indicated that in the initial segment, GJA1 associated with c-Src and TJP1, but not with PKAcat. In the caput, however, only a weak association was observed between GJA1 and TJP1. This indicates that GJA1 preferentially associates with its partners in the initial segment. Given the direct association and cellular localization of GJA1, c-Src and TJP1, it is possible that in control and orchidectomized animals given testosterone implants, c-Src acts as a negative regulator of GJIC [16, 32, 34, 47] and TJP1 may influence GJA1 incorporation into GJs [19, 48]. Further studies are necessary to address this possibility.

In conclusion, c-Src and PKAcat appear to be important mediators of signal transduction in the epididymis. Cellular Src may contribute to segment-specific physiological differences that are thought to be important for spermatozoal maturation. While c-Src and TJP1 associate with GJA1, they do not appear to be important regulators of GJA1 cellular localization, but may play a role in gap junction inhibition.

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FIGURE LEGENDS

Figure 1. Characterization of c-Src and PKAcat in the adult rat epididymis. Western blot analysis of tissue lysates from individual epididymal segments indicates multiple c-Src-family kinase members are expressed, as are the alpha and beta isoforms of the PKA catalytic subunit. IS: initial segment; CT: caput; CS: corpus; CA: cauda; T: testis positive control.

Figure 2. Localization of c-Src in the adult rat epididymis by immunocytochemistry. C-Src was observed in all four epididymal segments. C-Src is localized between basal (B) and principal (P) cells in all segments (white arrows), in the apical region of narrow cells (N) in the initial segment (white arrowheads), at the lateral plasma membrane in the caput and corpus (black arrowheads), cytoplasmic in the caput and corpus (open black arrow), in the microvilli of the caput, corpus and cauda (black arrows), the myoid layer of the corpus (double-headed black arrow) and the apical plasma membrane of the cauda (open white arrow). Lu: lumen; IT: intertubular space; S: spermatozoa. Magnifications are 1000x. PBS replaced the primary antibody for the negative control.

Figure 3. Localization of PKAcat in the adult rat epididymis by immunocytochemistry. PKAcat was observed in all four epididymal segments. PKAcat is localized between basal (B) and principal (P) cells in all segments (white arrows), in nuclei (asterix), the cytoplasm (open white arrows), intertubular space (IT) (black arrows) and spermatozoa (S) (black arrowhead). C: clear cell; Lu: lumen; Magnifications are 1600x. PBS replaced the primary antibody for the negative control.

Figure 4. Segment-specific expression of GJA1 in the initial segment (IS) and caput (CT) of sham-operated control (Ctrl), orchidectomy (Orch) and orchidectomy with testosterone maintenance (Orch + T) of adult rat epididymidis (A) by Western blot (n=3 per treatment group). Data are presented as the mean of three biological replicates normalized to actin ± SEM. P values of <0.05 were considered to be statistically significant. Orchidectomy decreases the expression of unphosphorylated GJA1 in both the initial segment and caput and is not reversed by testosterone maintenance. (B) The film was overexposed to visualize phosphorylated forms of GJA1 (P1 and P2). In the initial segment, P1 and P2 forms do not change between treatment groups, whereas the P1 form (GJA1 with one phosphorylation) is testicular factor-dependent in the caput. P0: unphosphorylated GJA1; P2: GJA1 phosphorylated on two amino acids.

Figure 5. Western blot analysis of total c-Src (A), active and inactive c-Src (B), PKAcat (C) and TJP1 (D) in the initial segment and caput of sham-operated control (Ctrl), orchidectomy (Orch) and orchidectomy with testosterone maintenance (Orch + T) of adult rat epididymidis (n=3 per treatment group). Data are presented as the mean of three biological replicates normalized to actin, with the exception of active and inactive Src, ± SEM. Student-Newman-Keuls Method was used for total c-Src in the caput (A). P values of <0.05 were considered to be statistically significant (asterix). Orchidectomy decreases the expression of total c-Src, PKAcat and TJP1 in both the initial segment and caput, whereas active c-Src is significantly increased in the caput by orchidectomy. Testosterone maintenance reverses the effect for active c-Src in the caput and partially restores total c-Src expression in the initial segment and caput and PKAcat expression in the caput.

Figure 6. Immunocytochemistry of c-Src and PKAcat in sham-operated controls, orchidectomy (Orch) and orchidectomy with testosterone maintenance (Orch + T) in the initial segment of the epididymis. C-Src localization was altered in orchidectomy to the apical region of the epithelium in some cells within a tubule cross-section (white arrowheads). The microvilli (black arrows) and the plasma membrane between basal (B) and principal (P) cells were immunoreactive (white arrows). C-Src was also found in the perinuclear region of some principal cells (white asterix). Testosterone maintenance did not alter microvilli staining; however, apical and perinuclear staining reverted to that observed in the controls, whereas plasma membrane staining between basal and principal cells was less than in controls. PKAcat immunoreaction was similar in all three treatment groups. Open arrows indicate cytoplasmic immunoreaction and black asterix indicate nuclear immunoreaction. Similar results were observed in the caput region for both c-Src and PKAcat (not shown). PBS replaced the primary antibody for the negative control. Magnifications are 1000x. N: narrow cell; IT: intertubular space; Lu: lumen; S: spermatozoa.

Figure 7. Segment-specific coimmunoprecipitation (CoIP) of GJA1 and its binding partners in sham-operated controls, orchidectomy (Orch) and orchidectomy with testosterone maintenance (Orch + T). In the initial segment (A), c-Src and TJP1 CoIPed with GJA1 in a treatment-independent manner, whereas there was no interaction between PKAcat and GJA1. (B) C-Src did not CoIP with GJA1 in the caput for any treatment group. A low level of TJP1 was detected to bind to GJA1. There was no interaction with GJA1 and PKAcat. Numbers indicate biological replicates. NC: Negative control; PC:

Positive control; NC^{C}: Negative control for control; NC^{O}: negative control for orchidectomy; NC^{O+T}: Negative control for orchidectomy with testosterone replacement; Beads: supernatant from Exactacruz beads boiled in 2X sample buffer.

Table 1. Antibodies and Western blot conditions used in this study

Protein	% Poly-acrylamide	Total Protein Loaded Per Well (μg)	Primary Antibody			Secondary Antibody		
			Source	Concentration (μg/mL)	Company	Source	Concentration (μg/mL)	Company
GJA1	10	150	Rabbit Poly	2	Santa Cruz	Anti rabbit HRP	0.2	Abcam
c-Src	8	50	Rabbit Mono	0.002	Cell Signaling	Anti rabbit HRP	0.04	Santa Cruz
p-Tyr-416-c-Src	8	50	Rabbit Poly	0.02	Cell Signaling	Anti rabbit HRP	0.2	Abcam
p-Tyr-527-c-Src	8	50	Rabbit Poly	0.1	Cell Signaling	Anti rabbit HRP	0.2	Abcam
PKAcat	10	50	Rabbit Poly	1	Santa Cruz	Anti rabbit HRP	0.2	Abcam
TJP1	6.5	70	Rabbit Poly	2.5	Zymed	Anti rabbit AP	0.8	Santa Cruz
Actin	6.5-10	50-150	Mouse Mono	1	Sigma	Anti mouse HRP	0.08	Santa Cruz

Table 2. Organ weights expressed as a percentage of the animal body weight

	% Weight of Epididymides	% Weight of Prostates	% Weight of Seminal Vesicles
Control	0.26 ± 0.019	0.11 ± 0.014	0.19 ± 0.036
Orchidectomy	0.15 ± 0.048	0.014 ± 0.0054*	0.074 ± 0.011*
Orchidectomy + T	0.27 ± 0.020	0.14 ± 0.018	0.19 ± 0.020

* $p<0.05$

Figure 1

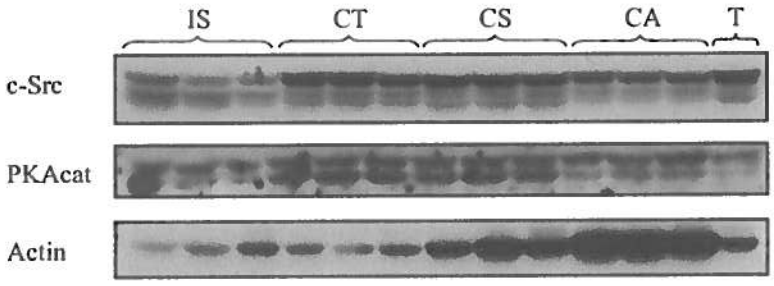


Figure 2

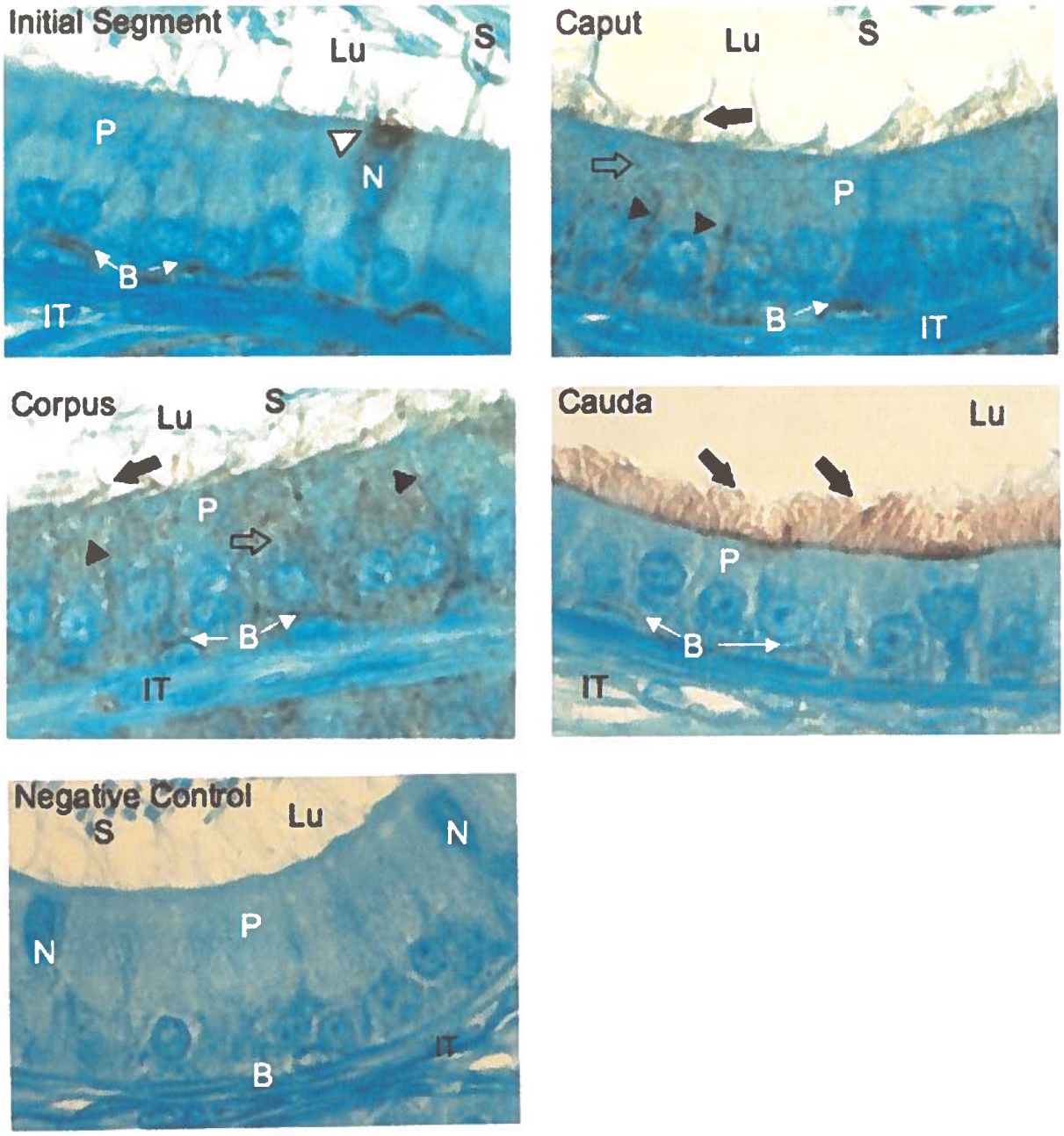

Initial Segment
Lu
S
P
N
B
IT
Caput
Lu
S
P
B
IT
Corpus
Lu
S
P
B
IT
Cauda
Lu
P
B
IT
Negative Control
S
Lu
N
P
N
IT
B

Figure 3

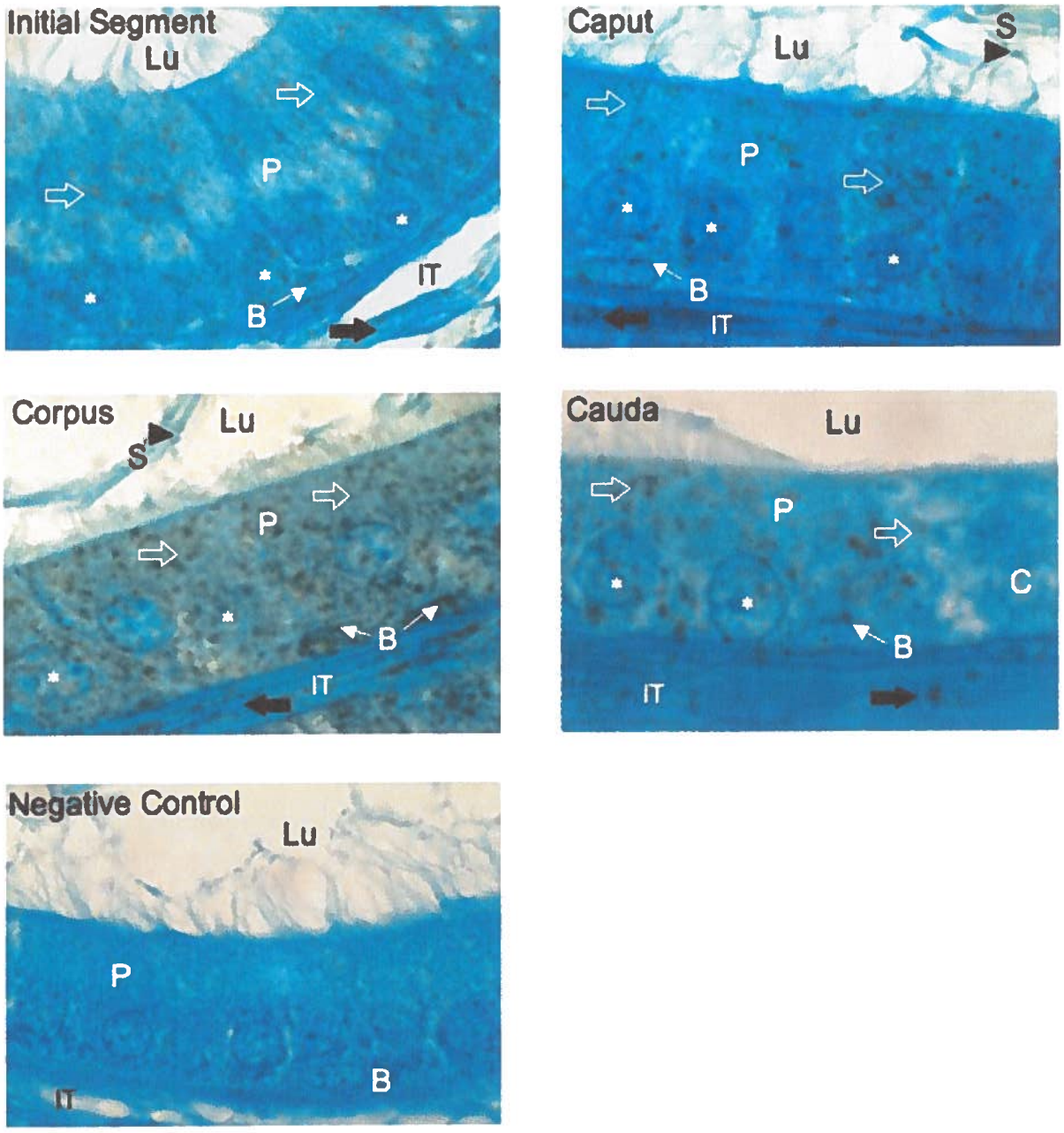
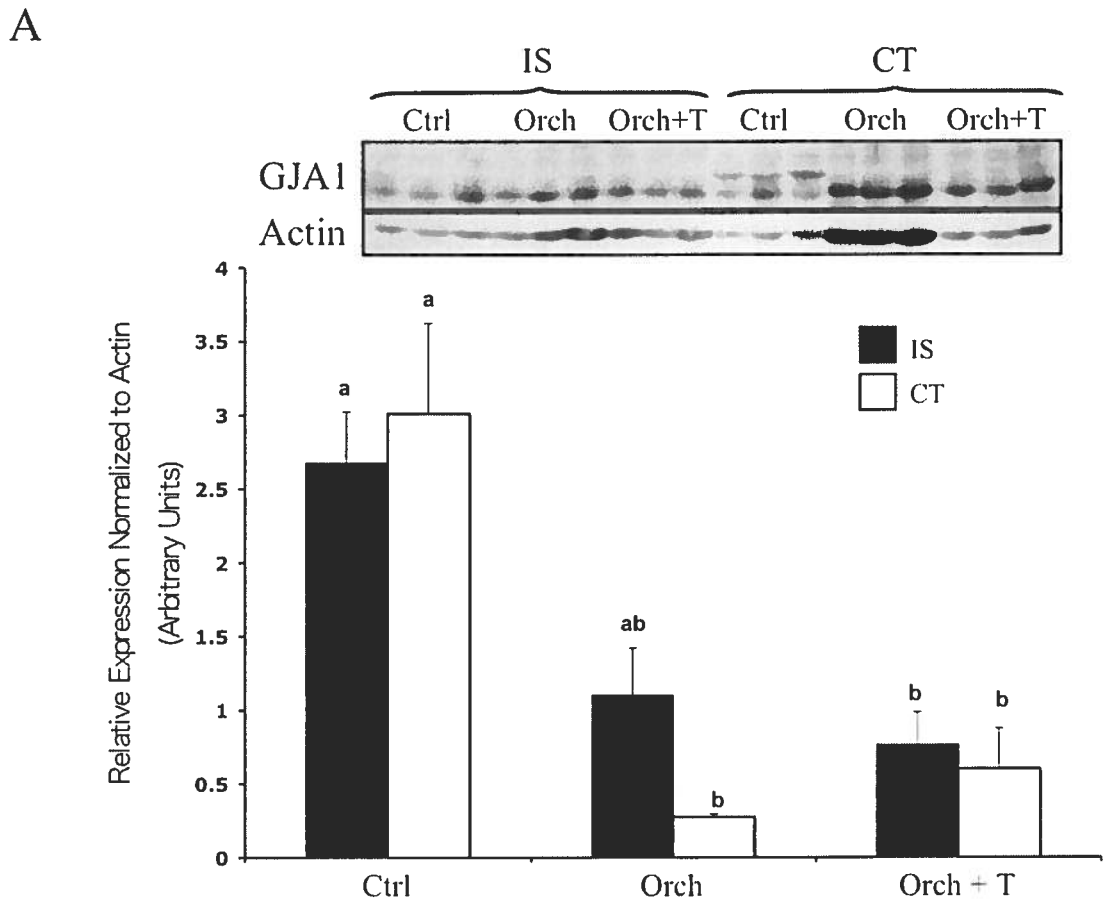
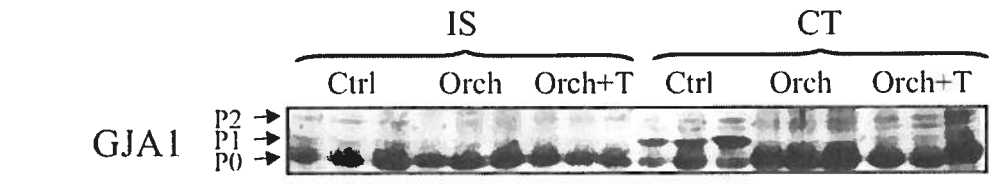

Initial Segment
Lu
P
IT
B
Caput
Lu
S
P
B
IT
Corpus
S
Lu
P
B
IT
Cauda
Lu
P
C
B
IT
Negative Control
Lu
P
B
IT

Figure 4


A
IS
CT
Ctrl
Orch
Orch+T
Ctrl
Orch
Orch+T
GJA1
Actin
Relative Expression Normalized to Actin
(Arbitrary Units)
4
3.5
3
2.5
2
1.5
1
0.5
0
a
a
ab
b
b
b
IS
CT
Ctrl
Orch
Orch + T


B
IS
CT
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Orch
Orch+T
Ctrl
Orch
Orch+T
GJA1
P2
P1
P0

Figure 5

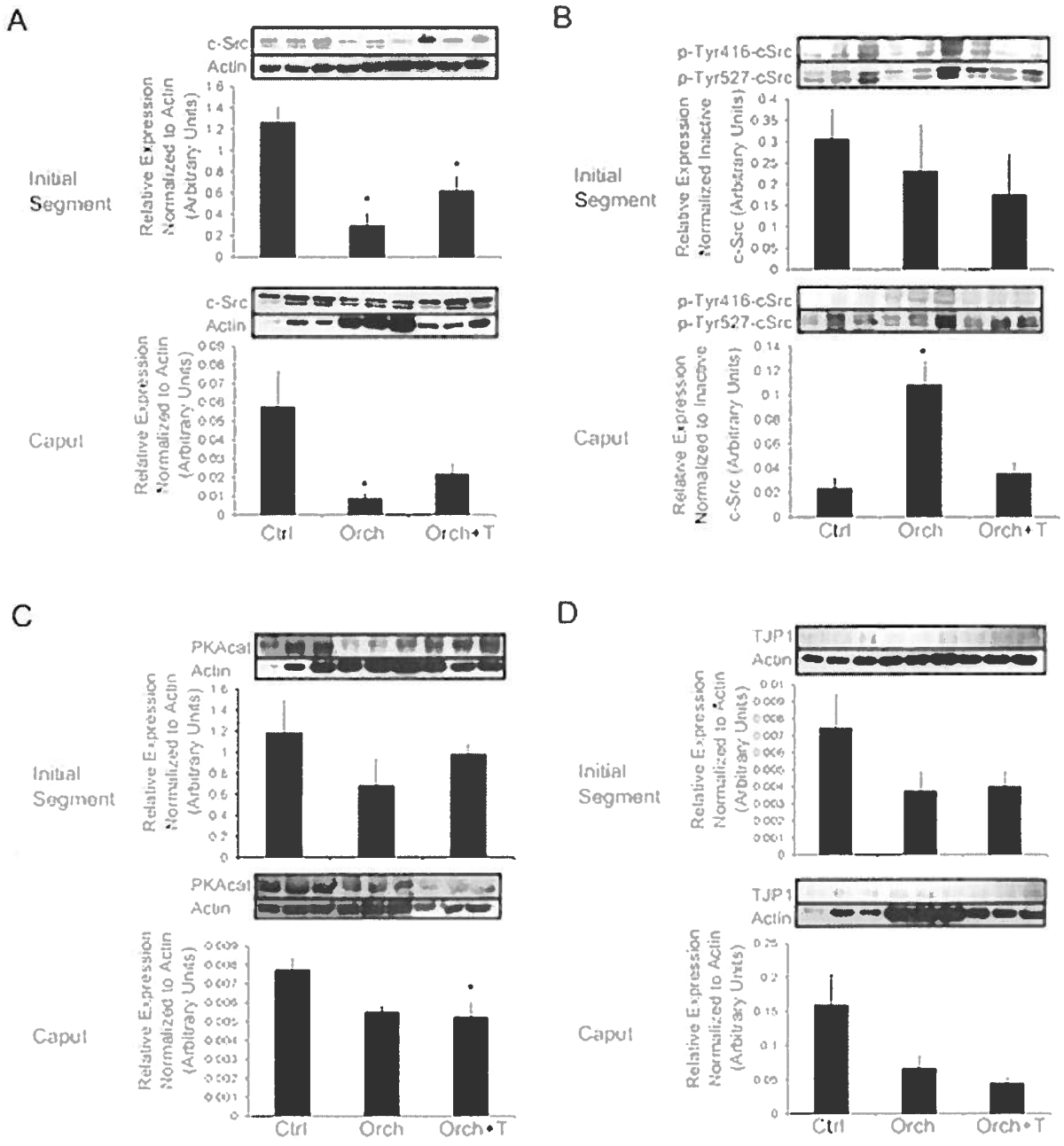

A
c-Src
Actin
Initial Segment
Relative Expression Normalized to Actin (Arbitrary Units)
Caput
Ctrl
Orch
Orch+T
B
p-Tyr416-cSrc
p-Tyr527-cSrc
Relative Expression Normalized Inactive c-Src (Arbitrary Units)
Relative Expression Normalized to Inactive c-Src (Arbitrary Units)
C
PKAcat
Actin
D
TJP1
Actin

Figure 6

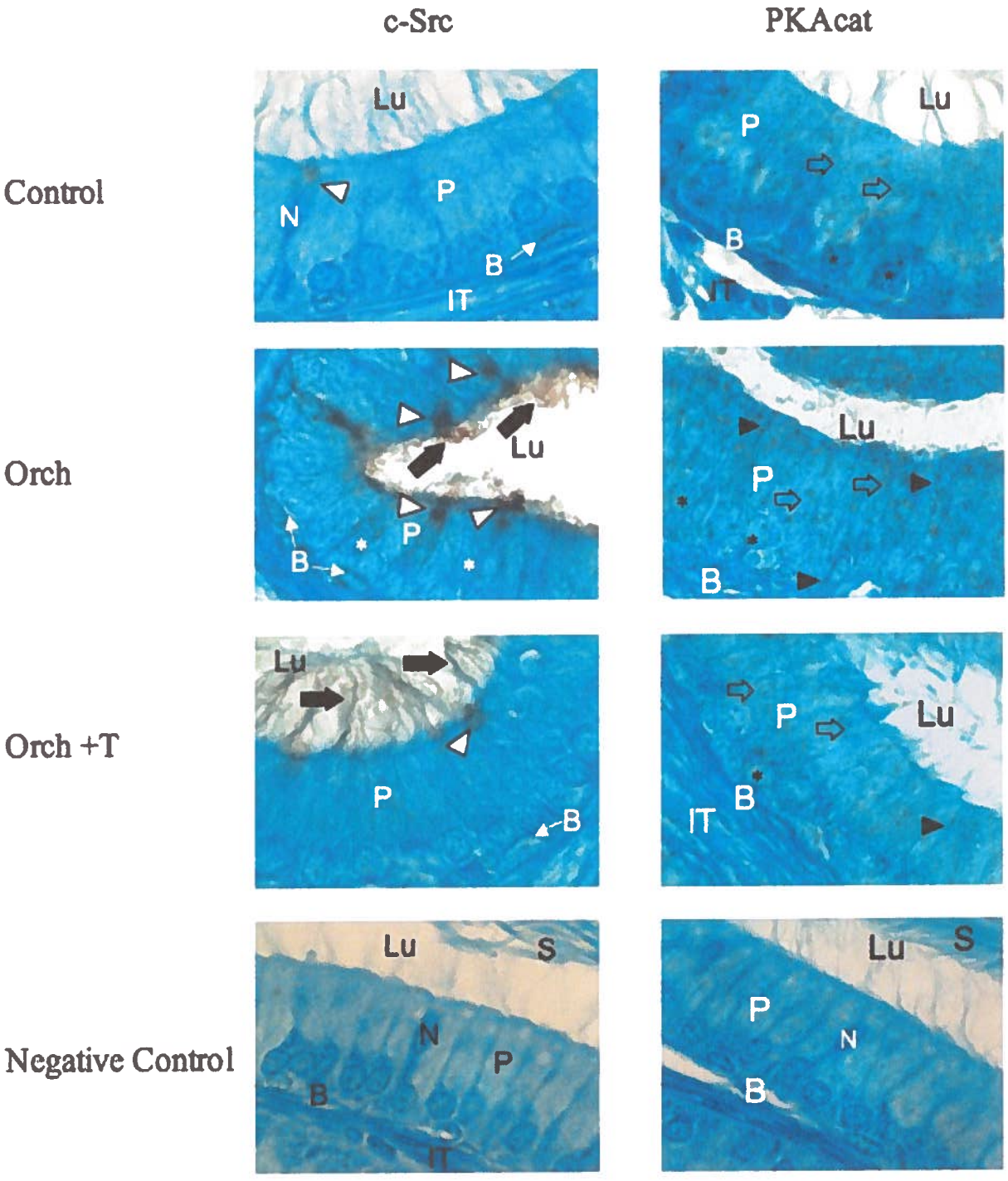
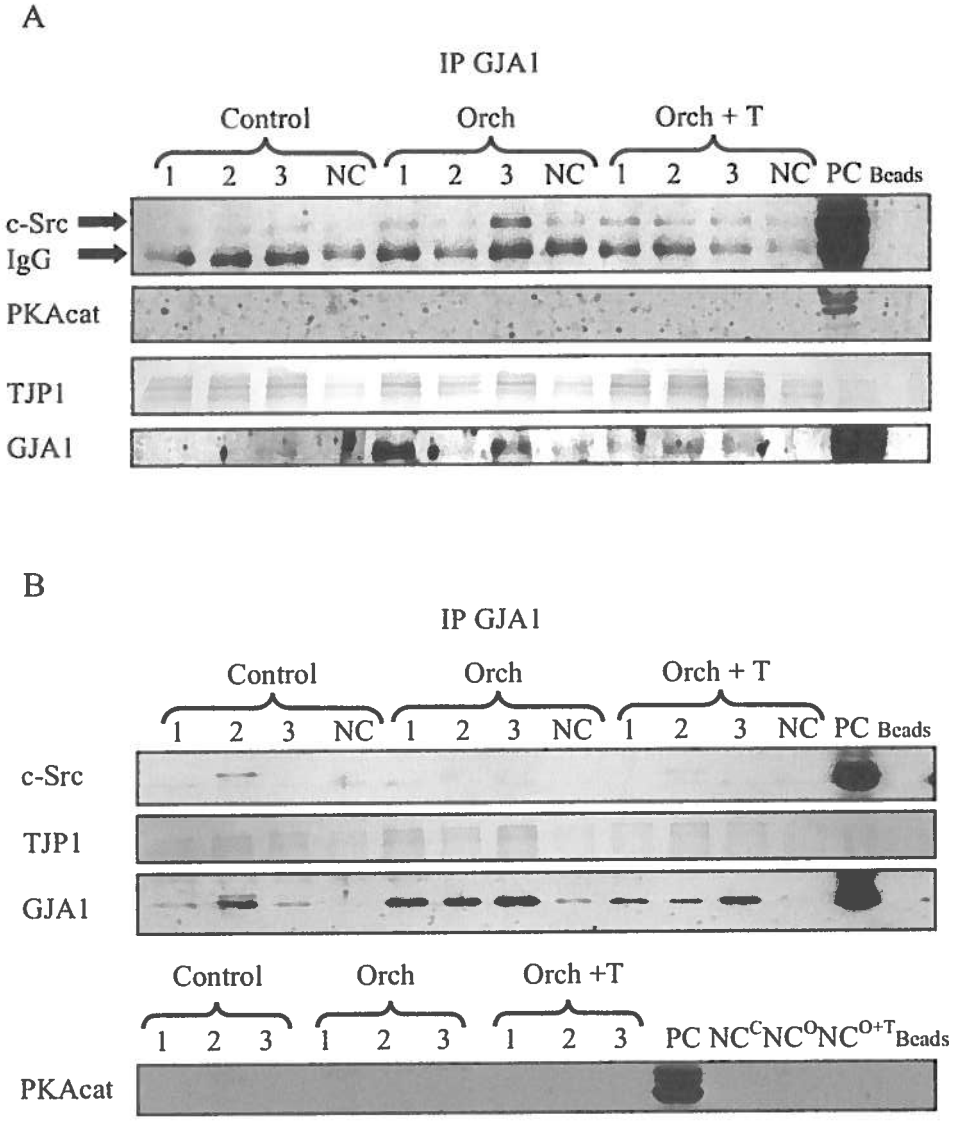


Figure 7


A
IP GJA1
Control
Orch
Orch + T
1 2 3 NC 1 2 3 NC 1 2 3 NC PC Beads
c-Src
IgG
PKAcat
TJP1
GJA1
B
IP GJA1
Control
Orch
Orch + T
1 2 3 NC 1 2 3 NC 1 2 3 NC PC Beads
c-Src
TJP1
GJA1
Control
Orch
Orch +T
1 2 3 1 2 3 1 2 3 PC NC^C NC^O NC^O+T Beads
PKAcat

CHAPTER 3: DISCUSSION AND CONCLUSIONS

This study investigated possible mechanisms for the androgen-dependent targeting of GJA1 in the initial segment of the rat epididymis. The results indicated that GJA1 targeting was independent of: its protein synthesis or phosphorylation state, the regulation of GJA1 binding proteins, or a direct association between GJA1 and c-Src, PKAcat or TJP1. Though a possible mechanism of GJA1 targeting could not be elucidated, the signalling effectors c-Src and PKAcat were identified in the epididymal epithelium for the first time. Additionally, GJA1, c-Src and PKAcat were regulated by a combination of testicular factors. Finally, the results indicated that GJA1 preferentially associated with c-Src and TJP1 in the initial segment.

Characterization of PKAcat and c-Src

While signalling effectors are generally thought to be important in epididymal regulation, few studies have identified signal transduction pathways. The present study demonstrated that c-Src and PKAcat were expressed throughout the epididymis, supporting the idea that functional signalling pathways are present (Tomsig et al., 2006; Cotton et al., 2008).

PKAcat immunostaining was fairly ubiquitous in all segments, except the corpus where the immunoreaction was much stronger. Since cAMP normally upregulates PKA by transcription, mRNA stability and translation (Skalhegg and Tasken, 1997), the intensity of PKAcat suggests that cAMP signalling is more prominent in this segment. Enzymatic assays would help to confirm this.

PKA is important for spermatozoal maturation in mouse and possibly human. Spermatozoa from homozygous *Pkacatα2* knockouts have defective forward motility (Skalhegg et al., 2002), while PKAcat α is localized to the midpiece of human, motile spermatozoa (Reinton et al., 2000). In the present study, PKAcat α was detected by Western blot and PKAcat was localized to spermatozoa. Therefore, PKAcat may also be important for rat spermatozoal motility.

C-Src localization had distinct and overlapping expression patterns in the epididymis. In all segments, c-Src was localized at the plasma membrane between basal and principal cells. The localization between heterologous cell types suggests that basal

and principal cells may communicate via signal transduction pathways. Indeed, studies have identified paracrine interactions in the epididymis (for instance Leung et al., 2004; Shum et al., 2008). In the caput and corpus, c-Src localized at the lateral plasma membrane suggesting that c-Src is capable of signal transduction between homologous cells. In the cauda, c-Src localized at the apical plasma membrane suggesting a role for lumicrine signalling in this segment. Immunocytochemical studies using phospho-specific antibodies will help to validate these possibilities.

C-Src was also localized to narrow cells, suggesting that it could play a role in degrading or recycling luminal proteins in this cell type; c-Src is found in endocytic vesicles and its kinase activity regulates endosomal trafficking (Sandilands and Frame, 2008). C-Src may also play a role in regulating the luminal pH. C-Src affects the assembly of a functional acidification apparatus in osteoclasts (Edwards et al., 2006). Additional support for an acidification role includes c-Src localization to microvilli with increasing intensity along the caput, corpus and cauda. Microvilli contain membrane pumps, transporters and channels involved in regulating the volume and pH of the epididymal epithelium and lumen (Primiani et al., 2007 and references within), which becomes increasingly acidic (Turner, 2002).

The c-Src antibody employed in this study did not immunoreact with spermatozoa in the epididymal lumen. This antibody recognizes the Src-related tyrosine kinase, c-Yes, which has been reported in human spermatozoa (Leclerc and Goupil, 2002). The absence of an immunoreaction in this study suggests that c-Yes is not expressed in rat spermatozoa. This is further supported by protein microarray data from this laboratory where c-Yes was undetected in the caput (Gravel et al. unpublished results). Thus, Src family members may represent an important distinction between human and rat spermatozoal maturation.

Functional studies on signal transduction pathways in the epididymis will be key in deciphering segment-specific physiological outcomes.

C-Src, PKAcat and TJP1 Regulation

The androgen regulation of c-Src, PKAcat and TJP1 was investigated to identify if a change in their expression could explain a change in GJA1 targeting. The results

indicated that neither c-Src, PKAcat nor TJP1 were solely androgen-dependent in the initial segment, as would have been expected if they influenced GJA1 intracellular localization. Yet, each GJA1 potential regulator demonstrated unique profiles of regulation in the initial segment and caput epididymidis.

C-Src expression was decreased after orchidectomy in both the initial segment and caput. Wong *et al.* (2005) used a sperm-sloughing model showing that androgen suppression upregulates c-Src expression in the adult rat testis. The difference in results between their study and ours may reflect the experimental methods used, or a means to differentially control signal transduction between the testis and epididymis. The latter possibility is substantiated by the androgen regulation of c-Src kinase activity.

Src kinase is activated when an agonist binds to the androgen receptor (Migliaccio et al., 2000; Kousteni et al., 2001; Zhoul et al., 2005). This increases cellular proliferation in both normal and cancerous cell lines such as Sertoli cells and prostate carcinoma-derived cells (LNCaP) (Migliaccio et al., 2000; Cheng et al., 2007). This activation of Src kinase is in contrast to the results from this study; in the initial segment, androgens did not influence c-Src activation, while in the caput androgens suppressed c-Src activity. The difference in Src regulation may represent an important mechanism for preventing cellular proliferation in the epididymis. Moreover, it may contribute to the low incidence of epididymal cancers compared to the prostate and testis.

Growth factor signalling and MAPK activation is limited by segmented boundaries in the initial segment (Tomsig et al., 2006). The present data support this, since c-Src activity was differentially regulated in the initial segment than the caput. In the initial segment, c-Src kinase activity was both androgen and testicular factor-independent. This suggests that either a non-androgenic circulating factor, or a mediator of stromal-epithelial signalling govern c-Src activity. Paracrine mediators of c-Src could include G-protein coupled receptors in stromal cells or receptor tyrosine or serine/threonine kinases. Numerous non-androgenic circulating factors exist. Techniques such as those employed by Tomsig *et al.* (2006) would be useful in determining whether paracrine or endocrine mechanisms regulate c-Src activity in the initial segment.

PKAcat expression was testicular factor-dependent in the caput. The regulatory and catalytic subunits of the PKA holoenzyme are regulated independently of one another, or in concert (Skalhegg and Tasken, 1997). A high dose of a DHT inhibitor down regulates PKA regulatory II β subunit gene expression in the rat caput (Henderson et al., 2004). Since PKAcat expression with orchidectomy plus testosterone maintenance was significantly different from controls, the present data suggest that PKAcat is not regulated by DHT in the caput. While together these data suggest that the PKA holoenzyme subunits are independently regulated, the gene expression data for the regulatory subunit need to be analyzed at the protein level. Given that cAMP is activated by GPCRs, it is likely that several GPCR effectors regulate PKA expression (Hur and Kim, 2002).

TJP1 expression was unchanged in both the initial segment and caput epididymidis of animals orchidectomized or orchidectomized and maintained with testosterone implants. The absence of TJP1 coincides with a compromised blood-epididymal barrier (BEB) in aging Brown Norway rats (Levy and Robaire, 1999). The results from this study, therefore, suggest that the BEB is unaffected by orchidectomy. This is in agreement with studies in other species (Cyr et al., 2007). Future studies on TJP1 localization in orchidectomized animals would help to confirm the state of the BEB.

GJA1 Regulation

Segment-specific GJA1 protein expression and phosphorylation was investigated to determine if these factors were influencing androgen-dependent, GJA1 targeting. GJA1 expression was testicular factor-dependent in the initial segment and caput, whereas GJA1 phosphorylation was testicular factor-dependent in the caput. The results suggested that neither GJA1 expression nor phosphorylation were regulating GJA1 localization.

Many hormones and growth factors control the expression and cellular localization of GJA1. Thus, although the testicular-factor-dependency of GJA1 protein expression was unexpected, it is not surprising that another component of the luminal fluid is involved in its regulation. The luminal fluid is comprised of a variety of hormones and growth factors including: estrogens, ANGII, FGFs and IGFs, all of which have been reported to regulate GJA1 in other tissues and species (Grummer et al., 1994; Hess et al.,

1997; Nadarajah et al., 1998; Kirby et al., 2003; Leung and Sernia, 2003; Jia et al., 2008). Deciphering key GJA1 regulator(s) will require specific *in vitro* and *in vivo* models directed at this question.

Association of GJA1 and its Binding Proteins

Direct associations between GJA1 and c-Src, PKAcat and TJP1 were investigated in a segment-specific manner to determine if GJA1 targeting was due to specific protein interactions. The results indicated that GJA1 associated with c-Src and TJP1 in the initial segment independently of the treatment group.

The evidence of a GJA1 interaction with TJP1 or c-Src was weak for some biological replicates, as GJA1 could not be detected. This is likely due to very low levels of GJA1, which required a greater amount of protein for the Western blots in the present study. Future studies investigating interactions between GJA1 and potential binding partners would benefit from *in vitro* assays where GJA1 can be tagged with either a His or glutathione *S*-transferase tag.

TJP1 has previously been reported to interact with the adherens junction protein, β-catenin, in young, rat epididymides (DeBellefeuille et al., 2003). This is the first study, however, to show that TJP1 can interact with a gap junction protein in the epididymis. Taken together, these data suggest that gap, tight and adherens junctions are intimately regulated in the epididymal epithelium, and that they are not mutually exclusive. Studying cross-junctional regulation will aid in understanding epididymal cell biology and how junctions contribute to creating the microenvironment.

Studies in other models demonstrate a direct interaction between GJA1 and c-Src and GJA1 and TJP1 and report the functional consequence as inhibition of gap junction intercellular communication (GJIC) (Giepmans and Moolenaar, 1998; Toyofuku et al., 1999; Giepmans et al., 2001a; Giepmans et al., 2001b; Duffy et al., 2004; Sorgen et al., 2004). The data from this study suggest that TJP1 and c-Src may mediate GJA1 GJIC in a segment-specific manner. Additionally, in orchidectomized animals it is tempting to speculate that the interactions also represent the endocytosis of apical GJA1 gap junctions (GJ) into annular junctions, as recently described by Gilleron *et al.* (2008). This is further supported by an absence of interaction between GJA1 and c-Src in the caput and a much

weaker GJA1-TJP1 association. Annular junctions have been reported in the epididymis (Cyr et al., 1995; Pelletier, 1995a).

An interaction between GJA1 and PKAcat was not observed in either segment or treatment group. TJP1 interacts with amino acid residues 364-382 of the GJA1 C-terminus, and this interacting site contains serine residues that are phosphorylated by PKAcat (TenBroek et al., 2001; Sorgen et al., 2004; Yogo et al., 2006). Thus, in this study TJP1 may prevent PKAcat from interacting with GJA1. It is also possible that GJA1 is a poor substrate for PKAcat, and that PKAcat may indirectly regulate GJIC via other kinases (TenBroek et al., 2001; Shah et al., 2002; Pahujaa et al., 2007).

The results from this study did not support a treatment-dependent association of GJA1 with c-Src or TJP1 or a role for these binding proteins in the apical targeting of GJA1. A recent publication by Shum *et al.* (2008) demonstrates that basal cells can send projections up to the lumen of the epididymis between principal cells, cross tight junctional strands and recreate the blood-epididymal barrier. While their study did not examine basal cells in the epididymis after orchidectomy, it suggests that the presence of GJA1 at the apical region of principal cells in orchidectomy could be an extension of GJIC between heterologous cell types and not altered targeting to homologous cell types. It is essential to re-examine the histology of basal cells in the context of orchidectomized animals before new studies exploring GJA1 targeting are undertaken. Regardless of which cell types communicate via GJA1, there are numerous possibilities as to why GJA1 localizes to the apical region of the initial segment epithelium with orchidectomy.

Perspectives on Mechanisms That Could Influence the Apical Localization of GJA1

GJA1 at the apical region of principal cells could be due to a compensatory mechanism. Perhaps another GJ at the apical region between principal cells is degraded from this site with orchidectomy, and thus GJA1 is recruited. While the original study on GJA1 regulation investigated this possibility using antibodies against GJB1, GJB2, GJA4, GJA5 (Cyr et al., 1996), since then, two new GJs have been reported in the epididymis (Dufresne et al., 2003). These more recent GJs, GJB4 and GJB5, similarly localize with GJA1 in other tissues, but they are differentially regulated (Goliger and Paul, 1994; Itahana et al., 1996). Therefore, it would be interesting to investigate the androgen

regulation of GJB4 and GJB5 in the epididymis to determine if GJA1 is indeed functioning in a compensatory capacity. Though it may be unlikely, since GJB4 and GJB5 are from different subfamilies than GJA1.

Claudin-1 is a tight junction (TJ) protein whose intracellular targeting is androgen-dependent at the apical region of principal cells in the rat initial segment (Gregory et al., 2001). Orchidectomy, results in the disappearance of claudin-1 from the region of TJs, but the same apical staining is present in testosterone maintained animals (Gregory et al., 2001). Thus, the appearance of GJA1 at the apical region of principal cells with orchidectomy is inverse to the disappearance of claudin-1 from this region. Duffy *et al.* (2000) demonstrated that interleukin-1 β (IL-1β) upregulates GJA1, but downregulates claudin-1 expression in human fetal astrocytes (Duffy et al., 2000). TJP1 expression, on the other hand, remained constant (Duffy et al., 2000). TJP1 expression was unchanged in the initial segment of orchidectomized animals (this study). Thus, the regulation of GJA1, claudin-1 and TJP1 in the initial segment of the rat epididymis parallels the IL-1β regulation in human astrocytes. The *Il-1* receptor mRNA is expressed at the apical region of mouse epididymal epithelia (Gomez et al., 1997) and detected by microarray analysis in the rat initial segment (Turner et al., 2007). Therefore, perhaps IL-1β plays a role in regulating junctional proteins in the rat initial segment. It would be interesting to study this possibility in the future.

GJA1 at the apical region of principal cells may be contributing to the formation of a junctional nexus and potentially aiding in the barrier and/or fence functions of TJs. Explanations for why this only occurs in the initial segment are several fold. Firstly, GJA1 may aid certain TJ components only found in the initial segment, such as claudin-10 (Guan et al., 2005). Secondly, GJA1 may compensate for the loss of a TJ component that only occurs in the initial segment, as with claudin-1 (Gregory et al., 2001). Lastly, the length of the TJ is much longer in the initial segment compared to the other epididymal segments (Cyr et al., 1995), which may be due to an increased luminal pressure (Cyr et al., 2007). Addressing these possibilities with *in vitro* models will be valuable for understanding junctional dynamics.

Since adherens and gap junctions are reciprocally regulated, it is also possible that GJA1 is contributing to adherens junction formation. Orchidectomized rats, however,

have decreased E-cadherin mRNA levels (Cyr et al., 1992), and cytoplasmic catenins (DeBellefeuille et al., 2003) throughout the epididymis. If reciprocal regulation was occurring, GJA1 staining at the plasma membrane would be expected to decrease and not increase (Meyer et al., 1992).

By seven days after orchidectomy the epididymal epithelium is still well differentiated. Many studies support a role for GJA1 in promoting differentiation. For instance, forced expression of GJA1 induces the differentiation of LNCaP cells (Mehta et al., 1999). The appearance of GJA1 in the testis correlates with germ cell differentiation in the guinea pig and mink (Pelletier, 1995b). Additionally, knockout mice demonstrate that GJA1 is required for postnatal expansion of germ cells (Roscoe et al., 2001), and for supporting Sertoli cell differentiation (Sridharan et al., 2007). Thus in the epididymis, perhaps recruitment of GJA1 to the apical region of principal cells contributes to maintaining a differentiated epithelium. This may only be required in the initial segment because of a stronger dependency on testicular inputs (Robaire et al., 2006). Specific markers of epididymal differentiation combined with immunocytochemistry studies would help to explore this possibility.

Conclusions

The results from this study provide credence to several ideas regarding epididymal cell biology:

1. C-Src and PKAcat appear to be important mediators of signal transduction in the epididymis, where c-Src may contribute to segment-specific physiological differences. This suggests that the epididymis relies on signal transduction pathways to differentially regulate the epithelium for spermatozoal maturation.
2. TJP1 associates with GJA1 in the initial segment independently of the treatment group. Thus, tight and gap junctions could form a junctional nexus in the epididymis.
3. C-Src and TJP1 associate with GJA1 in the initial segment, and may play a role in gap junction inhibition. This provides evidence that gap junctions are regulated in a segment-specific manner to contribute to epithelial diversity.

CHAPTER 4 : SYNTHÈSE DE LA MÉMOIRE REDIGE EN FRANÇAIS

1.0 Introduction

L'infertilité masculine est due à plusieurs facteurs comprenant les maladies et des mutations génétiques et moléculaires (Seshagiri 2001). Cependant, la cause est inconnue dans plus de la moitié des cas d'infertilité masculine (de Kretser 1997). Les spermatozoïdes produits par le testicule sont immatures jusqu'à ce qu'ils aient traversé l'épididyme. En effet c'est au cours du transit épididymaire qu'ils acquièrent leur motilité et leur pouvoir fécondant (Orgebin-Crist 1969). L'épididyme a plusieurs fonctions dont la concentration, le transport, la protection, la maturation et le stockage des spermatozoïdes (Robaire et Hermo 1988).

L'épididyme est un tubule hautement enroulé qui est généralement divisé en plusieurs segments, le segment initial, la tête, le corps et la queue, selon des critères histologiques et physiologiques (Robaire et Hermo 1988). On retrouve au sein de chaque segment un microenvironnement luminal spécifique composé de différentes molécules avec lesquelles les spermatozoïdes interagissent afin de devenir matures. Les spermatozoïdes sont alors entreposés dans la queue de l'épididyme, puis acheminés vers le canal déférent. Le canal déférent est ensuite relié à l'urètre qui débouche sur les organes génitaux externes.

Une couche de cellules musculaires lisses, nommée la couche myoïde, entoure l'épithélium pseudostratifié qui borde la lumière de l'épididyme. Le transport des spermatozoïdes par l'épididyme est possible grâce à la pression hydrostatique créée par le fluide testiculaire mais aussi grâce aux contractions péristaltiques du muscle lisse qui forcent les spermatozoïdes à se déplacer le long de l'épididyme (Robaire et Hermo 1988). Le tissu conjonctif est également vascularisé ce qui fournit un débit sanguin constant à l'épididyme et permet la livraison de substances endocriniennes. Chez le rat, la hauteur de l'épithélium diminue le long de l'épididyme alors que le diamètre du tubule augmente. De plus, la proportion de chacun des six types cellulaires présents dans l'épithélium diffère selon le segment.

Les cellules principales constituent le type cellulaire le plus abondant dans l'épididyme et sont en contact direct avec le compartiment luminal et la membrane

basale. Le segment initial et la tête comprennent le plus grand nombre de cellules principales alors que la queue en possède le plus faible nombre (Robaire et Hermo 1988). Le changement de la hauteur de l'épithélium est en grande partie dû aux différences de taille des cellules principales. De plus, il existe des jonctions serrées entre les cellules principales. Ces jonctions forment la barrière hémato-épididymaire qui permet de réguler le microenvironnement luminal et de protéger les spermatozoïdes contre les attaques du système immunitaire (Cyr et al. 2007). La création du microenvironnement luminal est aussi due aux sécrétions des cellules principales et la littérature actuelle suggère que ce processus est fortement régulé par différentes hormones et facteurs testiculaires (Robaire et al. 2006).

Les cellules basales se retrouvent dans chaque segment de l'épididyme. Leur nombre s'accroît le long de l'épididyme avec des proportions semblables dans le corps et la queue (Robaire et Hermo 1988). Ces cellules ont des projections cytoplasmiques qui s'insèrent entre les cellules principales adjacentes, et ce n'est que récemment qu'il a été montré que ces projections entrent en contact avec le compartiment luminal (Shum et al. 2008). Ces projections pourraient contribuer au contrôle du pH intraluminal ou à l'élimination des débris cellulaires provenant des spermatozoïdes (Seiler et al. 2000; Shum et al. 2008).

Les cellules étroites sont présentes uniquement dans le segment initial et dans la zone intermédiaire entre le segment initial et la tête proximale. Elles sont surtout abondantes dans la zone intermédiaire (Adamali et Hermo 1996). Le nom de ces cellules est dérivé du rétrécissement de leur cytoplasme vers la membrane basale; leur noyau est oval et situé dans la moitié supérieure de la cellule. Il y a trois fonctions proposées pour les cellules étroites: le contrôle du pH luminal, la dégradation des protéines dans la lumière de l'épididyme et la protection des spermatozoïdes (Adamali et Hermo 1996; Hermo et al. 2000; Hermo et al. 2005; Robaire et Hermo 1988; Andonian et Hermo 2003).

Les cellules apicales sont formées comme un gobelet et sont présentes dans le segment initial proximal et dans la zone intermédiaire (Adamali et Hermo 1996). Elles n'entrent pas en contact avec la membrane basale et possèdent quelques vésicules et endosomes apicaux (Adamali et Hermo 1996). Tandis que leur rôle précis est inconnu,

elles pourraient, comme les cellules étroites, contribuer à la protection des spermatozoïdes (Adamali et Hermo 1996; Andonian et Hermo 2003).

Les cellules claires sont présentes dans tous les segments à l'exception du segment initial et sont surtout abondantes dans la queue distale (Robaire et Hermo 1988). Leur rôle primaire est vraisemblablement endocytique, car des traceurs injectés dans la lumière de l'épididyme sont rapidement retrouvés dans les organelles des cellules claires impliqués dans l'endocytose (Hermo et al. 1988). Les cellules claires absorbent également le contenu des gouttelettes cytoplasmiques des spermatozoïdes, facilitant le processus de maturation (Robaire et Hermo 1988). Les cellules claires, comme les cellules étroites, sont impliquées dans l'acidification luminale (Hermo et al. 2000; Pastor-Soler et al. 2003).

Les cellules en halo se retrouvent le long de l'épididyme (Robaire et Hermo 1988). Les cellules en halo ont un noyau dense avec un cytoplasme clair et elles sont situées près du compartiment basal de l'épithélium (Robaire et al. 2006). Ces cellules sont composées de lymphocytes T, de lymphocytes T cytotoxiques et de monocytes chez des animaux adultes (Flickinger et al. 1997; Serre et Robaire 1999). Dans l'épididyme d'animaux âgés, les éosinophiles et les lymphocytes B sont également présents (Serre et Robaire 1998; Robaire et al. 2006).

Les études par Benoit dans les années 1920 ont démontré que les androgènes étaient nécessaires pour maintenir la structure et la fonction de l'épididyme (Benoit 1926). En fait, les niveaux des androgènes dans l'épididyme sont 10 fois plus élevés que dans le sang. Plusieurs modèles animaux ont été utilisés pour élucider le rôle des androgènes. Ces modèles incluent l'orchidectomie bilatérale (castration), l'orchidectomie avec un traitement à la testostérone, la ligature des canaux efférents (élimination des androgènes dans la lumière), le traitement avec des anti-androgènes et des études du développement embryonnaire et postnatal. Les conséquences d'un retrait des androgènes sont une diminuation du diamètre des tubules, une augmentation de l'espace interstitiel, un épaississement de la lamina densa de la membrane basale, des changements morphologiques des cellules principales et des variations d'expression de protéines et d'enzymes (passées en revue dans Ezer et Robaire 2002).

L'épididyme est régulé par les androgènes présents dans la lumière du tubule qui sont transportés par les canaux excréteurs et par les androgènes activement transportés à travers l'épithélium des fluides péritubulaires (Turner et Yamamoto 1991). Les androgènes proviennent des glandes surrénales périphériques et des cellules de Leydig situées dans l'espace interstitiel du testicule. Dans les cellules de Leydig, l'androstènedione, la testostérone et les hormones 5α- réduites, telles que la dihydrotestostérone (DHT), sont synthétisées à partir d'un précurseur, le cholestérol. Les hormones sont libérées des cellules de Leydig dans la circulation systémique par les vaisseaux sanguins et distribuées aux organes et aux tissus cibles, incluant l'épididyme. Les androgènes produits par les cellules de Leydig diffusent d'une façon restreinte à l'intérieur des cellules de Sertoli qui constituent l'épithélium séminifère (Turner 1991; Turner et Yamamoto 1991). Les androgènes d'origine testiculaire sont ensuite acheminés par les canaux excréteurs vers l'épididyme grâce à une protéine de transport, l'ABP (androgen binding protein) (Turner 1991). La protéine ABP est sécrétée par les cellules de Sertoli sous l'influence de l'hormone folliculo-stimulante (follicle stimulating hormone, FSH) et de la testostérone (Turner 1991). Les androgènes liés à la protéine ABP sont transportés jusqu'à l'épididyme et ce complexe est internalisé par les cellules principales dans le segment initial et dans la tête par un mécanisme médié par un récepteur. Une fois à l'intérieur de la cellule, la testostérone est libérée de la protéine ABP et est convertie par la 5α-réductase en DHT. La DHT est un androgène plus actif avec une affinité plus élevée pour le récepteur aux androgènes que la testostérone (passé en revue dans Ezer et Robaire 2002).

Les cellules de Sertoli sécrètent différentes molécules de signalisation dans la lumière des tubules séminifères qui sont ensuite transportées par les canaux excréteurs jusqu'à l'épididyme. De cette façon, le testicule régule l'épididyme par l'intermédiaire du compartiment luminal, d'où le nom de régulation lumicrine. Le fluide testiculaire intraluminal contient entre autres de l'eau, des ions, des petites molécules organiques, des protéines, des glycoprotéines et les spermatozoïdes, mais sa composition précise est inconnue (Robaire et Hermo 1988). Par conséquent, le terme «facteur testiculaire» est utilisé pour désigner un composant provenant du compartiment testiculaire et non pas de

la circulation sanguine. Les facteurs testiculaires incluent des protéines, des hormones, des facteurs de croissance, des facteurs mitogènes et des spermatozoïdes.

Les facteurs de croissance (growth factors, GF) favorisent la prolifération et la différenciation en liant et en activant des récepteurs à activité tyrosine kinase (receptor tyrosine kinase, RTK), ou sérine/thréonine, produisant l'activation de différentes voies de signalisation. Les testicules sont une source de facteurs de croissance. Si l'épididyme exprime le récepteur d'un de ces facteurs de croissance, il est probable qu'il soit impliqué dans la régulation lumicrine de l'épididyme (passé en revue dans Tomsig et Turner 2006). L'épididyme est lui-même une source de facteurs de croissance et peut donc utiliser la communication paracrine, autocrine ou juxtacrine.

Plusieurs facteurs de croissance et leurs récepteurs ont été identifiés dans l'épididyme, mais ils n'ont pas tous été identifiés comme fonctionnels. Par exemple, les membres de la famille des facteurs de croissance fibroblastique (fibroblast growth factor, FGF) 2, 4 et 8 sont présents dans le fluide testiculaire du rete testis chez le rat. Les différents récepteurs de FGF (FGFR) ont été également identifiés dans l'épididyme, comme FGFR-1 IIIcα et IIIcβ, et ont été localisés dans les cellules principales du segment initial (Kirby et al. 2003). Les ARN messagers de FGF1, 2 et 9, FGFR1-3 IIIb et IIIc ainsi que les protéines FGFR1 à 4 sont exprimées dans le segment initial chez le rat, mais leur localisation est inconnue (Kirby et al. 2003; Tomsig et al. 2006). Les ARN messagers de FGF10 et FGFR2 sont aussi présents le long de l'épididyme de rat (Henderson et al. 2006). D'autres facteurs de croissance ont été identifiés incluant le facteur de croissance 1 analogue à l'insuline (insulin-like growth factor 1, IGF-1), le facteur de croissance épidermique (EGF), le facteur de croissance transformant bêta (TGF-β), le facteur de croissance endothélial vasculaire (VEGF), le facteur de croissance nerveuse (NGF), le facteur de croissance dérivé des plaquettes (PDGF) et le facteur de croissance des hépatocytes (HGF) (passé en revue dans Tomsig et Turner 2006).

Le rôle précis des facteurs de croissance dans la régulation des fonctions épididymaires n'est pas encore bien connu. Bien que tous les segments de l'épididyme soient régulés par des facteurs testiculaires, le segment initial est le plus dépendant de la régulation par ces facteurs (Robaire et al. 2006). Pourtant, en dépit de l'exposition constante aux facteurs de croissance, les cellules dans le segment initial ne prolifèrent

pas. Il a été suggéré que les cellules puissent faire un cycle entre les états marche-arrêt en fonction de leur capacité à répondre aux facteurs de croissance. De cette façon, il y aurait une stimulation suffisante pour déclencher des cascades de signalisation sans que les cellules n'entrent dans le cycle cellulaire (Robaire et al. 2006). A quel point les effecteurs des facteurs de croissance et les hormones se chevauchent dans la régulation épididymaire contribue à la complexité de ce système. La discrimination des processus régulés par les facteurs testiculaires de ceux régulés par les androgènes peut se faire en utilisant un modèle animal d'orchidectomie avec un traitement à la testostérone.

L'orchidectomie et l'orchidectomie accompagnée d'un traitement de testostérone permet de distinguer la régulation par les facteurs testiculaires de la régulation par les androgènes en éliminant les contributions testiculaires du compartiment intraluminal épididymaire. Bien que beaucoup d'études aient observées des changements dans l'expression ou dans la localisation de certaines protéines suite à l'orchidectomie (par exemple Gregory et al. 2001; DeBellefeuille et al. 2003; Primiani et al. 2007), il est difficile d'avoir une image globale en raison des limitations techniques. L'élucidation des changements de l'expression protéique, particulièrement des protéines de signalisation cellulaire, aidera à déchiffrer les mécanismes épididymaires androgéno-dépendants.

Les messagers secondaires dans l'épididyme ont été très peu étudiés. Tandis que l'utilisation des microréseaux a permis d'identifier plusieurs composantes des voies de signalisation, peu d'études ont directement démontré la présence d'une voie de signalisation fonctionnelle (Tomsig et al. 2006; Pastor-Soler et al. 2008) bien que, plusieurs études aient fait référence à leur présence (Lan et al. 1998; Rodriguez et al. 2001; Cotton et al. 2008). Afin de comprendre la régulation épididymaire, il est essentiel de commencer à élucider les voies de signalisation qui sont impliquées dans la régulation de l'épididyme. Deux de ces voies de signalisation impliquent des récepteurs couplés aux protéines G (G-protein coupled receptor, GPCR) et les RTKs. Les GPCRs activent la voie de signalisation de l'adénylate cyclase, menant à l'activation de la protéine kinase dépendante de l'AMP cyclique A (cyclic AMP-dependent protein kinase A, PKA) et des non-récepteurs à activité tyrosine kinase (non-receptor tyrosine kinase, NRTK) telles que la protéine du proto-oncogène pp60 (cellular Rous sarcoma virus, c-Src). Ces deux composants de la signalisation cellulaire seront discutés plus en détail ultérieurement. Un

autre aspect de la régulation épithéliale implique la communication intercellulaire coordonnée par les jonctions lacunaires.

On pense que la communication intercellulaire par l'intermédiaire des jonctions lacunaires (gap junctional intercellular communication, GJIC) joue un rôle important dans l'acquisition et le maintien de la fertilité masculine, mais leur rôle exact est inconnu.

Les jonctions lacunaires (gap junction, GJ) forment une structure similaire à un pore entre les membranes plasmiques de cellules adjacentes pour permettre le passage de petites molécules, dont les messagers secondaires (Goodenough et al. 1996). Chaque jonction lacunaire est composée de deux connexons, un par membrane cellulaire, formés par un hexamère de six protéines transmembranaires, les protéines des jonctions lacunaires (GJP) antérieurement appelées connexines. Jusqu'ici, 20 GJPs ont été identifiées chez la souris et 21 chez l'humain (Sohl et Willecke 2003). Les GJPs sont nommées selon leur sous-groupe alpha (A), bêta (B), gamma (D) ou epsilon (E) en fonction de leur degré d'homologie, de la longueur du domaine cytoplasmique et de l'ordre de découverte (Sohl et Willecke 2003). Par exemple, GJA1 a été la première GJP identifiée dans le sous-groupe alpha. Les GJPs ont quatre domaines transmembranaires, deux domaines extracellulaires, une boucle cytoplasmique et des terminaisons N et C cytoplasmiques. Les différentes combinaisons de GJPs et de connexons confèrent les propriétés biochimiques et physiques spécifiques à chaque jonction. Plusieurs combinaisons sont possibles (Mese et al. 2007). Les GJs peuvent être trouvées à la membrane plasmique parmi beaucoup d'autres GJs, allant jusqu'à des milliers, qui forment une plaque.

Les GJs sont des structures dynamiques avec une demi-vie de 1 à 5 heures et sont donc constamment synthétisées et dégradées (Fallon et Goodenough 1981; Laird et al. 1991). Le transport du connexon à la membrane plasmique n'est pas entièrement élucidé, mais de nombreux types de vésicules intracellulaires semblent être impliqués dans ce mécanisme (Jordan et al. 1999; Thomas et al. 2005). Une fois insérés dans la membrane plasmique, les connexons peuvent communiquer à l'espace extracellulaire comme un demi-canal (Ebihara 2003; Goodenough et Paul 2003), ou diffuser latéralement aux régions de contact des cellules (Lauf et al. 2002). Les connexons peuvent former des GJs

seulement si les jonctions adhérentes sont déjà présentes (Jongen et al. 1991; Meyer et al. 1992).

L'internalisation des GJs implique plusieurs mécanismes probablement convergents dont la formation des jonctions annulaires (Laird 2006) et l'internalisation des GJs dans les vésicules recouvertes de clathrine par endocytose (Piehl et al. 2007). Bien que les deux mécanismes soient vraisemblablement différents (Laird 2006), des études ont démontré qu'ils pourraient être reliés (Leithe et Rivedal 2006; Nickel et al. 2008; Piehl et al. 2007; Gumpert et al. 2008). Ces deux mécanismes ont été identifiés dans l'épididyme (Cyr et al. 1995; Pelletier 1995; Hermo and Smith 1998) mais ils n'ont pas été associés à l'internalisation des GJs. La voie de dégradation des GJs est controversée et pourrait impliquer les voies lysosomale et protéosomale (passé en revue dedans Laird 2006).

Les GJs ont plusieurs rôles. Les GJs permettent la communication intercellulaire par les ions, les messagers secondaires, les petits métabolites et les acides aminés (Mese et al. 2007). Les GJs jouent aussi un rôle similaire à une protéine suppresseur de tumeur (Mesnil et al. 2005). Plusieurs études indiquent que des anomalies pathologiques causent ou sont causées par une localisation anormale des protéines de jonctions lacunaires affectant la communication intercellulaire (Mesnil et al. 2005). Récemment, il a été montré que la présence des GJs facilitent la fonction de barrière des jonctions serrées (Go et al. 2006; Kojima et al. 2002; Nagasawa et al. 2006).

De plus en plus de données démontrent que les jonctions serrées, adhérentes et lacunaires partagent des protéines communes d'échafaudage en plus de leur proximité spatiale ce qui amène l'idée d'une connexion des jonctions, initialement décrite vers la fin des années 1970 (par exemple Gros et al. 1978). Tandis que des interactions entre les composants des jonctions adhérentes et serrées ont été rapportées dans l'épididyme (DeBellefeuille et al. 2003), des associations avec les jonctions lacunaires n'ont pas encore été identifiées.

La protéine GJA1 est exprimée dans différents tissus et, par conséquent, est la plus étudiée. Beaucoup de tissus reproducteurs mâles expriment GJA1 incluant le testicule, la prostate et l'épididyme (passés en revue dans Pointis 2005). La régulation de

GJA1 est complexe et pourrait survenir à divers niveaux telles que la biosynthèse, l'oligomérisation, la localisation et la dégradation. Des études suggèrent que les hormones et leurs effecteurs jouent également un rôle dans ce processus.

Beaucoup d'hormones et de facteurs de croissance régulent GJA1. Par exemple, dans l'endomètre du rat, l'ovariectomie additionnée d'un traitement d'oestradiol augmente l'expression de GJA1 au niveau de l'ARN messager et de la protéine (Grummer et al. 1994). Dans le segment initial de l'épididyme de rat, GJA1 est régulée par les androgènes (Cyr et al. 1996). Normalement, GJA1 est localisée entre les cellules basales et principales. Chez les animaux orchidectomisés, GJA1 est aussi présente entre les cellules principales dans la portion apicale. Chez les animaux orchidectomisés et traités avec de la testostérone, GJA1 est présente uniquement entre les cellules basales et principales, comme dans les contrôles. Suite à l'orchidectomie l'expression de la protéine totale GJA1 augmente et GJA1 est hypophosphorylée (Cyr et al. 1996). Les mécanismes précis qui contrôlent le traffic de GJ sont inconnus, mais des études suggèrent que des kinases et les protéines liant les GJs soient impliquées dans ce processus. D'autres études ont montré que des facteurs de transcription et des kinases régulent également GJA1.

Les facteurs de transcription, Sp-1, Sp-3 et AP-1, activent le promoteur de GJA1 dans plusieurs types de cellules (Teunissen et al. 2003). Dans l'épididyme de rat, Sp-1 et Sp-3 activent le promoteur de claudine-1, une protéine des jonctions serrées qui est également régulée par les androgènes dans le segment initial de l'épididyme de rat (Dufresene et Cyr 2007; Gregory et al. 2001). Il est possible que les facteurs de transcription Sp1 et Sp3 soient impliqués dans la régulation de la transcription de *Gja1* dans l'épididyme de rat et/ou son expression androgéno-dépendante. Les facteurs de transcription c-Myc et HSP90, augmente l'expression de GJA1 (ARN messager et protéine) dans les cellules NIH3T3 qui exprime Ras constitutivement (Carystinos et al. 2003). C-*myc* est faiblement exprimé dans l'épididyme murin et n'est pas régulé par les androgènes ou par les facteurs testiculaires (Cornwall et al. 2001), suggérant qu'il ne joue pas un rôle dans la régulation de *Gja1* dans l'épididyme de rat.

GJA1 est une protéine phosphorylée, comme d'autres protéines des jonctions lacunaires. Beaucoup de kinases régulent GJA1. La plupart des kinases, dont les kinases c-Src, $p34^{Cdc2}$, protéine kinase C (PKC), kinases activées par les mitogènes (mitogen

activated protein kinase, MAPK) et protéine kinase B (PKB/Akt), régulent négativement GJA1 au niveau de la GJIC, de la conductibilité, de l'internalisation de GJs et du traffic des GJs. D'un autre coté, les kinases PKA et casein kinase 1 (CK1) régulent positivement GJA1 (passé en revue dans Lampe et Lau 2004; Park et al. 2006).

En plus des kinases, d'autres protéines de liaison de GJA1 ont été identifiées, comme les protéines de jonction serrée 1 et 2 (tight junction protein 1 and 2; TJP1, TJP2), la β-caténine, la drebine, les tubulines α et β, et les cavéolines 1 et 2 (passé en revue dans Laird 2006). La signification des interactions s'étend de l'inhibition de la croissance au transport des jonctions lacunaires (Laird 2006). Les protéines liant GJA1 qui régulent le transport intracellulaire de GJA1 et qui sont régulées par les androgènes seront décrites en détail.

C-Src est un proto-oncogène généralement connu pour favoriser la transformation et la cancérogénèse néo-plastiques. V-Src est l'oncogène muté de c-Src qui est constitutivement actif. C-Src appartient à une famille de non-récepteurs à activité tyrosine kinase qui est composée d'au-moins neuf membres (Sandilands et al. 2008). La famille des kinases c-Src a diverses fonctions biologiques dont l'adhérence des cellules, la modulation du transport des endosomes et des dynamiques de signalisation, la prolifération et la promotion des tumeurs (Sandilands et al. 2008). Les membres de cette famille ont une structure similaire avec des masses moléculaires se situant entre 52-62 kDa, avec c-Src à 60 kDa (Sandilands et al. 2008). Quand c-Src est inactif, il est phosphorylé au résidu tyrosine (Y) 527 ce qui maintient la protéine dans une conformation fermée. Après l'activation de c-Src, Y257 est déphosphorylé en permettant à c-Src de s'ouvrir et de libérer son domaine kinase, qui alors se phosphoryle au résidu Y416. C-Src se déplie pour permettre des interactions protéiques avec les domaines d'homologie Src 1 et 2 (SH2 et SH3), permettant à c-Src de phosphoryler ses substrats.

Comme lien entre les voies de GPCR et de RTK, Src peut intégrer les cascades de signalisation menant à la phosphorylation de GJA1 sur des résidus de tyrosine ou de sérine (Lampe et Lau 2004; Pahujaa et al. 2007; Solan et Lampe 2008). Plusieurs études ont étudié l'interaction directe de GJA1 avec c-Src ou v-Src. C-Src a également des

influences sur l'internalisation de GJA1 à l'intérieur des jonctions annulaires (Gilleron et al. 2008).

PKA est une kinase activée par les voies de signalisation qui augmente la 3',5'-cyclic-adénosine monophosphate (AMPc). L'holoenzyme PKA se compose de deux sous-unités régulatrices et de deux sous-unités catalytiques (Hansson et al. 2000). Deux AMPcs se lient directement à chaque sous-unité régulatrice, ce qui libèrent les sous-unités catalytiques de PKA (PKAcat) qui peuvent alors phosphoryler des substrats sur des résidus sérine ou thréonine. PKA a deux isoenzymes, nommées I et II, et chaque isoenzyme a différents isoformes. Les isoformes de la sous-unité de PKAcat se nomment α, β et γ; ainsi, les formes des sous-unités catalytiques sont: CIα, CIβ, CIγ et CIIα, CIIβ, et CIIγ qui ont une masse moléculaire d'environ 40 kDa. PKAcat est également nécessaire pour le traffic des protéines membranaires (Rodionov et al. 2003), plus spécifiquement pour le transport apical, et non pas pour le transport basal (Pimplikar et Simons 1994).

Les études utilisant des produits qui augmentent l'activité de l'AMPc ont prouvé que la phosphorylation de GJA1 et la GJIC sont augmentées suite à une plus grande biosynthèse et/ou à un traffic intracellulaire de GJA1 à la membrane plasmique (passés en revue dans Lampe et Lau 2004). PKAcat phosphoryle cinq résidus sérines du C-terminus de GJA1 (TenBroek et al. 2001; Yogo et al. 2006); cependant, l'importance de ces phosphorylations n'est pas claire et peut i) préparer GJA1 pour des phosphorylations séquentielles par d'autres kinases (Shah et al. 2002) ou ii) favoriser l'activité de canaux GJA1 (Yogo et al. 2006). Actuellement, il n'est pas clair si PKAcat joue un rôle direct ou indirect en régulant GJA1, mais en-dehors de CK1, c'est la seule kinase stimulatrice qui a été rapportée pour GJA1 et GJIC. De plus, Src et PKAcat régulent la phosphorylation des sérines 364 et 365 impliquées dans la régulation du traffic de GJA1 à ou dans la membrane plasmique (Solan et Lampe 2007; Solan et Lampe 2008).

TJP1 est une protéine d'échafaudage qui se lie avec des composants des jonctions serrées, adhérentes et lacunaires. Beaucoup d'études ont examiné l'association directe avec le C-terminus de GJA1 et le domaine PDZ2 de TJP1 (par exemple Giepmans et Moolenaar 1998; Toyofuku et al. 1998). Cependant, la pertinence fonctionnelle de cette interaction n'est pas entièrement comprise. Quelques études suggèrent que TJP1 régule la

taille et la stabilité des plaques de GJs composées de GJA1 (Laing et al. 2005; Hunter et al. 2005). D'autres études suggèrent que TJP1 joue un rôle dans l'internalisation de GJA1 (Segretain et al. 2004; Gilleron et al. 2008; Baker et al. 2008). TJP1 et c-Src semblent se concurrencer pour lier GJA1. GJA1 s'associe préférentiellement à c-Src à un faible pH intracellulaire et à TJP1 à pH intracellulaire élevé (Duffy et al. 2004). De plus, lorsque c-Src est lié au C-terminus de GJA1, cela empêche et renverse l'interaction entre GJA1 et TJP1 (Duffy et al. 2004; Sorgen et al. 2004). L'étude par Gilleron et autres (2008) a démontré que l'internalisation de GJA1 à l'intérieur des jonctions annulaires exige l'activation de c-Src et la rupture de l'interaction entre TJP1 et GJA1.

Dans l'épididyme de rat, GJA1, GJB1, GJB2, *Gjb4* et *Gjb5* sont exprimées (Cyr et al. 1996; Dufresne et al. 2003), mais peu d'information existe sur la régulation de ces GJs dans l'épididyme. Cependant, des études antérieures dans notre laboratoire ont démontré que la localisation de GJA1 est dépendante des androgènes testiculaires dans le segment initial (Cyr et al. 1996). Dans notre étude, nous supposons que le traffic de GJA1 dans le segment initial est provoqué a) par une augmentation segment-spécifique de la synthèse de la protéine GJA1, b) par des changements d'expression des protéines de liaison de GJA1 et/ou c) par des changements au niveau de l'interaction entre ces protéines et GJA1 suite au retrait des androgènes. Cette étude porte sur ces trois mécanismes possibles de régulation de GJA1 afin de i) mieux comprendre la régulation des GJs dans l'épididyme et ii) d'étudier les mécanismes du traffic intracellulaire de GJA1en utilisant un modèle *in vivo* unique.

2.0 Résultats et discussion

2.1 PKA et c-Src

Très peu de composants de la signalisation cellulaire ont été identifiés dans l'épididyme et même peu d'études ont identifié des voies fonctionnelles de signalisation. Il n'existe donc pas d'information sur l'expression ou la régulation de c-Src ou de PKAcat dans l'épididyme. Cependant des études suggèrent que ces deux composants soient régulés par les androgènes dans d'autres organes reproducteurs mâles (Wong et al. 2005; Sadar et al. 1999). Par conséquent, le premier objectif de cette étude était de caractériser c-Src et PKAcat dans l'épididyme de rat adulte afin de mieux connaître la

signalisation cellulaire épididymaire. Deuxièmement, l'étude visait à déterminer si l'expression ou la localisation de c-Src ou de PKAcat étaient régulées par les androgènes dans l'épididyme de rat.

PKAcat et c-Src ont été caractérisés dans les différentes régions de l'épididyme de rat adulte par immunocytochimie et par immunobuvardage de type Western. Les résultats ont montré l'expression de c-Src et de PKAcat le long de l'épididyme. De plus, l'anticorps dirigé contre c-Src a identifié des bandes multiples en immunobuvardage de type Western entre 50 et 60 kDa correspondant à plusieurs membres de la famille des tyrosines kinases Src. De même, l'anticorps de PKAcat permet d'identifier trois isoformes de la sous-unité. Les isoformes alpha et bêta de PKAcat (approximativement 40 kDa) ont été détectés dans les quatre segments de l'épididyme de rat adulte.

La localisation cellulaire de c-Src variait le long de l'épididyme. C-Src a été localisé à la membrane plasmique entre les cellules basales et principales dans chacun des segments épididymaires. Dans le segment initial, c-Src était également présent à la région apicale des cellules étroites. Dans la tête, c-Src a été détecté au niveau de quelques microvillosités des cellules principales, le long de la membrane plasmique de quelques cellules principales et faiblement dans le cytoplasme. La localisation de c-Src dans le corps était semblable à celle dans la tête; cependant, il y avait un un signal beaucoup plus fort le long de la membrane plasmique entre les cellules principales, au niveau des microvillosités des cellules principales et de la membrane plasmique apicale des cellules principales et claires. Une forte réaction cytoplasmique a été notée dans les cellules épithéliales et un faible signal dans les cellules du muscle du corps qui n'ont pas été observés dans les autres segments. Dans la queue, une forte immunoréaction a été observée pour c-Src au niveau des microvillosités et de la membrane plasmique apicale.

L'immunolocalisation de PKAcat était moins variable le long de l'épididyme. PKAcat était présente sous forme de petits points dans le cytoplasme, le noyau, la couche myoïde, l'endothélium, entre les cellules basales et principales, à la membrane plasmique latérale et sur les spermatozoïdes. La localisation de PKAcat suggère que cette protéine pourrait s'associer à des protéines d'échafaudage, comme les protéines d'ancrage aux protéines kinases A (A kinase anchoring protein, AKAP), ou aux vésicules intracellulaires (Rawe et al. 2004; Skalhegg et al. 2000).

L'expression de la protéine totale c-Src a été significativement diminuée suite à l'orchidectomie dans le segment initial et la tête et a seulement été partiellement maintenue dans la tête chez les rats orchidectomisés et traités avec de la testostérone. Cela suggère que l'expression de c-Src soit dépendante des androgènes et des facteurs testiculaires. Pour déterminer si les androgènes influençent l'activité de c-Src, des anticorps phospho-spécifiques contre les formes actives (p-Tyr-416) et inactives (p-Tyr-527) de c-Src ont été utilisés. Aucune différence significative n'a été observée dans le rapport de la protéine c-Src active sur la protéine inactive parmi les différents groupes d'animaux dans le segment initial. Cependant, dans la tête, le rapport augmentait significativement chez les animaux orchidectomisés. Chez les rats orchidectomisés et traités avec de la testostérone, le rapport de la protéine active sur la protéine inactive ne changait pas par rapport aux animaux témoins. Ainsi, alors que l'expression totale de c-Src était partiellement androgéno-dépendante dans le segment initial et la tête, l'activité de c-Src était régulée par les androgènes uniquement dans la tête. Les niveaux de PKAcat étaient plus faibles, bien que pas significativement, dans le segment initial et la tête des rats orchidectomisés. Chez les rats orchidectomisés et traités avec la testostérone, l'expression de PKAcat diminuait significativement dans la tête.

Afin de déterminer si les androgènes régulent le ciblage intracellulaire de c-Src et/ou de PKAcat dans le segment initial ou la tête, de l'immunocytochimie a été réalisée sur des sections d'épididymes de rats adultes des différents groupes d'animaux. Chez les animaux orchidectomisés, il y avait un signal intense de c-Src à la région apicale de cellules principales dispersées dans une section transversale d'un tubule du segment initial et de la tête. On a également détecté c-Src dans la région périnucléaire de quelques cellules principales qui n'ont pas démontré cette accumulation apicale. Les microvillosités étaient immunopositives, mais l'immunoréaction des cellules basales et principale était semblable aux animaux témoins. Chez les rats orchidectomisés et traités à la testostérone, les immunoréactions apicales et périnucléaires étaient semblables aux animaux témoins. Le signal entre les cellules basales et principales était beaucoup moins intense, mais l'immunoréaction au niveau des microvillosités a persisté. Il n'y avait aucun changement apparent au niveau de la localisation de PKAcat dans les différents segments épididymaires suite à l'orchidectomie.

Les analyses immunocytochimiques et par Western ont indiqué que PKAcat était présente dans tout l'épididyme, suggérant que PKAcat est impliquée dans différentes fonctions ou voies de signalisation dans l'épididyme. PKAcat est un effecteur en aval des processus médiés par un récepteur (Hur et al. 2002) mais a été également montré pour agir en tant que régulateur transcriptionel (Don et Stelzer 2002) et médiateur environnemental (Ciardiello et Tortora 1998). L'immunoréaction sous forme de points le long de l'épididyme suggère que PKAcat puisse s'associer à d'autres protéines, telles que les AKAPs (Rawe et al. 2004). Les AKAPs confèrent une spécificité à la signalisation de l'holoenzyme PKA en la séquestrant aux différents compartiments sous-cellulaires (Skalhegg et Tasken 2000). Les AKAPs sont exprimées dans les spermatozoïdes (Rawe et al. 2004; Moos et al. 1998; Vijayaraghavan et al. 1997), et dans les épididymes de souris (Turner et al. 2007).

Les niveaux d'expression de PKAcat diminuaient significativement dans la tête de l'épididyme des animaux orchidectomisés et traités avec de la testostérone, tandis qu'on n'a observé aucun effet dans le segment initial. La diminution de l'expression de PKAcat pourrait refléter les différences dans la réponse de l'AMPc, puisque l'AMPc est capable d'augmenter l'expression de PKA (Skalhegg et Tasken 1997). Il a été signalé aussi que les cellules épithéliales épididymaires isolés à partir de rats orchidectomisés ont une plus faible capacité de réponse à l'AMPc (Cheuk et al. 2000).

Plusieurs membres de la famille des kinases Src ont également été détectés par immunobuvardage de type Western dans chacun des quatre segments de l'épididyme. Cela suggère que c-Src pourrait être impliqué dans les cascades de signalisation dans toutes les régions épididymaires, bien que les analyses de c-Src actif dans le segment initial et la tête suggèrent que l'activation de c-Src soit segment-spécifique. Les différences dans la localisation de c-Src le long de l'épididyme soutiennent la notion que la régulation des voies de signalisation dans chaque segment épididymaire est unique et apporte une contribution spécifique à la maturation des spermatozoïdes (Turner 1991; Cornwall et al. 1990).

L'expression totale de c-Src diminuait significativement suite à l'orchidectomie dans le segment initial et la tête. Le traitement avec la testostérone permet de maintenir partiellement le niveau d'expression de c-Src, mais seulement dans la tête. Ce résultat

contraste avec ce qui a été démontré dans le testicule. En effet, Wong et autres (2005) ont rapporté que la suppression des androgènes entraîne une augmentation de l'expression de c-Src. Les anticorps phospho-spécifiques ont indiqué que le rapport de c-Src actif sur c-Src inactif est demeuré constant dans le segment initial dans les animaux contrôles (opération simulée), orchidectomisés, et orchidectomisés et traités à la testostérone alors que dans la tête ce rapport est significativement plus élevé uniquement dans les animaux orchidectomisés. Ces résultats indiquent qu'en dépit d'une diminution de l'expression de la protéine totale, l'orchidectomie ne change pas l'activité de la kinase c-Src dans le segment initial.

Plusieurs études démontrent que l'agoniste se liant au récepteur aux androgènes mène à l'activation de Src (Migliaccio et al. 2000; Kousteni et al. 2001; Zhoul et al. 2005). Cela augmente la prolifération cellulaire dans une variété de cellules normales et cancéreuses telles que les cellules de Sertoli et les cellules LNCaP dérivées d'un carcinome prostatique (Migliaccio et al. 2000; Cheng et al. 2007). Cette activation de Src contraste avec les résultats de notre étude; dans le segment initial, les androgènes n'ont pas influencé l'activation de c-Src, tandis que dans la tête les androgènes ont supprimé l'activité de c-Src. La différence dans la régulation de Src pourrait représenter un mécanisme important pour empêcher la prolifération cellulaire dans l'épididyme. Par ailleurs, il pourrait contribuer à l'incidence limitée des cancers épididymaires comparés à d'autres organes tels que la prostate et les testicules.

La signalisation des facteurs de croissance et l'activation de la voie MAPK est limitée par les septums du segment initial (Tomsig et al. 2006). Cela pourrait expliquer les données de notre étude puisque l'activité de c-Src était régulée différemment dans le segment initial et la tête. Dans le segment initial, l'activité du c-Src était indépendante des androgènes et des facteurs- testiculaires. Cela suggère qu'un facteur de circulation non-androgène, ou un médiateur de la signalisation entre l'épithelium et le compartiment interstitiel contrôle l'activité de c-Src. Les médiateurs de la communication paracrine de c-Src pourraient inclure des GPCRs, des RTKs ou des récepteurs à activité sérine/thréonine kinase situés dans le compartement interstitiel. De nombreux facteurs non-androgènes potentiels de la circulation existent. Les techniques comme celles

utilisées par Tomsig et autres (2006) seraient utiles pour déterminer si les mécanismes de communication paracrine ou endocrine régulent l'activité de c-Src dans le segment initial.

La présence de c-Src et de PKAcat entre les cellules basales et principales, où GJA1 a déjà été rapportée (Cyr et al. 1996), suggère qu'ils puissent directement réguler les jonctions lacunaires contenant GJA1. Ceci a été démontré dans d'autres tissus et espèces (Yogo et al. 2006; Giepmans et al. 2001; Duffy et al. 2004; Toyofuku et al. 1999; Pahujaa et al. 2007).

L'immunolocalisation de PKAcat n'est pas affectée par l'orchidectomie, suggérant qu'elle ne soit pas impliquée dans la régulation de la localisation de GJA1. Par contre, chez les rats orchidectomisés, c-Src a été retrouvé à la région apicale de quelques cellules principales, à la région périnucléaire des cellules principales où l'immunoréaction apicale était absente, et aux microvillosités. Chez les rats contrôles et orchidectomisés, c-Src est aussi localisée entre les cellules basales et principales. C-Src inactif est normalement retrouvé à la région périnucléaire des cellules liées au compartiment endosomal, alors que c-Src actif est retrouvé à la périphériede la cellule lié aux points de contact focaux (Sandilands et al. 2008). Puisque c-Src est localisé à la région apicale de quelques cellules principales chez les rats orchidectomisés, qu'il est plus actif dans les rats orchidectomisés que dans les rats contrôles et qu'il est associé avec GJA1 quand il est actif (Giepmans et al. 2001), il est possible qu'une association directe entre c-Src et GJA1 influence la localisation de GJA1.

2.2 TJP1

TJP1 est exprimé dans l'épididyme et est régulé par les androgènes au cours du développement postnatal et du vieillissement (DeBellefeuille et al. 2003; Levy et Robaire 1999). Par contre, chez le rat adulte, la régulation de TJP1 dans l'épididyme n'est pas connue. Le deuxième objectif de ce projet comprenait donc l'étude de l'androgène-dépendance de TJP1 dans l'épididyme de rat adulte.

Même si TJP1 était exprimé plus faiblement dans le segment initial et la tête d'épididymes de rats orchidectomisés et de rat orchidectomisés et traités avec de la testostérone que dans les rats contrôles, ces résultats n'étaient pas statistiquement significatifs. Il a déjà été démontré que TJP1 est un composant important des jonctions adhérentes et serrées dans l'épididyme de rat adulte (DeBellefeuille et al. 2003; Levy et

Robaire 1999). Chez le rat âgé, les niveaux plus faibles de TJP1 sont associés à une perte d'intégrité de la barrière hémato-épididymaire (Levy et Robaire 1999). Cela suggère donc que la barrière hémato-épididymaire n'est pas affectée par l'orchidectomie. C'est en accord avec des études réalisées chez d'autres espèces (Cyr et al. 2007). Les futures études sur la localisation de TJP1 dans les animaux orchidectimisés aideront à confirmer l'état de la BEB.

2.3 GJA1

Pour déterminer si le ciblage intracellulaire androgéno-dépendant de GJA1 est associé à un changement de synthèse de la protéine GJA1, une analyse par immunobuvardage de type Western a été réalisée avec des protéines du segment initial et de la tête des animaux contrôles (opération simulée), des animaux orchidectomisés, et des animaux orchidectomisés et traités avec de la testostérone. Les niveaux de la protéine GJA1 étaient semblables dans le segment initial et la tête de l'épididyme des différents animaux, sans différence significative entre les deux segments. Par contre, l'orchidectomie a entraîné une diminution de l'expression de GJA1 dans le segment initial et la tête (statistiquement significative dans la tête) mais cette baisse n'a pas été renversée par le traitement de testostérone. Cela suggère que même si le ciblage intracellulaire de la protéine GJA1 est androgéno-dépendant, son expression dépend de facteurs testiculaires autres que les androgènes. De plus, tandis qu'il n'y avait aucun changement apparent dans la phosphorylation de GJA1 dans le segment initial, la forme P1 phosphorylée est régulée par des facteurs testiculaires dans la tête.

Tandis qu'il a déjà été démontré que l'expression et la phosphorylation de GJA1 augmentent suite à l'orchidectomie, l'effet a été mesuré avec de la protéine d'épididyme total et ne prend donc pas en compte les possibles différences entre les segments (Cyr et al. 1996). Dans cette étude, des immunobuvardages de type Western ont été réalisés sur des protéines du segment initial pour déterminer si l'orchidectomie influençait les niveaux de GJA1. La tête a été utilisé comme contrôle, puisque le ciblage intracellulaire de GJA1 n'est pas modifié dans ce segment suite à l'orchidectomie. Les résultats ont indiqué qu'il y avait une diminution significative d'expression de la protéine GJA1 dans la tête des rats orchidectomisés et dans le segment initial et la tête des rats

orchidectomisés et traités avec de la testostérone. Ces résultats suggèrent donc que les facteurs testiculaires régulent l'expression de la protéine GJA1 et de sa forme phosphorylée P1 dans la tête. Plusieurs facteurs de croissance règulent GJA1 dans d'autres tissus (Reuss et Unsicker 1998) et pourraient donc jouer un rôle dans la régulation des jonctions lacunaires au niveau de l'épididyme (Tomsig et Turner 2006). La phosphorylation de GJA1 n'a pas été modifiée dans le segment initial, indiquant que la phosphorylation n'influençe pas le ciblage intracellulaire de GJA1 dans cette région.

Les facteurs testiculaires, incluant les androgènes, régulent l'expression et la localisation cellulaire de GJA1. Le fluide luminal testiculaire est composé d'hormones et de facteurs de croissance tels que les estrogènes, l'angiotensine II, FGF et IGF-1 qui régulent GJA1 dans d'autres tissus et espèces (Grummer et al. 1994; Hess et al. 1997; Nadarajah et al; 1998; Kirby et al. 2003; Leung et Sernia 2003; Jia et al. 2008). L'identification des principaux régulateurs de GJA1 exigeront des modèles *in vitro* et *in vivo* spécifiques à chacun des régulateurs.

2.4 Co-immunoprécipitation

Finalement, cette étude essaye de déterminer si c-Src, PKAcat et TJP1 sont des régulateurs potentiels du ciblage intracellulaire de GJA1 qui est dépendant des androgènes et segment-spécifique dans l'épididyme de rat. Une association directe entre GJA1, c-Src, PKAcat et TJP1 a été étudiée.

Une co-immunoprécipitation a été réalisée en utilisant un extrait protéique du segment initial et de la tête des animaux contrôle (opération simulée), orchidectomisés, et orchidectomisés et traités avec de la testostérone. Dans le segment initial, une association directe a été observée entre GJA1 et c-Src ainsi qu'entre GJA1 et TJP1, mais pas entre GJA1 et PKAcat dans les animaux contrôles et traités. Dans la tête, aucune interaction n'a été observé entre GJA1 et c-Src. Par contre, une interaction entre TJP1 et GJA1 a été observée dans la tête, bien que celle-ci semble être beaucoup plus faible que celle observée dans le segment initial. Aucune association entre GJA1 et PKAcat n'a été mise en évidence. Les observations étaient les mêmes dans les différents groupes d'animaux.

Les études de co-immunoprécipitation ont indiqué que dans le segment initial, GJA1 se lie à c-Src et à TJP1, mais pas à PKAcat. Cependant, dans la tête, on n'a observé

qu'une association faible entre GJA1 et TJP1 ce qui indique que GJA1 s'associe préférentiellement à ces protéines dans le segment initial et non pas dans la tête. Ces résultats suggèrent que chez les rats témoins et les animaux orchidectomisés et traités à la testostérone c-Src agisse en tant qu'un régulateur négatif de GJIC (Giepmans et al. 2001; Duffy et al. 2004; Toyofuku et al. 1999; Sorgen et al. 2004) et que TJP1 pourrait influencer l'incorporation de GJA1 dans les GJs (Laing et al. 2005; Hunter et al. 2005). Des études supplémentaires seraient nécessaires pour vérifier ces hypothèses.

Il a déjà été démontré que TJP1 interagit avec la protéine des jonctions adhérentes, la β-caténine, dans les épididymes de jeunes rats (DeBellefeuille et al. 2003). Par contre, notre étude démontre pour la première fois que TJP1 peut interagir avec une protéine des jonctions lacunaires, GJA1, dans l'épididyme de rat. Dans l'ensemble, ces données suggèrent que les jonctions lacunaires, serrées et adhérentes sont intimement régulées dans l'épithélium épididymaire, et que ces jonctions interagissent entre elles. L'étude des interactions entre les différents jonctions facilitera la compréhension du fonctionnement de l'épididyme et de la contribution des jonctions à créer le microenvironnement intraluminal.

Des études dans d'autres modèles démontrent une interaction directe entre GJA1 et c-Src ainsi qu'entre GJA1 et TJP1 qui a pour conséquence fonctionnelle l'inhibition de la GJIC (Toyofuku et al. 1999; Giepmans et al. 2001; Duffy et al. 2004; Sorgen et al. 2004, Giepmans et al. 1998). Les données de cette étude suggèrent que TJP1 et c-Src puissent être des médiateurs de la GJIC basée sur GJA1 d'une façon segment-spécifique. De plus, chez les animaux orchidectomisés, on pourrait spéculer que ces interactions jouent un rôle dans l'endocytose de GJA1 au niveau des jonctions annulaires tel que récemment décrit par Gilleron et al. (2008). Cette hypothèse est soutenue par une absence d'interaction entre GJA1 et c-Src dans la tête qinsi qu'une faible association entre GJA1 et TJP1. La présence de jonctions annulaires a été rapportée dans l'épididyme (Cyr et al. 1995; Pelletier 1995), mais il reste à établir si les GJs sont éliminées de la membrane plasmique épididymaire par l'intermédiaire de ce mécanisme.

Aucune interaction n'a été observée entre GJA1 et PKAcat. TJP1 s'associe aux résidus d'acides aminés 364-382 du C-terminus de GJA1, et ce site d'interaction contient les résidus sérine qui sont phosphorylés par PKAcat (TenBroek et al. 2001; Sorgen et al

2004; Yogo et al 2006). Ainsi, TJP1 pourrait empêcher PKAcat de s'associer à GJA1. Il est également possible que GJA1 soit un pauvre substrat pour PKAcat, et que PKAcat régule indirectement GJIC par l'intermédaire d'autres kinases (TenBroek et al 2001; Shah et al 2002; Pahujaa et al. 2007).

Les résultats de cette étude n'ont pas établi une association traitement-dépendante de GJA1 avec c-Src ou TJP1 ou un rôle pour c-Src et TJP1 dans le ciblage apicale de GJA1. Une publication récente par Shum et al. (2008) démontre que les cellules basales sont parfois pourvues de projections s'étendant jusqu'à la lumière de l'épididyme entre les cellules principales, sans affecter l'intégrité de la barrière hémato-épididymaire. Cette étude suggère que la présence de GJA1 à la région apicale des cellules principales dans l'épididyme d'animaux orchidectomisés pourrait aussi être due à la présence de projections de cellules basales. Il est essentiel de réexaminer l'histologie des cellules basales dans le cadre de l'orchidectomie avant que de nouvelles études portant sur le ciblage intracellulaire soient réalisées.

3.0 Conclusions

Cette étude a examiné, au niveau du segment initial de l'épididyme du rat adulte, les mécanismes possibles impliqués dans le ciblage intracellulaire androgéno-dépendant de la protéine GJA1. Les résultats ont démontré que le ciblage intracellulaire de la protéine GJA1 était indépendant de son état de synthèse ou de phosphorylation, de la régulation des protéines se liant à GJA1 ou d'une association directe avec c-Src, PKAcat et TJP1. Bien qu'il n'a pas été possible de mettre en évidence les mécanismes impliqués dans le ciblage intracellulaire de GJA1, cette étude est la première à identifier les effecteurs de signalisation c-Src et PKAcat dans l'épididyme. C-Src et PKAcat semblent être des effecteurs importants de la signalisation cellulaire dans l'épididyme. C-Src pourrait également contribuer aux différences physiologiques entre les segments qui sont vraisemblablement importantes pour la maturation des spermatozoïdes. En plus, nous avons démontré que c-Src, GJA1 et PKAcat sont régulés par les facteurs testiculaires. Finalement, les résultats ont indiqué que GJA1 se lie préférentiellement à c-Src et à TJP1 dans le segment initial, suggérant qu'ils puissent jouer un rôle dans l'inhibition des jonctions lacunaires dans cette région.

Cette étude supporte plusieurs idées concernant la biologie des cellules épithéliales épididymaires:

1. La création et le maintien du microenvironnement luminal épididymaire exigent l'implication de plusieurs voies de signalisation permettant de réguler différentiellement les compartiments épithéliaux.
2. Les jonctions lacunaires et serrées interagissent entre elles dans l'épididyme de manière spécifique au segment et à l'âge. Les jonctions lacunaires pourraient être impliquées dans les fonctions des jonctions serrées et vice-versa.
3. Les jonctions lacunaires sont régulées d'une façon segment-spécifique afin de contribuer à la diversité épithéliale.

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