EXPRESSION AND REGULATION OF AQUAPORINS IN THE MALE REPRODUCTIVE TRACT AND ITS ROLE IN FLUID HOMEOSTASIS

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To my mentor and friend....

"The better is the enemy of the good"

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LIST OF ABBREVIATIONS

ALH: amplitude of lateral head displacement

AQP: aquaporin

BCF: beat cross frequency

CASA: computer assisted sperm analysis

CFTR: cystic fibrosis transmembrane regulator

DES: diethylstilbestrol

ED: efferent ductile

EDL: efferent duct ligation

ER: estrogen receptor

ERKO: estrogen receptor knockout

Elong: elongation

GPC: glycerophosphorylcholine

GST: glutathione-S-transferase

IVOS: integrated visual optical system

LIN: linearity

NHE: sodium-hydrogen exchanger

STR: straightness

VAP: smoothed path velocity

VCL: track velocity

VSL: straight line velocity

WT: wild type

% slow: percentage of slow sperm

% rapid: percentage of rapid sperm

ABSTRACT

Aquaporins (AQPs) are transmembrane protein channels that allow the rapid passage of water across an epithelium at low energy requirement. With the discovery of expression of multiple AQPs in different tissues, we undertook a systematic analysis with several other members of the AQP family on Bouin-fixed tissues of the male reproductive tract employing light microscope immunocytochemistry. In the testis, AQP-0 expression in the seminiferous epithelium was restricted to Sertoli cells and to Leydig cells. The epithelial expression of AQP-10 was restricted to the microvilli of the nonciliated cells and cilia of ciliated cells of the efferent ducts. AQP-3 expression was localized exclusively to basal cells of the epididymis. Taken together the data indicate a cell type and tissue specific expression for AQP-0, -3 and -10 in the testis, efferent ducts and epididymis, as well as differential regulating factors for the expression of AQP-3 in basal cells.

Estrogens play an important role in the efferent ducts (EDs), where α-estrogen receptors (αER) have been localized. Mice deficient in this receptor are infertile, and the effect appears to be due in part to retention of water at the level of the EDs. We examine the role of the αER on aquaporins (AQPs) in the EDs and on sperm counts and sperm motility in WT and αERKO mice. Mice fed lab chow (contains phytoestrogens), versus a casein diet (no phytoestrogens) were also compared. Epithelial staining for AQP-1, AQP-9 and AQP-10 prominent in WT mice, was dramatically reduced in αERKO mice. No additional changes in immunolocalizations were noted as a consequence of diet. Computer-assisted sperm analyses indicated that more sperm were present in the cauda epididymidis of αERKO mice on lab chow diet comparfed to those fed casein. All sperm motility parameters were altered in αERKO mice, and more so in those fed lab chow, suggesting that phytoestrogens function to promote sperm motility.

The cystic fibrosis transmembrane conductance regulator (CFTR) had been localized in the efferent duct. In the epididymis, reaction product was visualized over the apical plasma membrane of principal cells, but not their microvilli, along with a diffuse weak apical cytoplasmic reaction. Examination of orchidectomized rats with or without testosterone supplementation revealed an intense apical/supranuclear reaction in the form

of small vesicular structures . Taken together these data suggest that a luminal factor derived from the testis regulates the targeting of CFTR-containing vesicles to the apical plasma membrane of principal cells.

RÉSUMÉ

Les aquaporines (AQPs) sont des canaux transmembranaires qui permettent le passage rapide de l'eau au travers d'un épithélium, et ce, à bas coût énergétique. Avec la découverte que différentes aquaporines étaient localisées dans différents tissus, nous avons systématiquement analysé toutes les aquaporines en utilisant l'immunocytochimie sur des tissus fixés au Bouin. Dans les testicules, AQP-0 est exprimé dans les cellules de Sertoli and de Leydig. L'expression épithéliale de AQP-10 est restreinte aux microvillis des cellules non-ciliées et aux ciles des cellules ciliées. AQP-3 est localisée exclusivement dans les cellules basales de l'épididyme. Ensembles, ces résultats indiquent une spécificité cellulaire et tissulaire dans l'expression des aquaporines susmentionnées. Nous démontrons aussi le rôle d'un facteur luminal dans l'expression de AQP-3 dans les cellules basales de l'épididyme.

Les estrogènes jouent un rôle important dans les ductules efférents, où leurs récepteurs alpha sont localisés. Les souris déficientes dans l'expression de ces récepteurs sont infertiles secondairement à une rétention hydrique au niveau des ductules efférents. Nous examinons le rôle des récepteurs alpha auprès des aquaporines exprimées dans les ductules efférents ainsi que les comptes spermatozoïdaux et les paramètres de mobilité dans des souris « knockouts ». Des souris se nourrissant de « chow » laboratoire (contient des phytoestrogènes) et d'autres de caséine (sans phytoestrogènes) ont été comparées. L'immunoréaction prominente observée pour les AQP-1, -9 et –10 dans les souris normales est dramatiquement réduite dans les souris « knockouts ». Pas de changements additionnels ont été notés en conséquence à l'administration des différentes diètes. L'analyse des spermatozoïdes par analyse assistée par ordinateur (CASA) nous indique une augmentation du nombre au niveau de l'épididyme caudal dans les souris « knockouts » nourries au « chow » de laboratoire. Tous les paramètres de mobilité sont diminués dans les « knockouts » et de façon beaucoup plus compromettante chez celles nourries au « chow » de laboratoire suggérant que les phytoestrogènes présents stimulent la mobilité cellulaire.

Le régulateur de conductance transmembranaire absent dans la fibrose kystique (CFTR) est localisé dans les ductules efférents. Dans l'épididyme, une immunoréaction a

été visualisée au niveau de la membrane apicale des cellules principales, sans réaction sur leurs microvillis, ainsi qu'une réaction diffuse apicale cytoplasmique. L'analyse des souris orchidectomisées avec ou sans supplémentation en testostérone révèle une réaction apicale supranucléaire vésiculaire. Ces résultats suggèrent qu'un facteur luminal influencerait la fusion des vésicules contant le CFTR avec la membrane apicale des cellules principales de l'épididyme.

INTRODUCTION

The male reproductive tract is a complex organ whose ultimate goal is to produce fertile sperm to achieve man's most primitive function, reproduction. The sperm are produced in the testis through a process called spermatogenesis. They then travel through the efferent ductules, where water reabsorption concentrates the sperm. If this fails, diluted sperm do not interact with epididymal secretions and cannot become motile and fertile. Therefore, the rationale behind the work presented in this thesis is that water reabsorption is essential for fertility.

Hence, the first part of this study is dedicated to the assessment of aquaporin channel expression in different regions of the male reproductive tract. Sharing of embryological origin and the importance of water homeostasis in the renal excretory system, have led us to use this organ as a model in our research on aquaporins in the male reproductive tract. Previous studies had shown that three aquaporins were expressed in the male tract. Knowing that there are 11 members to the aquaporin family, we took a systematic approach and addressed all other aquaporins.

Upon localizing many other members of the aquaporin family, we used the paradigms that estrogen regulated water reabsorption in the male and that the α-estrogen receptor knockout (αERKO) mice were infertile. We therefore looked into the role of estrogen in regulating aquaporin channel expression.

After finding that estrogen had a role to play in aquaporin expression, we decided to undertake the analysis of sperm counts and sperm motility parameters using computer assisted sperm assessment (CASA) technology. This allowed us to provide insight into the pathophysiology of infertility.

At the same time, we were interested in the definition of water homeostasis which is the balance between water reabsorption and secretion. Water homeostasis is a tightly achieved balance between these two very complex mechanisms. Water reabsorption was studied via estrogens and aquaporins. The cystic fibrosis transmembrane regulator (CFTR) is a chloride channel, that provides the osmotic gradient for water secretion in many epithelial systems. We therefore decided to investigate CFTR localization in the

male reproductive tract with focus on the epididymis, where water homeostasis is a key player in the achievement of fertility.

CHAPTER 1

Literature Review

A. MALE REPRODUCTIVE TRACT ANATOMY AND FUNCTION

TESTIS

The testes are formed from the primordial germ cells that migrate from the yolk sac to the genital ridges of the mesonephros, where they form the primary epithelial or medullary cords in association with somatic cells from the genital ridges.

The testes in the adult are paired ovoid organs encapsulated in a fibrous capsule called the tunica albuginea. The testis is made up of seminiferous tubules and interstitial spaces. Interstitial spaces are located between seminiferous tubules and contain all the blood and lymph vessels and nerves of the testicular parenchyma (Fawcett et al., 1969; Clark, 1976). Also present in these spaces are Leydig cells, mast cells (Nistal et al., 1984; Hermo and Lalli, 1978; Christensen et al., 1976) and macrophages (Niemi et al., 1986; Miller et al., 1984).

The walls of the seminiferous tubules are formed by the seminiferous epithelium. The seminiferous epithelium is responsible for the production of the spermatozoa. This epithelium is composed of two cell types: germ cells that evolve into the gametes and the sustentacular cells or Sertoli cells.

CELL TYPES OF THE TESTIS

Sertoli cells

Structure

The Sertoli cell was discovered by Enrico Sertoli in 1865. It is a tall columnar cell whose lateral as well as apical cell membranes present complex infoldings (Wong and Russel, 1983). The cytoplasm contains abundant profiles of smooth endoplasmic reticulum, numerous mitochondria, a well developed Golgi apparatus (Schulze, 1974; Ross, 1976; Vogl et al., 1983) and numerous vesicles that belong to the endocytic

apparatus (Hermo et al., 1994). Cytoskeletal elements are very abundant in the cytoplasm (Fawcett, 1975, 1977) which is in keeping with one of the main functions of the Sertoli cell, the structural support of the developing germ cells. There are also abundant contractile elements in the cytoplasm which help the Sertoli cell alter its shape in relation to the 14 stages of the spermatogenic cycle (Leblond and Clermont, 1952). The lateral cell membranes of adjacent Sertoli cells form occluding junctions with each other (Flickinger and Fawcett, 1967; Russell and Peterson, 1985; Fawcett et al., 1970) subdividing the lumen of the seminiferous tubules into two concentric compartments (Fawcett, 1973). The basal compartment is narrower and located basal to the zonulae occludentes and surrounds the adluminal compartment.

Function

The functions of the Sertoli cells are numerous. Postnatally, the Sertoli cell plays an essential role in spermatogenesis. It secretes a variety of products (Griswold, 1003) that provide the developing germ cells with a unique environment.

One of the most important roles of the Sertoli cell is the physical and nutritional support of the developing germ cells. The infoldings of the lateral and apical cell membranes accommodate the penetration of the elongating spermatids (Wong and Russell, 1983). The functions of the Sertoli cell vary according to the stages of the cycle of spermatogenesis.

The zonulae occludentes of these cells establish a blood-testis barrier (Russell, 1078) that isolates the adluminal compartment from the connective tissue influences, thereby protecting the developing gametes from the immune system. The blood-testis barrier also serves the function of maintaining a gradient of ions, small molecules and proteins between blood and tubular fluid in order to create the unique environment needed for the proper development of the germ cells. Just before the spermatozoa are released, Sertoli cells phagocytose the excess cytoplasm (the residual bodies) from the spermatids. The Sertoli cell also secretes a fructose-rich fluid that nourishes, and at the time of spermiation, transports the spermatozoa to the genital ducts. This fluid is produced at a rate of approximately 10-20µl per gram of testis per hour (Voglmayer et

al., 1967, 1970; Waites and Einer-Jensen, 1974; Free and Jaffee, 1979) and is continuous without diurnal variation. This fluid has ten times more potassium than blood plasma. Many researchers have tried to elucidate the process by which fluid is secreted by the Sertoli cell. It is suggested that the fluid comes from the vascular channels underlying the epithelium of the seminiferous tubules. However, the tight junctions between adjacent Sertoli cells do not allow the movement of water between the cells (Nicander, 1967; Fawcett et al., 1970; Dym and Fawcett, 1970; Dym, 1973). The junctions of the blood-testis barrier would however permit the establishment of an osmotic gradient on the luminal side of the junctions (Setchell et al., 1969; Setchell, 1970). As in the standing osmotic gradient model of Diamond and Tormey (1966), a Na^+K^+ATPase present in the basal domain of the cell would pump potassium from the interstitial space into the cell, which would then diffuse into the intercellular spaces. Water would then follow this osmotic gradient into the intercellular spaces and into the lumen of the tubules.

Germ cells

Germ cells are the cells that undergo spermatogenesis to produce the male gametes. Spermatogenesis depends upon the unique environment formed in the seminiferous tubules by the Sertoli cells and the blood-testis barrier (Setchell and Waites, 1975). The production of the spermatozoa may be divided into three steps: a) stem cell renewal by mitosis, b) the reduction of the chromosome number by meiosis and c) the metamorphosis of the germ cell into an organized motile structure (spermiogenesis)

Leydig cells

Structure

The most prominent feature of this cell's cytoplasm is the very extensive smooth endoplasmic (sER) reticulum. In the rat, up to 39% of the cytoplasm may be occupied by profiles of sER (Kerr et al., 1979). The cytosol also contains lipid droplets bounded by a

membrane of approximately 50A (the typical lipid bilayer is approximately 75A). The droplets consist primarily of cholesterol and neutral fats (Johnson, 1979).

Function

Leydig cells function in secreting androgens in a regulated fashion (Eik-Nes and Hall, 1965; Christensen and Mason, 1965). Androgens are responsible for the development and maintenance of the internal and external genitalia, secondary sex characteristics, development of the musculoskeletal system, feedback inhibition of the hypothalamopituitary axis and stimulation of spermatogenesis. The substrate for androgen production is cholesterol (Hall, 1963). Leydig cells can synthesize their own cholesterol or can import it from blood plasma in lipoproteins (Hall, 1970, 1979). They use LDL (Freeman, Ascoli, 1983) and HDL (Chen et al., 1980) as a source of cholesterol that they store in the cytoplasm in lipid droplets (Christensen, 1975). Indirect evidence also suggests that Leydig cells as well as Sertoli cells are involved in the synthesis and secretion of estrogens (Dorrington, Armstrong, 1975; DeJong et al., 1974; Tcholakian and Steinberger, 1979).

Postnatal development of the testis

The development of the testis begins with the formation of sex cords composed of immature Sertoli cells and primordial germ cells (Clermont and Huckins, 1961). During fetal life and a short time after birth, the sex cords contain a greater number of Sertoli cells. The proliferative activity of the Sertoli cells declines steadily to complete cessation at postnatal days 14 to 16 in the immature rat to establish the non-dividing adult population (Clermont and Perey, 1957; Clermont and Leblond, 1953; Steinberger and Steinberger, 1977). At approximately the same time that fetal Sertoli cells are actively dividing (just before birth), the fetal Leydig cells reach maximum numbers.

The inter-Sertoli-cell tight junctions that form the blood-testis barrier appears at days 16 to 19 after parturition, which is after the cessation of Sertoli cell division and the onset of spermatogenesis (Connell, 1980; Pelletier and Friend, 1983).

At postnatal day 18, the lumen of the seminiferous cords forms (Vitale et al., 1973; Tindall et al., 1975) and the Sertoli cells begin to secrete substances into the lumen.

EFFERENT DUCTS

Anatomy of the duct

The efferent ducts consist of 4 to 20 tubules (Hemeida et al., 1978; Nistal and Paniagua, 1984) that arise from the rete testis and come together to form a single highly convoluted duct, the epididymis in certain animals including the rat, mouse and some guinea pigs (Cooper and Jackson, 1972; Guttroff et al., 1992; Jones and Jurd, 1987; Lewis-Jones et al., 1982; Reid and Cleland, 1957).

The epithelium of the efferent ducts is composed of ciliated and nonciliated cells (Byers et al., 1985; Hermo and Morales, 1984; Hermo et al., 1988; Hamilton, 1975; Hess and Bassily, 1988; Hoffer and Greenberg, 1978; Jones and Jurd, 1987; Ramos and Dym, 1977). The transition from a rete testis epithelium to that of the efferent ducts is very abrupt, changing from low cuboidal to low columnar (Amann et al., 1977; Robaire and Hermo, 1988).

Cell types of the efferent ducts

Nonciliated cells

The nonciliated cells possess a brush border with well-developed microvilli (Hamilton et al., 1977). Immediately beneath the cell surface, membrane bound tubules are found (Hermo et al., 1988; Robaire and Hermo, 1988). Occasionally, these apical tubules are seen connected to endosomes in the apical cytoplasm of the cells (Wrobel, 1972; Robaire and Hermo, 1988). Below these endosomes, multivesicular bodies are present and in the supranuclear region, lysosomes can be seen (Robaire and Hermo, 1988).

Ciliated cells

The ciliated cells of the efferent ducts are noted to possess the organelles typical of ciliated cells elsewhere in the body (Hoffer, 1972).

Functions of the epithelium

The most obvious function of the efferent ducts is not only a conduit transporting spermatozoa from the testis to the epididymis, but also the reabsorption of fluid that is constantly secreted from the seminiferous epithelium (Crabo, 1965; Mason and Shaver, 1952).

An essential role played by the efferent ducts is that of water reabsorption. The epithelium of the efferent ducts reabsorbs between 50% and 96% of the fluids secreted by the seminiferous tubules (Crabo, 1965; Djakiew and Jones, 1983; Howards et al., 1975; Jones, 1981; Jones and Jurd, 1987; Levine and Marsh, 1971; Turner, 1984). There still remains controversy regarding the mechanism by which water is reabsorbed from the lumen of the efferent ducts. Some researchers suggest that endocytosis is the main mechanism by which fluid is moved across the epithelium (Goyal et al., 1980, 1981, 1988; Hoffer et al., 1972; Flickinger et al., 1978). Jones and Jurd (1987) have shown that the rate of absorption of water from the efferent ducts could not be accounted for by endocytosis alone. Crabo and Gustafsson (1964), Crabo (1965), Montorzi and Labiano (1970), Levine and Marsh (1971), Jenkins et al. (1980), Turner (1979, 1984) and Hinton and Turner (1988) have shown that the concentration of sodium decreases from the rete testis to the caput epididymidis because this ion is reabsorbed in the efferent ducts. This decline in sodium concentration was concomitant with the reabsorption of fluid from the lumen as evidenced by the increase in sperm concentration. Levine and Marsh (1971) have shown that sodium is reabsorbed against its electrochemical gradient (demonstrating the presence of an enery-dependant pump) and that water reabsorption was secondary to the transepithelial transport of this electrolyte. Hinton and Turner (1988) and Wong et al. (1978) have postulated that fluid reabsorption in the efferent ducts is dependant of the transepithelial transport of chloride. Like sodium, chloride is transported against its

electrochemical gradient and its concentration decreased along the length of the excurrent duct (Crabo, 1965); Levine and Marsh, 1971). Hohlbrugger (1980) has demonstrated that chloride reabsorption is concomitant with fluid reabsorption.

The cells of the efferent ducts have also been involved in the internalization of macromolecules (Jones, 1987; Koskimies and Kormano, 1975; Olson and Hinton, 1985). Hermo and Morales (1984), Hermo et al. (1985) and Morales and Hermo (1983) have shown that the internalization of macromolecules would proceed via fluid-phase, adsorptive and receptor-mediated endocytosis.

The main function of the efferent ducts is to reabsorb the majority of the fluid entering the duct from the rete testis. The non-ciliated cells of the epithelium possess a well developed endocytic apparatus and are greatly involved in the internalization of large molecules. Fluid reabsorption in the ducts is possible secondary to the active transport of electrolytes.

Regulation of the efferent ducts

It is well known that the male reproductive tract is greatly dependant on the presence of androgens. Recently, however, the role of estrogen in male reproductive tract has become more important than previously known. Estrogens have been localized in high concentrations in rete testis fluid and in semen (Claus et al., 1987, 1992; Eiler and Graves, 1977; Free and Jaffe, 1979) and estrogen receptors have been reported in the male reproductive tract of several species (Danzo and Eller, 1979; Murphy et al., 1980; Younes and Pierrepoint, 1981; Schleicher et al., 1984; Tekpetey and Amann, 1988; Toney and Danzo, 1988; West and Brenner, 1990; Iuchi et al., 1991; Goyal et al., 1997). Hess et al. (1997) have demonstrated that estrogen receptor-α (ERα) was expressed in the efferent ducts o0f the rat at a concentration nearly 3.5 times of that in the rat uterus. Mice lacking the ERα (ERKO) were shown to have numerous abnormalities along the entire male duct (Hess et al., 2000) including a deficiency in fluid reabsorption in the efferent ducts (Hess et al., 1997).

EPIDIDYMIS

After spermatogenesis, the sperm leave the seminiferous tubules and are transported to the epididymis via the efferent ducts. The sperm entering the epididymis are not mature and are not capable of fertilizing an ovum. The journey through the epididymis infers motility and fertilizing ability to the spermatozoa (Robaire and Hermo, 1988).

Anatomy of the duct

The epididymis is a highly convoluted tube that extends from the efferent ducts to the vas deferens. It is divided into five regions based on the different cell types present in these regions and the frequency of their occurrence. The first region that receives the spermatozoa from the efferent ducts is the initial segment. The sperm then go into the intermediate zone, the caput, corpus and finally the cauda where the sperm may be stored for a short period of time (Robaire and Hermo, 1988).

Cell types of the epididymis

The main cell type present throughout the duct is the principal cell. It is an active secretory cell with extensive endoplasmic reticulum and a well developed Golgi apparatus. There is also the basal cell that is present in all the epididymal regions. It contains glutathione S-transferases to protect the epithelium from harmful electrophiles. Other cell types include the narrow cell, present only in the initial segment. The narrow cell is a specialized cell that pumps H^+ ions into the lumen to acidify it. Finally, there are clear cells that are present in the caput, corpus and cauda, their numbers increasing from the caput to the cauda. These cells are very active resorptive cells displaying a very prominent endocytic apparatus (Robaire and Hermo, 1988; Hamilton, 1975; Hermo et al., 1994).

Functions of the epithelium

The main function of the epididymis is to create a fluid microenvironment that is appropriate for sperm maturation. The tight junction between principal cells (Suzuki and Nagano, 1978; Greenberg and Forssmann, 1983; Hoffer and Hinton, 1984) of the epithelium form a blood-epididymis barrier that allows the formation of a luminal environment distinct from blood plasma. The blood-epididymis barrier has been functionally demonstrated by several researchers (Crabo and Gustafsson, 1964; Jendins et al., 1980); Turner et al., 1984) showing that the composition of luminal fluid with regard to the concentration of inorganic and organic substances was very different from that of blood.

The sperm transit in the epididymis imparts on them the ability to fertilize an ovum. It has been discovered that passage through some part of the cauda is essential for acquiring fertilizing capability (Orgebin-Crist and Olson, 1984). The sperm also acquire motility in the epididymis but the mechanism of this acquisition is still unknown. Although the sperm are motile and capable of fertilization, there are luminal proteins secreted by the epithelium that maintain the sperm quiescent (Usselman and Cone, 1983; Turner and Giles, 1982; Carr and Acott, 1984; Kirchhoff, 1999).

Secretion

The epididymis can secrete into its lumen a variety of ions, small organic molecules and glycoproteins. The main secretory cell of the epithelium is the principal cell. It can synthesize small molecules or can take them up from the circulation, transport and secrete them into the lumen. The lumen also contains proteins that differ from those present in blood plasma (Alumat et al., 1971; Amann et al., 1973; Olson and Hinton, 1985; Turner et al., 1979). A number of these proteins adhere to and interact with the sperm and have therefore been implicated in sperm maturation and the acquisition of fertilizing ability (Orgebin-Crist and Jahct, 1978) and sperm motility (Turner and Giles, 1982; Kirchhoff, 1999).

Absorption

As previously stated, 90% of the fluid entering the efferent ducts from the rete testis is absorbed by the epithelium of the efferent ducts and the proximal region of the epididymis (Crabo, 1965; Levine and Marsh, 1971). Wong (1990) has performed numerous studies on water reabsorption in the distal regions of the epididymis. It is suggested that a standing osmotic gradient is established across the epithelium to move water from the lumen to the interstitial space and into the vascular channels. The osmotic gradient is established by passive diffusion of movement of sodium across the apical surface of the epithelium followed by its active transport across the basolateral membrane of the cells. The active transport across the basolateral membrane is thought to be via a Na^+K^+ATPase located on these membranes (Byers and Graham, 1990). The sodium gradient created draws water and Cl^- from the lumen. This movement is electroneutral and is probably due to the secretion of H^+ and K^+ into the lumen (Wong, 1990). Levine and Marsh (1971) offered supporting evidence for this model when they measured the concentrations of different ions in the lumen of the epididymis and found that the concentration of Na^+ and Cl^- decreased from the caput to the cauda and that the concentration of K^+ increased.

Regulation of the epididymis

Androgens regulate the growth and development of the epididymis as well as the microenvironment produce in the lumen of the duct (Orgebin-Crist, 1996). The epididymis receives androgens both from the circulation and from testicular fluid (Orgebin-Crist, 1996). Various epididymal functions are regulated by factors coming from the testes that enter the epididymal lumen (Fawcett and Hoffer, 1979). These factors are probably secreted by the Sertoli cells (Robaire and Ewing, unpublished observations). Water resorption has been shown to be dependant not only on androgens (Wong and Yeung, 1977) but also aldosterone (Au et al., 1978; Turner and Cesarini, 1983) and affected by a number of diuretics (Jenkins et al., 1983; Wong and Lee, 1982).

Postnatal development of the efferent ducts and epididymis

Elevated levels of androgens are present during the period of development of the cells of the epididymis and they have been shown to affect the differentiation of the epididymal epithelial cells (Orgebin-Crist et al., 1975).

At postnatal day 21, the epithelial cells of the male duct are undifferentiated (Hermo et al., 1992). At postnatal day 39 in the efferent ducts, the nonciliated cells begin to take on a more differentiated appearance (Hermo et al., 1992) and resemble adult cells by day 49. In all regions of the epididymis, principal cells differentiate by day 39. At that same age, narrow cells in the initial segement take on adult appearance and the clear cells in the cauda also resemble adult cells. However, clear cells in the caput and corpus regions differentiate only by day 49 (Hermo et al., 1992).

B. THE IMPORTANCE WATER TRANSPORT

Water is an important component of all biological systems. Membrane water transport is an activity that is shared by many cells of the body. Water has the ability to simply diffuse through the membrane lipid bilayer. However, this process is costly both in time and energy. It is known that diffusion of water through membranes is driven by ionic gradients which require energy-dependent processes and therefore could become costly when water channels are not present. In some tissues, the cells require water transport to be more rapid. It is now believed that when water needs to cross a non-fenestrated endothelium or an epithelium with tight junctions, is does so at a fast rate and with low energy cost, only if the cell membrane contains an adequate number of specific water channels (Wintour, 1997). These water channels have been collectively termed the aquaporins.

AQUAPORINS

Gene structure of aquaporins

Five members of aquaporin family posses genes that are similarly organized. The genes for the mammalian AQPs 1, 2 and 5 all have a large first exon encoding the amino terminal of the molecule and exons 2, 3 and 4 encoding segments of the carboxy terminus (Inase et al., 1995; Lee et al., 1996; Lu et al., 1996; Tsukaguchi, 1999). Although their amino-acid sequences contain variability, their intron-exon boundaries are located at identical sites in the genes. In contrast to these four members, AQPs 3, 7, 8 and 9 are coded for by genes that possess 6 exons (Echevarria et al., 1994; Ishibashi et al., 1994, 1997; Ma et al., 1994). The gene for AQP-4 also differs from the classical gene structure. It contains in its amino terminus an additional exon (exon 0) without homology to other aquaporin genes (Lu et al., 1996). Analysis of the sequence of the genes of all members of the Major Intrinsic Protein family, to which all aquaporins belong, has revealed that the first and second half of the genes are homologous (Park and Saier Jr, 1996; King and Agre, 1996). The genes for the different aquaporins have been mapped to chromosomal

loci in the human genome. AQPs 0, 2, 5 and 6 (Lee et al., 1996; Lu et al., 1996; Ma et al., 1996; Mulders et al., 1996) have been mapped to chromosome 12p13 and AQPs 3 and 7 have been located at chromosome 9q13 (Ishibashi et al, 1995, 1998) suggesting that subfamilies of these water channels are located in gene clusters.

Protein structure of the channels

As previously said, the first exon of the aquaporin gene is translated into the amino terminus of the protein while the other exons code for segments of the carboxy terminus (King and Agre, 1996). The structure of the aquaporin molecules has been extensively studied. The molecular weight of the aquaporins ranges between 25 and 30 kDa in their non-glycosylated states. Hydropathy plots revealed that the channels are made up of 6 membrane-spannig domains (King and Agre, 1996; Preston and Agre, 1991) connected by 5 loops (A-E). Both the amino and carboxy termini are intracellular (King and Agre, 1996; Bai et al., 1996; Preston et al., 1991, 1994; Shi et al., 1995) Loops B and E contain a conserved NPA (Asn-Pro-Ala) box present in all members of the MIP family. Point mutagenesis studies indicate that the NPA motif is implicated in the water permeating function of the aquaporins (Bai et al., 1996; Jung et al., 1994; Kuwahara et al., 1997; Shi and Verkman, 1996) and that it may in fact be physically present inside the aqueous pore (Cheng et al., 1997; Lee et al., 1997; Walz et al., 1997). It was also observed that the first half of the molecule is homologous to the second half and that they were oriented 180° to each other (Preston et al., 1994). This internal symmetry allows for bi-directional flow of water through the pore (Meinild et al., 1998).

Sequencing of the proteins revealed N-linked glycosylation sites that receive a large polylactosaminoglycan (Verkman and Mitra, 2000; Van Hoek et al., 1995). However, glycosylation does not appear to be necessary for aquaporin function or membrane targeting (Baumgarten et al., 1998). Phosphorylation sites have also been found on some aquaporins (King and Agre, 1996) suggesting that they may be regulated by phosphorylation as is the case for AQP-2.

The current model for pore formation suggests that loop B folds into the lipid bilayer from the cytosolic face of the membrane and that loop E folds from the

extracellular face. The overlap of loops B and E domains between the leaflets of the membrane create an aqueous pore 3.8 A in diamete (Zhang et al., 1993), for the passage of water in a single file. This structure is referred to as the « hourglass model » (Jung et al., 1994; King and Agre, 1996), describing the shape of the channel. Hydrodynamic studies, freeze fracture and electron microscopic studies have revealed that aquaporins associate in the membrane to form homotetramers (King and Agre, 1996; Verbavatz et al., 1993; Walz et al., 1994; Mitra et al., 1995) and even larger aggregates in the case of AQP-4 whose homotetramers aggregate in the membrane in larger orthogonal arrays of particles (OAPs) (Ren et al., 1999; Verbavatz et al., 1997; Yang et al., 1996). Four independently functional pores (Verkman et al., 1996) associate non-covalently in the membrane to form the homotetramers with 50% of the subunits being glycosylated (Van Hoek et al., 1993, 1995). It is not yet clear whether different aquaporins can form heterotetramers in membranes of cells where more than one type of the channel is found (Jiang and Goodenough, 1996).

Tissue distribution of the known members

So far, 11 aquaporins (AQP-0 to AQP-10) have been identified and localized in several tissues of the body. AQP-0 is located in the eye lens epithelium (Wintour, 1997; Ishibashi et al., 1997; Nielsen et al., 1993) and its presence ther is thought to maintain the desiccation and transparency of the lens (Nielsen et al., 1993). First identified from erythocyte membranes as CHIP-28 (channel forming integral membrane protein of 28 kDa), AQP-1 has since been localized in many other tissues of the body, namely the choroids plexus of the brain, the proximal convoluted tubules and the descending limb of Henle's loop in the kidney, in the eye, lung, bile duct, vascular endothelium and others (Ishibashi et al., 1997; Nielsen et al., 1993a, b, 1995; Brown et al., 1993). Brown et al. (1993) using frozen sections and undiluted anti-AQP-1 antibody reported a patchy expression of AQP-1 over the apical and basolateral membranes of principal cells of the region of the ampulla of the vas deferens. Andonian and Hermo (1999) reported expression of AQP-1 over the endothelium of vascular channels and capillaries in the lamina propria. AQP-2 is expressed mainly in the kidney, in the cytoplasm and apical

pole of collecting duct cells (Nielsen et al., 1998; Sasaki et al., 1994; Uchida et al., 1994). AQP-3 is found mainly in the basolateral cell membrane of the principal cells of the collecting tubules, in the epithelium of the stomach, colon, (Echevarria et al., 1996; Ma et al., 1994) lungs, brain, skeletal muscle and eye (Ma et al., 1994). AQP-4 is localized in many tissues, namely the stomach, skeletal muscle, retina, lung, intestine, kidney and most importantly in various organs of the CNS (Ishibashi et al., 1997; Nielsen et al., 1997; Lee et al., 1997). Immunohistochemical experiments localized AQP-5 on the apical membrane of serous gland cells and not mucous gland cells. It was also intensely expressed on the microvilli in the intercellular canaliculi of serous glands. Other tissues where the protein is expressed are the corneal epithelium of the eye, apical membranes of acinar cells of the lacrimal gland and the apical membrane of type I pulmonary epithelial cells (Funaki et al., 1998). AQP-6 was isolated from kidney. Formerly known as hKID, it is mercury-sensitive and forms a water selective pore (Ma et al., 1996). Although its physiological function in the kidney is at present unknown, it is though to participate in water transport. This speculation is based on the fact that dehydration caused upregulation of the protein in the rat (Dibas et al., 1998). The gene encoding AQP-7 was identified from rat testis where it is most abundantly expressed. Other tissues expressing this channel are the heart and kidney, and weaker expression occurs in skeletal muscle and brain (Ishibashi et al., 1997). In situ hybridization of testis with antisense probe showed staining of the cells of the late stages of spermatogenesis located at the inner surface of seminiferous tubules (Ishibashi et al., 1997). AQP-8 is a second aquaporin abundantly expressed in testis (Ishibashi et al., 1997). A weaker band was found in the liver. AQP-9 is the last known member of the aquaporin family. Using Northern Blots, Kuriyama et al. (1997) localized AQP-9 in adipose tissue, with much fainter bands detected in heart, kidney and small intestine. Ishibashi et al. (1997) localized AQP-9 mainly in peripheral leukocytes, with weaker bands in liver and still weaker bands in lung and spleen.

Functions of the channels

Aquaporins are proteins that form an aqueous pore in membranes of cells that require rapid movement of water across their membranes. It is suggested that ion pumps in the membranes of the cells move electrolytes across the cells and that water follows the gradient thus created. Therefore, the channels facilitate the diffusion of water across the lipid bilayer. Furthermore, the aggregation of AQP-4 into large OAPs suggests that it may be involved in a bulk siphoning mechanism (Yang et al., 1996). AQPs 0, 1, 2, 4, 5, 6 and 8 from water selective pores (Fushimi et al., 1993; Ishibashi et al., 1994; Ma et al, 1994) whereas AQPs 3, 7 and 9 have been demonstrated to be permeable not only to water, but to glycerol and urea (Echevarria et al., 1994, 1996; Ishibashi et al., 1994, 1997) and AQP-9 has been shown to permeate even larger neutral solutes as well (Tsukaguchi et al., 1998). In these 3 aquaporins, water and the different solutes appear to pass through the pore of the channel and not between monomers of the tetramer. The channels (with the exception of AQP-2) appear to be constitutively expressed in the membranes where they are located and no gating mechanism has been elucidated to explain the selectively of the pores. All but two of the aquaporins (AQP-4 and AQP-7) are inhibited by mercurial compounds (King and Agre, 1996; Preston et al., 1993), which do not inhibit water's diffusion across the membranes. Aquaporin 2 in the kidney is responsible for the resorption of water from the collecting ducts under the regulation of vasopressin (AVP). AVP binds its V2 receptor and this increases the expression of the water channel on the apical plasma membrane (DiGiovanni et al., 1994; Nielsen et al., 1995). When the AVP signal ceases, the AQP-2 molecules are internalized back into the cell where they reside in apical vesicles until the next AVP signal (Katsura et al., 1996).

Diseases associated with aquaporins

Numerous diseases states have been associated with mutations in aquaporins or the lack of the molecule altogether. AQP-0 has been implicated in maintaining the desiccation and transparency of the lens of the eye. Lack of this channel has been implicated in the formation of cataracts in animal models (King and Agre, 1996; Shiels and Basnetts, 1996).

Since AQP-1 was found to be responsible for the protection of erythrocytes from osmotic shock, it was thought that the lack of this channel would be pathological if not lethal to the organism. After extensive worldwide searches of blood banks, a handful of individuals have been found who lack a functional AQP-1 (King and Agre, 1996). These five (Colton-null) persons are virtually AQP-1 knockouts. Surprisingly, the individuals present no overt pathological phenotype (King and Agre, 1996). Whether other water channels compensate for the lack of AQP-1 is at present unknown. Interestingly, the five Colton-null persons are female. This poses the question: does the lack of AQP-1 cause a more severe phenotype in males? This still remains a mystery. However, animal models for the lack of AQP-1 do display a defect in their urine concentrating ability (Verkman and Mitra, 2000).

Mutations in AQP-2 cause a sever form of nephrogenic diabetes insipidus (NDI) (van Lieburg et al., 1995; Deen et al., 1995) whose clinical hallmark is the excretion of large volumes of dilute urine (King and Agre, 1996). AQP-3 null mice, as in AQP-1 knockouts also display difficulty in concentrating their urine (Verkman and Mitra, 2000). The lack of AQP-4 may be implicated in pseudotumor cerebri, which is believed to result from abnormal CSF resorption (King and Agre, 1996). Lack of AQP-5 in animals leads to defects in saliva and tear production. More studies must be performed to determine the pathologies associated with the other aquaporins.

Prospects for new drug discovery

Aquaporins have been suggested to be important in the search for new drugs. Aquaporin inhibitors might serve as aquaretics in hypertension and congestive heart failure, inhibitors of brain edema following head trauma and regulators of intracranial and intraocular pressure (Verkman and Mitra, 2000). Aquaporin activators or aquaporin gene replacement might be useful in glandular hypofunction such as in Sjorgren's syndrome and in water diuretic states (Verkman and Mitra 2000). AQP-1 has also been associated with angiogenesis in tumor formation (Lanahan et al., 1992). This information may prove valuable in the fight against cancer.

C. A POTENTIAL ROLE FOR ESTROGENS IN THE MALE REPRODUCTIVE TRACT

Estrogen is considered to be the « female » hormone, whereas testosterone is considered the « male » hormone. In fact, testosterone's metabolite, 5α-dihydrotestosterone (DHT), is recognized as the primary hormone that regulates epididymal functions (Orgebin-Christ et al, 1975; Robaire and Viger, 1995). However, both hormones are present in both sexes. Thus sexual distinctions are not qualitative differences, but rather result from quantitative divergence in hormone concentrations and differential expressions of steroid hormone receptors. In males, estrogen is present in low concentrations in blood, but can be extraordinarily high in semen, and as high as 250 pg ml^{-} in rete testis fluids (Ganjam et al, 1976; Free et al, 1979), which is higher than serum estradiol in the female (Smith et al, 1975). Estrogen in the rete testis fluid is now thought to be derived from the conversion of testosterone to estradiol by P450 aromatase found in germ cells of the testis and spermatozoa traversing the reproductive tract, as well as in Leydig cells and Sertoli cells of the testes. It is well known that male reproductive tissues express estrogen receptors (Cooke et al, 1991; Greco et al, 1993, Schleicher et al, 1984; West et al, 1990), in particular, the efferent ductule region, where ERα is abundant. However, ERα and ERβ are both found in various regions of the epididymal duct and vas deferens. So, what does estrogen do in the male reproductive system? An answer to this question remains incomplete at this time, as we are only now beginning to reveal some of the potential functions through the use of knock-out mice and pure antiestrogen compounds.

We will try to elucidate some of the possible roles of estrogen by covering the following topics : 1) Estrogen source and concentrations, 2) Estrogen receptors, 3) Estrogen and development, 4) Estrogen functions in efferent ductules, 5) New data on the regulation of water reabsorption by estrogen.

Estrogen source and concentration

In the males, estrogen is produced in sizable quantities in the testis, as well as the brain (Roselli et al, 1997). In fact, estrogen is synthesized in the male reproductive tract by at least three different cell types; Sertoli, Leydig and germ cells, and is therefore found in extremely high concentrations in the semen of several species (Claus et al., 1992, 1987; Free and Jaffe, 1979, Ganjam and Amann, 1976).

Early studies reported that the primary source of estrogen in the male was Sertoli cells of the testis (van der Molen et al., 1981). In the adult testis, Leydig cells express P450 aromatase and actively synthesize estradiol at a rate much greater than that seen in the adult Sertoli cells (Payne et al., 1976; Carreau et al., 1999). Currently, a growing body of evidence indicates that germ cells also synthesize estrogen, and possibly serve as the major source of this steroid in the male reproductive tract (Nitta et al, 1993; Hess et al, 2001). Thus, it appeared from this early work that sperm could serve as mobile endocrine units, capable of producing estrogen that would target estrogen receptors (ER) downstream from the testis.

Estrogen receptors

In 1975, Danzo suggested that estrogen might be capable of binding to receptors in the epididymal epithelium and serve some type of function in the male. Estrogen receptors have been detected in the male reproductive tract of numerous species (Schleicher et al., 1984; Cooke et al., 1991b; Greco et al., 1993). Estrogen receptors come in two isoforms, alpha (ERα) and beta (ERβ), in the male reproductive tract, and their distribution has been found to vary greatly between species. For purposes of simplicity, emphasis will be put on their distribution in the rat.

Autoradiographic techniques were initially used for specific localization of ^{3}H-estradiol binding in tissues. Using this technique, Schleicher and coworkers (1984) found very strong labeling of the efferent ductules and initial segment of the epididymis, with lesser binding to the distal tract. Knowing that there are two isoforms to the receptor, they wouldn't be able to conclude what isoform radiolabeled-estrogen was binding to. Therefore, other approaches necessary to separate the two ER subtypes.

Using immunocytochemistry (ICC), estrogen receptors have been localized for both subtypes and their distribution broadly defined. In the rat at 90 days of age, the efferent ductule epithelium was strongly positive for ERα. immunostaining. Other epithelia along the epididymis showed negligible reaction. In the vas deferens, anti- ERα antibody gave no reactivity in the epithelium (Hess et al., 2002).

The discovery of a second form of ER (ERβ) further complicates the interpretation of earlier data from estrogen binding studies. ERβ has now been found in testis, efferent ductules, epididymis and prostate (Kuiper et al., 1996, Hess et al., 2002). However, function for ERβ in the male reproductive tract awaits further investigation, as the ERβ knockout mouse is fertile and appears to have a normal testis and epididymis (Krege et al., 1998). Phenotypic expressions of the ERα knockout mouse are much more dramatic, and the mouse is in fact infertile.

Estrogen and development

The effects of diethylstilbestrol (DES) on development and function of the male reproductive tract illustrates the potentially serious pathological responses to a potent synthetic estrogen. These include the following: cryptorchid testis, testicular atrophy, distension with overgrowth of the rete testis and formation of adenocarcinoma, distension of the efferent ductules, inhibition of aquaporin-1 expression in the efferent ductule epithelium, underdevelopment of the epididymis and vas deferens and formation of cysts, sperm abnormalities, sperm granulomas, coiling of the vas deferens, inflammation of the prostate, and other changes that could affect fertility and male reproductive functions (review by Klinefelter and Hess, 1998). All of this information argues for the fact that regulation of estrogen levels in the male reproductive tract is crucial for its normal development. Although a precise biochemical mechanism to account for DES-induced abnormal development is lacking, data has shown that in utero exposure to DES alters the expression of steroid hormone receptors (Sato et al., 1994). However, the question remains, what it is the function of estrogen in the adult male?

Estrogen functions in efferent ductules

Composed of columnar, nonciliated principal cells and ciliated cells with kinocilia, both having a well developed endocytotic system with features specialized for fluid reabsorption, the efferent ductule epithelium is recognized as a fluid-transporting (Clulow et al., 1998). The movement of water through these ductules involves several pathways, including paracellular flow and the use of apical and basolateral aquaporin water channels for transcellular movement (Fisher et al., 1998). In fact, active ion transport creates a gradient for water transport from lumen to vascular compartment, i.e. water reabsorption.

People hypothesized that estrogen might be the major effector in regulating water reabsorptive processes, crucial for the normal development of sperm. Three discoveries led to this hypothesis: 1) the estradiol concentration in rete testis fluid is relatively high, approximately 250 pg/ml (Free and Jaffe, 1979), 2) germ cells and sperm contain P450 aromatase and actively convert androgens to estrogen (Nitta et al., 1993; Hess et al., 1995), and 3) ERα is abundant in efferent ductule epithelium (Hess et al., 1997b). Because, the efferent ductules function to reabsorb nearly 90% of the luminal fluids, it was logical to suggest that estrogen could regulate this major physiological event in the male tract.

To test this hypothesis, the ERα gene knockout mouse (ERKO) (Lubahn et al., 1993) was evaluated for histological changes in efferent ductule epithelium, fluid reabsorption and fluid dynamics in the testis over time. The ERKO male is infertile and shows the following aberrations. The testes become atrophic at about 150 days. Sperm from the ERKO male are abnormal and sperm concentrations are significantly reduced in the epididymis (Eddy et al., 1996). Rete testis in ERKO males is dilated and protrudes into the testis (Hess et al., 1997a). Downstream from the rete, the efferent ductules are swollen (Hess et al., 1997a) with an increased luminal diameter in the ERKO ductules and reduced height in epithelial cells (Hess et al., 2000).

From these observations, two hypotheses were formed to explain how the disruption of ERα could cause fluid to accumulate in the ERKO testis. The first hypothesis involved excessive fluid reabsorption from the efferent ductule lumen, which would increase the concentration of sperm and cause luminal contents to become

compacted. This rapid response would induce and occlusion of the efferent ducts, which would produce fluid buildup in the testis and subsequent backpressure atrophy of the testis. The second hypothesis suggested that an opposite mechanism would cause an inhibition of reabsorption and possibly a net inward flux of water into the ductal lumen. This excessive accumulation of fluid in the lumen would overload the ductal system. Thus, the inhibition of reabsorption would also cause the accumulation of fluid in the lumen, which would subsequently cause backpressure atrophy of the testis.

All of the changes observed in the structure of the efferent ductule epithelium were consistent with a decrease in fluid reabsorption observed in the ERKO males. Thus, the apical surface of this absorbing epithelium appeared to be transformed into a non-absorbing lining, when ERα was lacking. To test this hypothesis, the initial segment epididymis was surgically cauterized to occlude the terminal end of the efferent ductules. Testis weight 48h post-surgery was increased 30% more in ERKO than in wild-type males (Hess et al., 1997a). Also, an *in vitro* experiment where the tubular ends of the efferent ductules were ligated with fine suture suggested that inhibition of reabsorption was at play. Efferent ductules from wild-type males were capable of rapidly reabsorbing the luminal fluid, resulting in a collapse of the ductule walls; however, the luminal area of ERKO ductules did not collapse, but instead showed a dramatic increase in the area (Hess et al., 1997a). Thus, ERKO mice appear to follow the second hypothesis for inducing fluid accumulation in the male tract. Therefore, a functional ERα is involved in the regulation of fluid transport in the male reproductive tract, and responsible for increasing the concentration of sperm as they enter the epididymis.

New data on the regulation of water reabsorption by estrogen: the sodium-hydrogen exchanger-3 (NHE-3)

One of the key effector proteins in the generation of an ion-gradient for fluid reabsorption is the sodium-hydrogen exchanger-3 (NHE-3) present on the apical plasma membrane of efferent ductule epithelium. Therefore, it was hypothesized that an examination of mice deficient in this gene would reveal the more important proteins regulating fluid reabsorption. The NHE-3 knockout was therefore generated and

morphologically analysed. This knockout presented almost identical morphological aberrations compared with the ERKO in that it has dilated rete testis and efferent ductules (Zhou et al., 2001). It was therefore suggested that estrogen might be playing a key role in regulating the expression of NHE-3. So Northern blot analysis was performed to test mRNA levels for the ion transporter and assess its expression in the ERKO male. As predicted, NHE-3 mRNA levels was reduced nearly 6-fold in ERKO (Zhou et al., 2001).

An additional parameter needs to be explained at this point. The following question needs to be asked: What if the structural and functional aberrations found in the ERKO mouse resulted from developmental deficit and not from the lack of function of the ERα?. To answer that question, a functional synthetic estrogen receptor antagonist, namely *ICI 182,780,* was used to assess that matter. In fact, immunocytochemical localization studies for the NHE-3 protein expression was studied using such antiestrogen treatments and comparing those results to the ERKO mouse. It was found that ICI-treated mice no longer expressed the NHE-3 protein, as did the ERKO mouse (Zhou et al., 2001).

Interference with this physiological process or inhibition of the expression and function of this critical gene leads to male infertility. Hence, a basic molecular mechanism by which estrogen controls fluid reabsorption in efferent ductules of male reproductive tract had been uncovered. One of its primary function is expression of NHE-3 gene, which regulates the exchange of Na^+ and H^+ in mediating water transport and the concentration of sperm in the epididymis, and thus fertilizing ability of sperm. Knowing that estrogen is such a potent hormone, is it safe to say that it can possibly be involved in other aspects of male reproduction, i.e. water reabsorption in the efferent ductules?

D. CONCLUSION

The physiological process of fluid reabsorption in the efferent ducts is critical for the transport of sperm to the initial segment of the epididymis as a concentrated entity so as to aquire fertilizing capability by having access to all the maturational factors secreted by the epithelium of the epididymis. A role for estrogen in such a process has been uncovered by numerous findings, i.e. NHE-3 expression.

In conclusion, estrogen is important in the regulation of the male reproductive tract, with clear evidence pointing to a direct effect on the function of the efferent ductule epithelium. However, the current litterature leaves the mechanisms of estrogen action in this resorptive epithelium, as well as in the epididymis and vas deferens, unsettled and even confusing. Although, a lot has been done on the possible role of ERα in mediating an estrogen response, we are missing important steps towards a comprehensive elaboration of the role of estrogen in the male as nothing much is known about the ERβ. Thus, answers to these questions will likely come from new studies that use *in vitro* methods or *in vivo* treatments with pure antiestrogens that target the epithelium from the lumen, rather than from the blood. Regardless, it is now well established that the loss of estrogen receptor activity in the male reproductive tract interferes with its normal function, and it appears that estrogen may be required for normal fertility in the male. Are we looking at the next solution for contraception in the male?

CHAPTER 2

Cell type and tissue specific expression of aquaporins -0, -3 and -10 in the testis, efferent ducts and epididymis of adult rats

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ABSTRACT

Aquaporins (AQPs) are transmembrane protein channels that allow the rapid passage of water across an epithelium at low energy requirement, though they also transport glycerol, urea and solutes of various sizes. At present 11 members of the AQP family of proteins have been described in mammals, with several being localized to the testis (AQP-7, 8), efferent ducts (AQP-1, 9) and epididymis (AQP-1, 9) of adult rats. With the discovery of expression of multiple AQPs in different tissues, we undertook a systematic analysis of several other members of the AQP family on Bouin-fixed tissues of the male reproductive tract employing light microscope immunocytochemistry. In the testis, AQP-0 expression in the seminiferous epithelium was restricted to Sertoli cells and to Leydig cells of the interstitial space; no reaction was observed in the efferent ducts or epididymis. In the case of Sertoli cells, a semicircular or pie-shaped pattern of staining was noted with only a quarter or half of the Sertoli cells of a given tubule showing a reaction product. Furthermore, while Sertoli cells at stages VI-VIII of the cycle showed intense staining, those at stages IX-XIV were least reactive, with Sertoli cells at stages I-V revealing intermediate levels of reaction product. The epithelial expression of AQP-10 was restricted to the microvilli of the nonciliated cells and cilia of ciliated cells of the efferent ducts, however, the endothelial cells of vascular channels of the efferent ducts and epididymis were also intensely reactive. AQP-3 expression was localized exclusively to the epididymis, where intense staining was noted exclusively over basal cells. Examination of orchidectomized rats revealed that AQP-3 expression was abolished over basal cells, and that it was greatly diminished after efferent duct ligation. As the reaction was not fully restored in orchidectomized animals supplemented with high levels of testosterone, it is suggested that AQP-3 expression in basal cells is regulated in part by testosterone, in addition to a luminal factor emanating from the testis. Taken together the data indicate a cell type and tissue specific expression for AQP-0, -3 and -10 in the testis, efferent ducts and epididymis, as well as differential regulating factors for the expression of AQP-3 in basal cells.

INTRODUCTION

While it is known that the transport of water across the lipid bilayer of cell membranes occurs by simple diffusion, it has been demonstrated that many mammalian tissues require a more rapid transport in and out of cells. As a consequence, protein water channels, referred to as aquaporins (AQPs), have evolved in the cell membranes of a variety of different tissues (Preston and Agre, 1991; Wintour, 1997; Verkman and Mitra, 2000; Schrier and Cadnapaphornchai, 2003). AQPs are homologous to the major intrinsic protein superfamily of integral membrane proteins and are assembled in plasma membranes as homotetramers. Each monomer has a molecular weight of approximately 30kD and consists of 6 membrane-spanning α-helical domains with its' own distinct pore to allow the bi-directional transport of water (King and Agre, 1996; Wintour, 1997; Verkman and Mitra, 2000). To date, 11 AQPs (0-10) have been found in different tissues, and they have been divided into 2 groups based on their permeability properties: the water-selective AQPs and the aqua-glyceroporins, which, in addition, also permeate urea, glycerol and other uncharged molecules (Preston and Agre, 1991; Deen and van Os, 1998; Borgnia et al., 1999; Sansom and Law, 2001; Hatakeyama, 2001).

AQPs are expressed throughout the mammalian body and have been studied extensively (Verkman and Mitra, 2000; Neilson et al., 2002; Schrier and Cadnapaphornchai, 2003). Many are tissue, region and even cell specific, and more than one AQP can be expressed on the same cell type (King and Agre, 1996; Echevarria and Ilundain, 1998; Verkman and Mitra, 2000; Neilson et al., 2002). While hormones regulate some AQPs, others are constitutively expressed (Verkman and Mitra, 2000; Neilson et al., 2002; Schrier and Cadnapaphornchai, 2003). Alteration in expression of AQPs has been shown to result in a variety of pathological states (King et al., 2000; Verkman and Mitra, 2000; Neilson et al., 2002; Schrier and Cadnapaphornchai, 2003).

The transport of water in the male reproductive tract is essential for its various functions. In seminiferous tubules of the testis, water is secreted into the lumen by Sertoli cells in order to create the fluid environment essential for maintaining spermatogenesis and in serving as the vehicle to move sperm from the testis and through the efferent ducts into the epididymis (Setchell et al., 1969). In the efferent ducts, up to 90% of the

testicular luminal fluid is reabsorbed and fluid is constantly reabsorbed and secreted along the epididymis to concentrate the sperm so that they can have the proper environment to become fertile and motile (Ilio and Hess, 2002; Wong et al., 2002).

In the male reproductive tract, the distribution and regulation of several members of the AQP family have been studied in some detail (Brown, 1993; Andonian and Hermo, 1999; Pastor-Soler et al., 2001; Nihei, 2001; Badran and Hermo, 2002). In the rat, AQP-1 and -9 have been localized to epithelial cells of the efferent ducts, and AQP-9 is expressed in principal and clear cells of the epididymis in a region specific manner (Fischer et al., 1998; Elkjaer et al., 2000; Pastor-Soler et al., 2001; Badran and Hermo, 2002). AQP-1 was also localized to the endothelial cells of vascular channels throughout the efferent ducts and epididymis (Badran and Hermo, 2002). In the testis, AQP-9 was localized to Leydig cells of the interstitial space, and AQP-8 to Sertoli cells of the seminiferous epithelium (Nihei, 2001; Badran and Hermo, 2002). Various studies have revealed that estrogen or testosterone does not regulate expression of AQP-1 over the microvilli of nonciliated cells, and that expression of AQP-9 in principal cells of the epididymis is dependent on different factors in different epididymal regions (Zhou et al., 2001; Badran and Hermo, 2002).

Since many members of the AQP family are widely expressed in a given tissue, e.g. the kidney, where they perform a variety of important functions, and considering the similar mesonephric embryological origin of regions of the male excurrent duct and the kidney, we undertook to examine the distribution of several other AQP family members in the different cell types of the efferent ducts and epididymis, as well as the testis. Multiple expressions of AQPs in a given tissue may suggest diverse functions, as well as the overall importance of AQPs in that tissue. In the testis, AQPs may play an indirect role in maintaining spermatogenesis, while in the epididymis it may provide the proper luminal environment for the transport and maturation of sperm.

In the present study, we examine the immunocytochemical localization of AQP-0, -3 and -10 in the different cell types of the testis, efferent ducts and epididymis of normal adult rats. In addition, the regulation of AQP-3 expressed in the epididymis was examined in efferent duct ligated and orchidectomized rats with or without testosterone supplementation. The results demonstrate that a cell and tissue specific expression was

noted for AQP-0, -3 and -10 in the control adult testis, efferent ducts and epididymis, as well as differential regulating factors for the expression of AQP-3 in the epididymis.

MATERIALS AND METHODS

Animals and experimental protocols:

Adult male Sprague Dawley rats (350-450g, 3-4 months of age) were obtained from Charles River Laboratory Ltd (St. Constant, Quebec) and were subdivided into five groups. The first group consisted of four normal adult control animals. Bilateral ligation of the efferent ducts (EDL) constituted the second group. After an intraperitoneal injection of sodium pentobarbital (Somnitol, MTC Pharmaceuticals, Hamilton, Ontario), the testes and epididymides of adult rats were exposed through an incision of the anterior abdominal wall. Using a dissecting microscope, a ligature was placed around both right and left efferent ducts at a site close to and further removed from the rete testis, with care being taken to avoid interference of the blood vessels entering the testis. The interval between the two ligatures was then excised to ensure that no sperm or fluids would enter the epididymis from the testis. The animals (four per interval) were sacrificed at 3, 7, 14 and 21 days following surgery. Bilateral orchidectomy constituted the third group. After anaesthesia, both testes of each rat were removed after a ligature was placed around the efferent ducts and testicular blood vessels. The animals (four per interval) were sacrificed at 3, 7, 14 and 21 days after surgery. Bilaterally orchidectomized rats that received three 6.2cm testosterone-filled implants constituted the fourth group. Testosterone-filled polydimethyl-siloxane (silastic) implants were prepared according to the method of Stratton et al. (1973) and have well-characterized steroid release rates (Brawer et al. 1983). The latter mimic epididymal (18.6 cm) testosterone levels, which are 10 times greater than blood levels (Turner, 2002). To ensure that the newly made capsules would have a constant testosterone release rate and that the initial surge of testosterone release would be complete at the time of implantation in orchidectomized rats, additional carrier rats were implanted with the testosterone implants prior to the start of the experiment. These implants were removed from the carrier rats 3 days later, cleaned, and inserted subcutaneously on the backs of experimental animals at the time of orchidectomy. Subsequent to anaesthesia, both testes were removed from each rat and the implants placed subcutaneously immediately after orchidectomy. The rats (four per interval) were sacrificed 14 and 21 days after surgery. The fifth group consisted of four sham-operated

animals, two of which received three empty 6.2 cm-long implants, with all rats being sacrificed 14 and 21 days after initiation of the experiment. All experimentation was carried out with minimal stress and discomfort being placed on the animals both during and after surgery as set up by the guidelines and approval of the McGill University Animal Care Committee.

Tissue preparation for light microscope immunocytochemistry:

In the case of control animals, the testes, efferent ducts and epididymides of each rat were fixed by perfusion with Bouin's fixative via the abdominal aorta for 10 minutes. For experimental animals, only the epididymides were perfused-fixed. Following perfusion, the various tissues were removed and the epididymides cut so that given sections would include all the major regions of the epididymis, i.e. the initial segment, intermediate zone, caput, corpus and cauda (Hermo et al., 1991). The tissues were then immersed in Bouin's fixative for 72 hours, after which they were dehydrated and embedded in paraffin.

Immunoperoxidase staining of sections was carried out according to the procedure of Oko and Clermont (1989). Polyclonal, affinity-purified anti-AQP-0, -3 and -10 antibodies were tried at different dilutions (1:50-1:200) in Tris-buffered saline (TBS), pH 7.4; with the 1:100 dilution showing the optimal reaction for the type of fixation and immunostaining method used. The anti-AQP-0 and -10 antibodies were obtained from Alpha Diagnostics International (San Antonio, Tx). The anti-AQP-3 antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, Ca). The antibodies, well characterized and specific for their respective peptide, were purified from ascites fluid by Protein G chromatography and raised against a 17 amino acid synthetic peptide for AQP-0 (Shiels et al., 1996), an 18 amino acid synthetic peptide for AQP-3 (Ishibashi et al., 1994) and a 17 amino acid synthetic peptide for AQP-10 (Hatakeyama et al., 2001), all within the carboxy termini of the proteins. They were supplied as 1 μg/mL solutions in phosphate-buffered saline (PBS), pH 7.4, with 0.2% bovine serum albumin as a stabilizer. The antibody solutions also contained 15 mM sodium azide as a preservative.

Paraffin sections, 5μm thick, were deparaffinized in Histoclear (Diamed Lab Supplies Inc, Mississauga, ON, Canada) and hydrated in a series of graded ethanol solutions. During hydration, residual picric acid was neutralized in 70% ethanol containing 1% lithium carbonate, and endogenous peroxidase activity was abolished in 70% ethanol containing 1% (vol/vol) H_2O_2. Once hydrated, the tissue sections were washed in distilled water containing glycine to block free aldehyde groups.

After rinsing with tap water and PBS, sections were incubated in normal blocking serum (Vectastain Elite ABC kit, Vector K-6101; Vector Laboratories, Burlingame, CA) for 30 min and then with the polyclonal anti-AQP antibodies, diluted to 1:100 with PBS. Sections were then washed three times with PBS and incubated with biotinylated secondary antibody (ABC kit) for 30 min. After washing three times with PBS, sections were incubated with the ABC reagent for 30 min and finally washed again three times with PBS. Visualization of the stain was achieved by incubating sections with 0.05% diaminobenzidine tetrahydrochloride (Bio Fx Laboratories, Owings-Mills, MD) until the desired staining intensity was achieved. Slides were rinsed with tap water for 5 min and then counterstained with 0.1% methylene blue. Passing them through a graded ethanol series dehydrated the tissues. Thereafter, the tissue sections were mounted on glass slides with Permount for observation. Negative controls were obtained by omission of the primary antibody or use of normal rabbit serum.

RESULTS

EXPRESSION OF AQP-0, -3 AND -10 IN CONTROL ANIMALS

In the case of AQP-0, no noticeable reaction was observed in the efferent ducts or epididymis, unlike the intense staining noted in the testis. In the interstitial space, Leydig cells were intensely reactive, and this was observed throughout the entire interstitial space (Figs. 1a-c). In the seminiferous epithelium, a cell-type specific reaction was noted, that was restricted to given areas of the epithelium as well as to specific stages of the spermatogenic cycle. The only cell type to express AQP-0 was the Sertoli cell. These cells were identified as such, since the reaction product radiated in a stellate manner from the base of the epithelium towards the lumen (Figs. 1a-d), comparable to that seen for specific Sertoli cell markers (Hermo et al., 1991). However, in any given cross section of a seminiferous tubule, only one half or less of the circumference of the tubule revealed intensely reactive Sertoli cells, leaving the other Sertoli cells encompassing that tubular cross section either weakly reactive or completely unreactive (Figs. 1a-c).

In addition, this pattern of staining was restricted to specific stages of the cycle. At the early stages (stages I-V), only about one quarter of the tubular circumference revealed intensely reactive Sertoli cells (Fig. 1a). This was accentuated at stages VI-VIII, where intensely reactive Sertoli cells enveloped approximately one half of the tubular circumference (Fig. 1b). At later stages (IX-XIV), a similar staining pattern was observed, but Sertoli cells at these stages were either weakly reactive or completely unreactive (Fig. 1c). Thus AQP-0 expression in the seminiferous epithelium was restricted to Sertoli cells and was visualized in the different cross sections of seminiferous tubules, as a semicircular or pie shaped pattern of staining that was also dependent on the different stages of the cycle.

In the efferent ducts, intense staining for the anti-AQP-10 antibody was noted over the microvilli of the nonciliated cells as well as the cilia of ciliated cells (Fig. 1e). While there was no reaction over the epithelial cells of the entire epididymis, the endothelium of vascular channels of the intertubular spaces of the efferent ducts and epididymis were intensely reactive.

In the testis and efferent ducts, there was no expression of AQP-3, other than background levels of staining. However, in the epididymis, AQP-3 was intensely expressed in the epithelium in a cell specific manner, but not restricted to any given epididymal region (Figs. 2a, b). The cell type that revealed an intense immunoperoxidase reaction product was the basal cell (Figs. 2a, b). The latter reside at the base of the epithelium, and the reaction clearly defined the boundaries of these cells leaving their nuclei completely unstained. The thin lateral processes of basal cells were also stained and at times appeared as thin bands or islands of cytoplasm detached from their main cell body (Figs. 2a, b). On occasion, processes of these cells extended towards, but did not contact the lumen (Fig. 2b). Throughout the entire epididymal duct, there was no reaction over the epithelial principal, clear or narrow cells. Likewise sperm in the lumen were completely unreactive (Fig. 2a).

When the various tissues were immunostained by omitting the primary antibody or when treated with normal rabbit serum, there was no reaction over any areas of the tissue, including the epithelium, interstitial space of the testis, efferent ducts and epididymis, as well as sperm in the lumen (Fig. 2a inset).

REGULATION OF AQP-3 IN THE EPIDIDYMIS

At the different time intervals after orchidectomy, the epididymal epithelium was completely devoid of reaction product. In contrast to the intense staining of basal cells seen in control animals, no reaction was noted over these cells (Fig. 2c). In 14 day orchidectomized animals supplemented immediately with high levels of testosterone, a reaction was restored to basal cells, however, not to the degree of that seen in control animals (Fig. 2d). Efferent duct ligation, at the different time intervals, also resulted in a loss of reactivity over basal cells (Fig. 2e), but it was not as dramatic as that seen in orchidectomized animals; however, basal cells were not as reactive as that seen in testosterone supplemented animals. Thus, efferent duct ligation revealed levels of reactivity over basal cells that were intermediate to the complete absence of staining noted in orchidectomized animals and the moderate reaction noted in testosterone supplemented-orchidectomized animals.

DISCUSSION

In the testis, AQP-0 was localized to Sertoli cells of the seminiferous epithelium and Leydig cells of the interstitial space, but there was no specific reaction for AQP-3 or -10 in any cell type (Table 1). The finding of AQP-0 in Sertoli cells is in one sense interesting, since these cells also express other AQPs. Indeed, in the rat testis, AQP-8 is expressed exclusively in Sertoli cells and is not present in germ or Leydig cells (Badran and Hermo, 2002). In the case of AQP-8, the reaction was not specific to certain stages of the spermatogenic cycle, and all Sertoli cells enveloping a given tubule were reactive. This is in contrast to the distribution noted for AQP-0. Firstly, the reaction over Sertoli cells was most intense at stages VI-VIII, with stages I-V showing moderate reactivity and stages IX-XIV showing the weakest reaction. Thus like many other proteins that are expressed by Sertoli cells, a cyclic variation is observed according to the different stages of the cycle (Parvinen et al., 1993; Griswold, 1993). This variation often reflects important functions for these proteins in accordance with events occurring during spermatogenesis. Secondly, the staining pattern for Sertoli cells, with the anti-AQP-0 antibody, at the various stages of the cycle was semicircular in appearance, with only half the Sertoli cells encompassing the circumference of a given tubule being reactive. This was especially evident at stages VI-VIII, when Sertoli cells were maximally reactive. Such a staining pattern has not been described for Sertoli cells with other well-known markers (Hermo et al., 1991, 1992; Hermo et al., 1994). The absence of staining over half of the Sertoli cells of seminiferous tubules at these stages suggests either that these cells do not express AQP-0 or that the unreactive cells may be out of synchrony in their expression with the other Sertoli cells at a given moment in time. While not previously described for Sertoli cells, such a pattern of staining is well recognized for the principal cells of the epididymis, which with some antibodies and in given tubular cross sections show both intensely, moderately and weakly reactive cells, a pattern described as checkerboard in appearance (Hermo et al., 1991, 1998; Rankin et al., 1992).

In the testis, Sertoli cells carry out a variety of functions, many of which are related to events taking place during spermatogenesis (Russell, 1993). One of their functions is to continuously produce fluid that bathes the developing germ cells and

which serves as the vehicle for sperm to enter the epididymis (Setchell et al., 1969; Voglmayr et al., 1970; Hinton and Setchell, 1993). In this regard, expression of AQP-8 already described in Sertoli cells (Badran and Hermo, 2002) would be involved in the transport of water from the interstitial space into the lumen and this would occur at all stages of the cycle. However, the finding of AQP-0 in these cells and maximally expressed at stages VI-VIII could also assist in this function. Stages VI-VIII correlate with the period of time immediately prior to and precisely when the elongating spermatids are being released into the lumen. Thus the presence of two AQPs at this time point during the cycle may greatly facilitate the transport of water into the lumen and hence the movement of sperm out of the seminiferous tubules. A rationale for the semicircular pattern of AQP-0 expression in Sertoli cells is not at present clear. However, it may be suggested that at the time of spermiation, not all the elongating spermatids of a given tubule are released at the same time, thus accounting for the asynchronous staining pattern of AQP-0 in Sertoli cells. Further experimentation involving AQP-0 and its role in Sertoli cells is required.

In the testis, AQP-0 was also noted over Leydig cells. The latter are the steroid secreting cells occupying about 75% of the cellular interstitial space compartment (Wing and Christensen, 1982). Leydig cells have also been shown to express AQP-9 (Tsukaguchi et al., 1999; Elkjaer et al., 2000; Nihei et al., 2001; Badran and Hermo, 2002). As for other tissues, including Sertoli cells, Leydig cells express more than one AQP. While both AQP-9 and AQP-0 may maintain water equilibrium in these cells, AQP-9 is also involved in the passage of solutes such as polyols, purines and pyrimidines (Tsukaguchi et al., 1999). Alternatively, these two AQPs may also transport steroids, a major function of Leydig cells, out of these cells, although such a function for AQPs has yet to be documented.

In the testis, AQP-8 along with AQP-0 now appears to reside in Sertoli cells and AQP-9 and -0 in Leydig cells. The presence of more than one AQP in the same cell type suggests the importance of water transport in that cell, or alternatively that AQPs serve more than one function. The expression of AQP-0, also known as major intrinsic protein-26 (MIP26), has been well documented in the lens fiber of the eye (Shiels et al., 1996). The lens specific AQP-0 represents up to 80% of total lens membrane protein. Defects in

MIP26 are cause of autosomal dominant cataract. AQP-0 is a 263 amino acid transmembrane protein than contains six transmembrane domains, where both the N and C-termini are predicted to be cytoplasmic. However, AQP-0 belongs to the family of AQPs that are highly selective for water and not to the aqua-glyceroporin family of AQPs, i.e. AQP-3, -7, and -9 that, in addition to water, also transport glycerol and urea (Verkman and Mitra, 2002). It remains to be determined why different AQPs reside in Sertoli and Leydig cells.

The efferent ducts have a major role in reabsorbing water that enters the lumen of these ducts from the seminiferous tubules of the testis. In fact the nonciliated epithelial cells resorb about 50-90% of the fluid coming from the testis (Crabo, 1965; Setchell and Brooks, 1988; Clulow et al., 1998). In terms of the distribution of members of the AQP family thusfar, both AQP-1 and -9 are expressed on the nonciliated cells of the efferent ducts (Brown et al., 1993; Fischer et al., 1998; Pastor-Soler et al., 2001). However, while anti-AQP-1 antibody decorated the microvilli and basolateral plasma membranes of the epithelial nonciliated cells, as well as the cilia of ciliated cells, anti-AQP-9 antibody was restricted to the microvilli of nonciliated cells and was not present on ciliated cells (Badran and Hermo, 2002). AQP-1 also stained endosomes whereby water could be removed from these structures as they evolved into smaller, denser and compact lysosomes (Badran and Hermo, 2002). In the present study, AQP-10 was noted solely on the microvilli of the nonciliated cells, not on their basolateral plasma membranes or endosomes, and it was not detected in ciliated cells. AQP-10, first identified in human absorptive epithelial cells of the small intestine, encodes a 264-amino acid protein with high sequence identity with AQP-3, -7 and -9 (Hatakeyama et al., 2001). However, unlike the aqua-glyceroporin family of AQPs, AQP-10 is not permeable to urea and glycerol (Hatakeyama et al., 2001).

In the efferent ducts, water resorption in nonciliated cells requires an apically located Na+/H+ exchanger, isoform NHE3, and a Na+/K+ ATPase localized to their basolateral plasma membranes (Ilio and Hess, 1992; 1994; Hansen et al., 1999; Leung et al., 2001). The standing osmotic gradient created by these pumps and the expression of AQP-1, -9 and -10 would then allow the passage of water from the lumen into the intertubular space. The requirement of AQP-1, -9 and -10, all decorating the microvilli of

nonciliated cells of the efferent ducts, suggests the need for rapid movement of water across these cells. However, the presence of more than one AQP in a given cell type also suggests that these AQPs perform other as yet unknown functions. It has yet to be demonstrated if the pore size of the different AQPs are similar or not and this may also influence the passage of large movements of water across the cell as well as other molecules. Indeed, the finding of more than one AQP in a given tissue is not a rare phenomenon. Indeed, in the kidney and intestine, as examples, several members of the AQP family of proteins have been localized often showing cell type and region specificity with more than one AQP being present on the same cell type (King and Agre, 1996; Wintour, 1997; Nielsen et al., 2002).

As for AQP-1 (Badran and Hermo, 2002), the endothelial cells of vascular channels of the efferent ducts and epididymis were also stained with the anti-AQP-10 antibody. Thus once water is transported across the epithelium, it would move into the vascular channels by means of both AQP-1 and -10 to maintain water equilibrium in these tissues. In the efferent ducts, the removal of water concentrates sperm in the lumen of the initial segment, which is of small size, as compared to the lumen of the remaining epididymal regions. In this way, the secretory products of the principal cells would have more access to the sperm surface enabling them to acquire their maturational characteristics, as the initial segment is where sperm begin to become fertile (Cooper, 1995). The infertility of male mice in the αERKO mouse model system, which results in the retention of water and a diluted sperm concentration in the initial segment is indicative of the importance of water removal in the efferent ducts (Lubahn et al., 1993; Eddy et al., 1996; Hess et al., 1997).

While AQP-10 was present in the efferent ducts it was not expressed in the testis or epididymal epithelium. In the epididymal epithelium, AQP-9 has been localized to the microvilli of principal cells, with the most intense reaction being noted in the initial segment and cauda regions (Elkjaer et al., 2000; Pastor-Soler et al., 2001;Badran and Hermo, 2002). In addition, AQP-9 was expressed in clear cells, but only of the cauda region (Badran and Hermo, 2002). Thus the expression of AQP-9 appears to be region specific in the case of principal and clear cells. There was no expression of AQP-1 or -8 in the epididymal epithelium (Badran and Hermo, 2002). In contrast, in the present study,

AQP-3 was detected only in the epididymis and not in the testis or efferent ducts. The cell type that was reactive was the basal cell, and this was noted in all epididymal regions.

In the epididymis, water continues to be reabsorbed from the lumen (Levine and Marsh, 1971; Setchell and Brooks, 1988; Wong et al., 2002). The finding of AQP-3 in basal cells suggests that there is an intricate cooperative removal of water from the epididymal lumen that would appear to involve the different cell types. For example, in the initial segment, AQP-9 is found on principal cells, AQP-1 on myoid cells enveloping the tubules and endothelial cells of vascular channels (Badran and Hermo, 2002). With the present finding of AQP-3 on basal cells, this would suggest that water is rapidly transported from the epididymal lumen across the entire width of the epithelium, including myoid cell layers to eventually reach the lumen of vascular channels. AQP-3 expression in basal cells does not appear to be unique to the epididymis. Indeed basal cells of the epithelium of the trachea also express AQP-3 (Nielsen, 1997). In addition, AQP-3 has also been demonstrated in various cell types and regions of the kidney and gastrointestinal tract (Shrier and Cadnapaphornchai, 2003). AQP-3 is a 31.4 kDa protein, with 285 amino acids, that plays a major role in water and urea exit mechanisms in the collecting duct cells of the kidney. It is also highly permeable to glycerol, hence a member of the aqua-glyceroporin family of proteins (Ishibashi et al., 1994). Thus while AQP-3 in basal cells of the epididymis may move water across the epithelium, it may also transport glycerol. Indeed, glycerol is a component of the epididymal fluid at concentrations of about 1.15 mM, with epididymal sperm utilizing glycerol to produce CO2 (Cooper and Brooks, 1981). Intraluminal glycerol is a metabolic substrate for epididymal sperm. Glycerol is derived from glycerylphosphorylcholine (GPC) through the activity of GPC cholinephosphohydrolase. GPC is found at high concentrations in epididymal fluids and the epididymis (Dawson and Rowlands, 1958), where it is synthesized presumably within principal and basal cells and transported to the lumen where it would be utilized by sperm. Thus the presence of AQP-3 in basal cells along with the expression of AQP-9 in principal cells may lead to the efficient transport of glycerol, as well as GPC, to the epididymal lumen where they could play a functional role in sperm maturation.

Basal cells reside in a variety of different epithelial tissues but these cells have not been studied in much detail. Recent findings on these cells in the epididymis suggest that they have a unique structural appearance and perform a variety of functions. In the adult epididymis, these cells do not divide and thus are not stem cells. In this tissue, basal cells are small hemispherical cells residing on the basement membrane and not in apparent contact with the lumen of the duct, even though, on occasion, they send thin processes apically (Robaire and Hermo, 1988). However, basal cells possess attenuated thin foot like processes that extend along the basement membrane in such a way as to collectively encompass a large portion of the circumference of each tubule (Veri et al., 1993). Thus they form a barrier, albeit an incomplete one, between the epididymal lumen on the one hand and blood vessels and other contents of the intertubular space on the other. They can, therefore, to a degree effectively eliminate potentially harmful substances emanating from the blood, trying to access the sperm in the lumen. In this context, basal cells express various antioxidants (Nonogaki et al., 1992; Papp et al., 1995) and metallothioneins (Cyr et al., 2001). Basal cells also express connexin-43 with neighbouring principal cells, a gap junctional protein by which these two cell types can communicate information with one another (Cyr et al., 1996), in addition to the adhesion molecules, catenins (Cyr et al., 2003). The finding of AQP-3 expression adds another twist to the ever-growing functions that basal cells can perform that now includes water transport across the epididymal epithelium.

Regulation of AQP-3 in the epididymis

In the present study, an examination of the regulation of AQP-3 expression in the epididymis revealed that basal cells became unreactive in the absence of testicular factors (Table 2). The intensity of the reaction with the anti-AQP-3 antibody was also considerably reduced after efferent duct ligation suggesting that circulating levels of testosterone alone could not maintain AQP-3 expression at control levels and that a luminal factor emanating from the testis was also needed. This was confirmed in orchidectomized animals that were supplemented with high levels of testosterone. In such cases, the reaction was enhanced above that seen for efferent duct ligated animals, but not

to the degree seen in control animals. It was thus suggested that AQP-3 expression in basal cells was dependent in part on testosterone and in part on a luminal testicular factor. In comparison, regulation studies of AQP-9 in the adult rat epididymis revealed cell type and region specificity. In the initial segment, where it was intensely expressed on the microvilli of principal cells in controls, a dependence of both testosterone and a lumicrine factor was noted for maximal expression (Badran and Hermo, 2002). A similar situation was also reported for clear cells of the cauda epididymidis (Badran and Hermo, 2002). It remains to be determined whether or not the lumicrine factor regulating AQP-9 and AQP-3 expression in principal and clear cells and basal cells, respectively, is the same or different for each cell type. Nevertheless, AQP-9 staining in principal cells of the caput, corpus and cauda regions was not modified from controls after efferent duct ligation or orchidectomy, suggesting no dependence on testicular factors for its expression in these regions (Badran and Hermo, 2002). Thus while there are some similarities in the pattern of regulation for AQP-9 and AQP-3 in the various epithelial cell types, differences are also apparent.

In the epididymis, various factors come into play in regulating epididymal functions at both the gene and protein levels (Robaire and Hermo, 1988; Orgebin-Crist, 1996; Ezer and Robaire, 2002; Cornwall et al., 2002). This includes the dependence of androgens on some epididymal functions, but not on others (Ezer and Robaire, 2002; Cornwall et al., 2002). However, in addition to the regulation mediated by androgens, factors emanating from the testis that enter the epididymis via the lumen of the duct, defined as lumicrine factors, also play a role in regulating epididymal functions (Hinton et al., 1998, Cornwall et al., 2002). Indeed, ligation of the efferent ducts induces changes in epididymal structure and gene and protein expression (Cornwall et al., 2002). Lumicrine factors derived from the testis regulate several proteins synthesized by the epididymis (Garrett et al., 1991; Cornwall et al., 2002; Lareyre et al., 2001; Hermo and Andonian, 2003).

Information regarding the regulation of the function of basal cells is scanty. In their expression of Yb_1-GST, basal cells show a differential region specific response. In the corpus region, Yb_1-GST expression in basal cells is regulated by testosterone, but in the proximal initial segment, expression is regulated by a lumicrine factor. These data

differ dramatically from that obtained for the Yf-GST subunit, where its expression in basal cells was unaltered after orchidectomy, efferent duct ligation or hypophysectomy, indicating that neither testicular nor pituitary factors governed Yf-GST expression in basal cells (Hermo and Papp, 1996). Thus basal cells of different regions show differential responses to the absence of androgens or testicular lumicrine factors in their expression of a given GST as well as between different GSTs. The expression of metallothionein by basal cells, although detected in all epididymal regions, was shown to be androgen dependent according to specific regions (Cyr et al., 2001). In the present study, basal cell expression of AQP-3 appears to be dependent on both testosterone and a lumicrine factor. Although this is the first demonstration of the dependence of regulating factors of different origins on basal cells, at the mRNA level, gamma glutamyl-transpeptidases, which show multiple transcripts, have been shown to be differentially regulated by androgens and/or lumicrine factors in the different epididymal regions (Palladino and Hinton, 1994). Thus the dependence of several regulating factors is not uncommon for epididymal cell functions.

In summary, the present study indicates that expression of AQP-0, -3 and -10 is cell, tissue and region specific in the testis, efferent ducts and epididymis of adult rats. In addition, both testosterone and lumicrine factors appear to regulate the expression of AQP-3 in basal cells of the epididymis.

Table 1. Expression of aquaporins (AQPs) -0, -3 and -10 in the testis, efferent ducts and epididymis of normal adult animals

Aquaporins	Testis[1]		Efferent ducts[2]		Initial segment[3]			Caput epididymidis[4]				Corpus epididymidis				Cauda epididymidis			
	S	L	NC	C	P	B	Vc	P	B	Cl	Vc	P	B	Cl	Vc	P	B	Cl	Vc
AQP-0	+*	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AQP-3	-	-	-	-	-	+	-	-	+	-	-	-	+	-	-	-	+	-	-
AQP-10	-	-	+	+	-	-	+	-	-	-	+	-	-	-	+	-	-	-	+

[1]S indicates Sertoli cell; L, Leydig cell
[2]NC indicates nonciliated cell; C, ciliated cell
[3]P indicates principal cell; B, basal cell; Vc, endothelial cells vascular channels
[4]Cl indicates clear cell

(+) indicates presence of reaction; (-), absence of reaction

* Sertoli cells were reactive in a semicircular pattern of expression and were restricted to specific stages of the spermatogenic cycle

Table 2. Regulation of aquaporin-3 (AQP-3) in basal cells of the epididymis *

	Normal	Orchidectomy (O)	Orchidectomy + testosterone (O+T)	Efferent duct ligation (EDL)
All regions	+++	-	++	+

The number of plus signs is directly proportional to the extent of reactivity, with (+) being weak, (++) being moderate, (+++) being strong and (-) indicating absence of reaction

* All experimental conditions (O, O+T, EDL), were analyzed in 3, 7, 10 and 14 day animals.

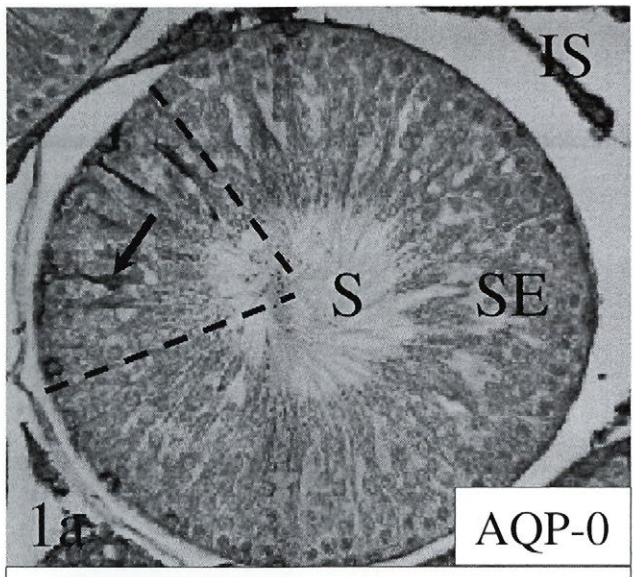
Figs. 1a-c: Cross sections of seminiferous tubules of the adult rat testis at stages IV-V (a), VII (b) and XII (c) of the cycle of the seminiferous epithelium immunostained with an anti-AQP-0 antibody. In the various tubular cross sections, the immunoperoxidase reaction product (arrows) radiates in the form of well-separated threads or bands from the base of the seminiferous epithelium (SE) to the lumen, reminiscent of a reaction over Sertoli cells. Similar to other well-described Sertoli cell markers, the reaction appears to be stage specific, with the most intense reaction being noted over early (a) and mid (b) stages of the cycle and the weakest reaction over late (c) stages of the cycle. However, unlike the staining pattern for Sertoli cells noted with other antibodies, only a quarter (a) or half (b) of each tubular cross section at stages I-VIII reveals reactive Sertoli cells with the remaining Sertoli cells appearing weakly reactive or unreactive. At stages IX-XIV, Sertoli cells also show a segmented reaction but the reaction is weak over these cells. An intense reaction also envelops each seminiferous tubule, suggesting a reaction over the cells comprising the limiting membrane. Cells of the interstitial space (IS) also demonstrate an intense reaction. Note the absence of a reaction over the tails of spermatids (S) in the lumen. (a-c) 262X

Fig. 1d: High power light micrograph of a portion of a seminiferous tubule of an adult rat testis at stage II-III of the cycle immunostained with an anti-AQP-0 antibody. Sertoli cells extending from the base of the seminiferous epithelium (SE) to the lumen show an intense reaction (arrows). Note absence of reactivity over germ cells (GC). 1048X

Figs. 1e and inset: Efferent ducts at low and high (inset) magnification of an adult rat immunostained with an anti-AQP-10 antibody. The microvilli of the nonciliated cells (nc, arrowheads) and cilia of ciliated cells (c), open arrows) present an intense immunoperoxidase reaction product. The luminal contents (L) are devoid of a reaction. IT, intertubular space. (e) 420X; (inset) 840X

Figs. 2a, b: Initial segment (a) and corpus (b) epididymidis of a control adult rat immunostained with an anti-AQP-3 antibody. In each epididymal region, the immunoperoxidase reaction hovers over the unreactive nuclei (**n**) of basal cells (curved arrows). Thin ribbons of reaction product also stretch along the base of the epithelium (notched open arrows), but there is an absence of reaction over the principal cells (P). Some basal cells show processes extending towards the lumen (notched open arrows). Primary antibody incubation was omitted and used as a negative control for the reaction (inset). (a, inset) 262X; (b) 420X

Figs. 2c-e: Corpus epididymidis of 14 day orchidectomized(c), orchidectomized supplemented with testosterone (d) and efferent duct ligated (e) adult rats. In (c), a complete absence of reaction is evident over the entire epithelium, including principal (P) and basal cells (curved arrows). In (d), reactivity is restored to basal cells (curved arrows), but not to the degree of intensity of that seen in control animals. In (e), a reaction appears over basal cells, but it is in no way comparable to that seen in control animals or in orchidectomized animals supplemented with testosterone. L, lumen; IT, intertubular space. (c) 262X; (d,e) 420X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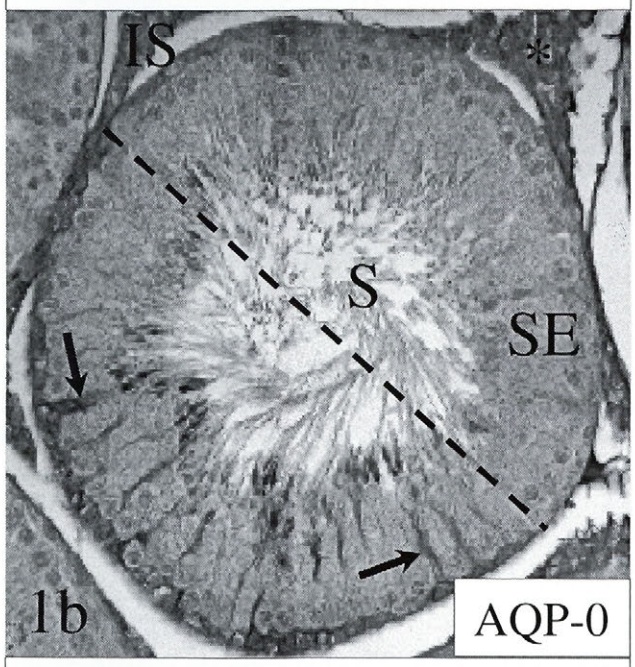
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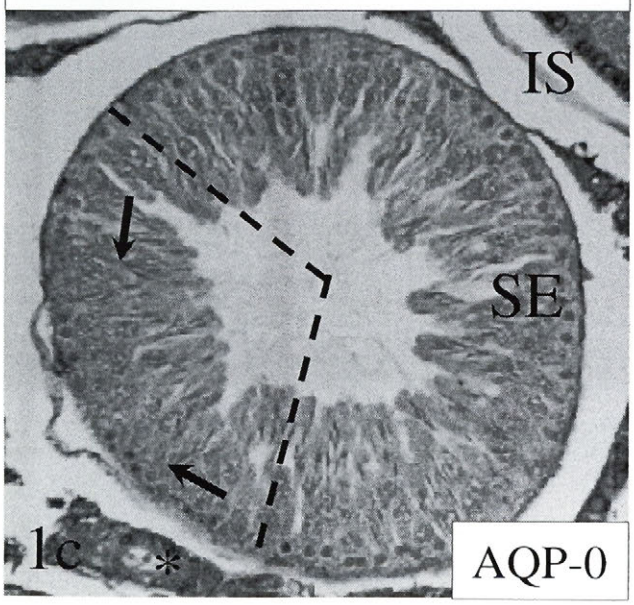
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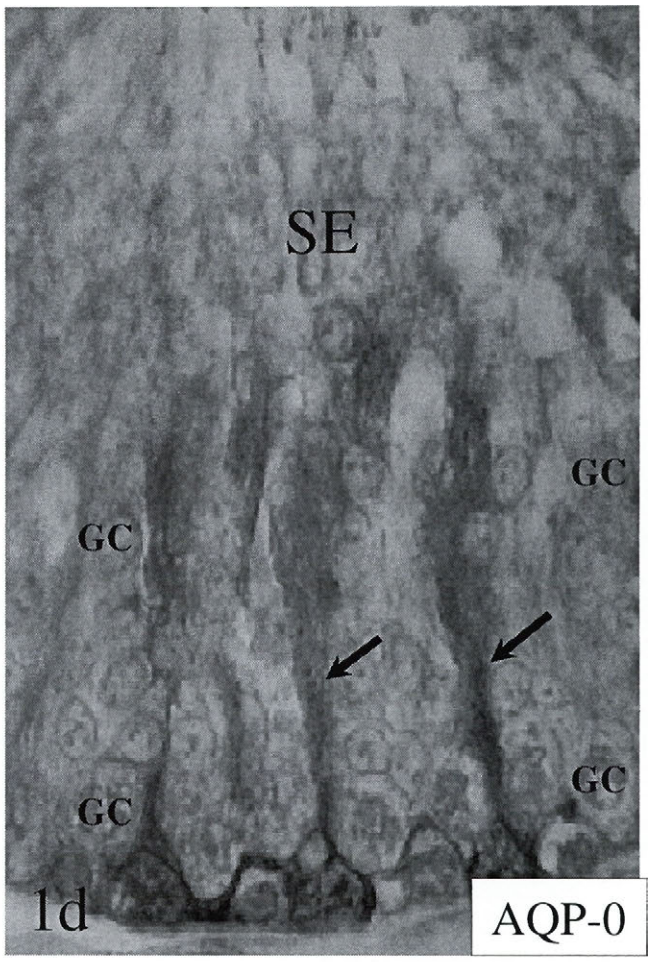
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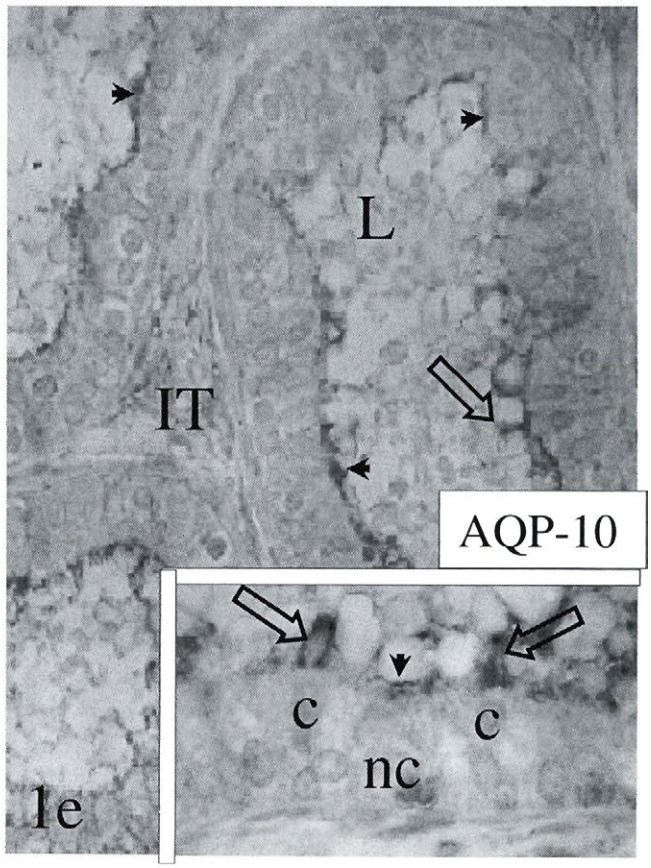
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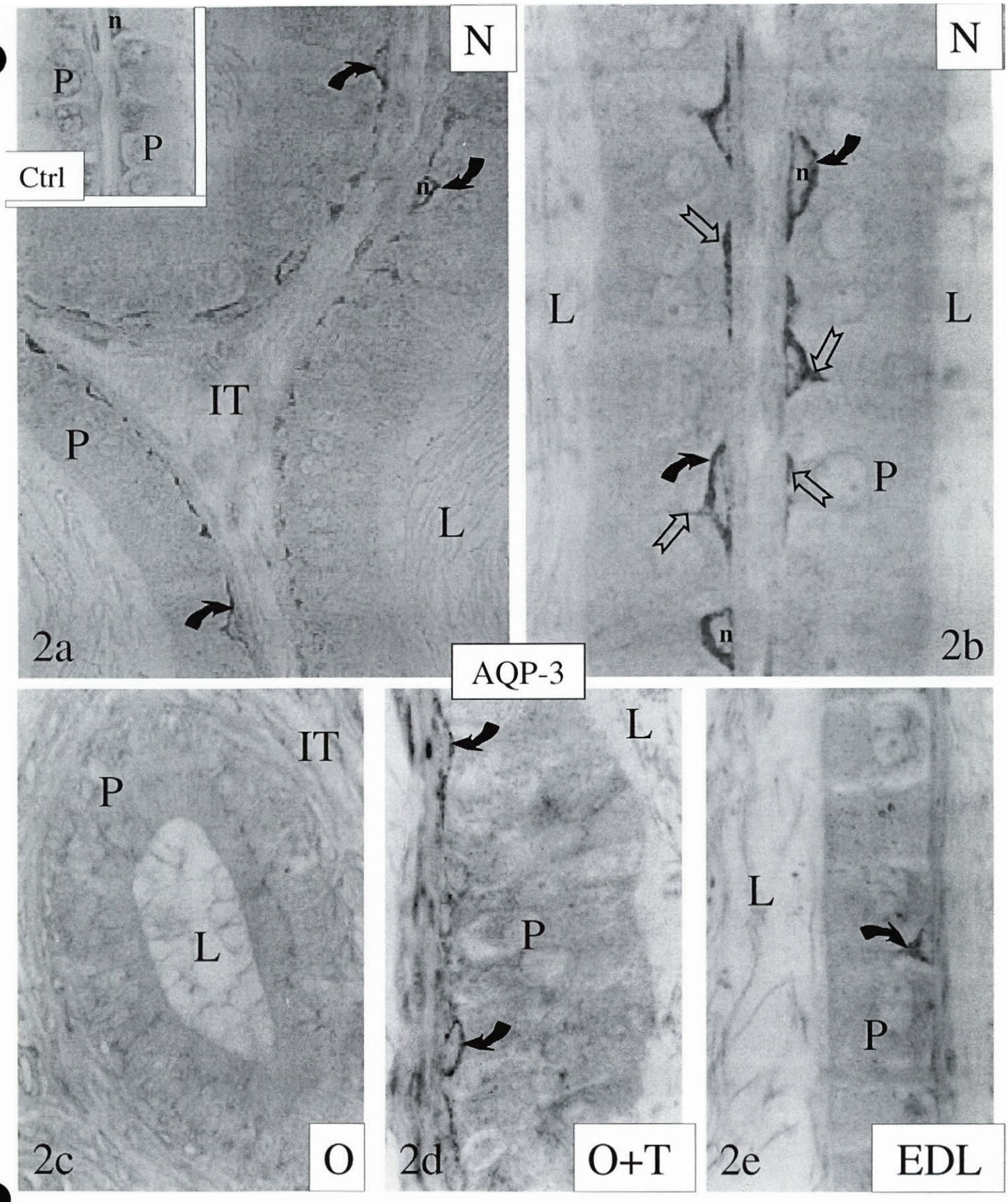
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CHAPTER 3

Effects of Lab Chow and Phytoestrogen-Free Casein Diets on Sperm Counts, Sperm Motility, and Expression of Aquaporins-1, -9 and -10 in the Efferent Ducts of αERKO Adult Mice

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ABSTRACT

Estrogens play an important role in the male reproductive tract, and this is especially so for the efferent ductules, where α-estrogen receptors (ERα) have been localized. Mice deficient in ERα (αERKO mice) are infertile, and the effect appears to be due in part to retention of water at the level of the efferent ductules. In the present study, we examined the consequences of ERα deletion on the distribution of certain aquaporins (AQPs), water protein channels, in the efferent ductules and on sperm numbers and motility. In addition, the effects of feeding mice a regular lab chow diet, which contains phytoestrogens, known to affect male reproductive tract functions, and a casein diet, which lacks phytoestrogens, were also assessed. Light microscope immuno-localizations of AQP-1 and AQP-9 revealed dramatic reduction and patchier staining in αERKO mice with distal areas of the efferent ductules being more affected than proximal areas. Immunostaining for AQP-10, present on cilia of ciliated cells in wild type mice, was reduced in αERKO mice, concomitant with reactivity over the microvilli of some nonciliated cells, which were never reactive normally. The latter finding suggests regulation at the transcriptional or translational level in wild type mice and suggests in αERKO mice, a compensatory role for AQP-10 due to the dramatic reduction in AQP-1 and -9 expression in the nonciliated cells. No other changes in immunolocalizations were noted as a consequence of diet. Computer-assisted sperm analyses demonstrated a 62% reduction in cauda epididymal sperm/ml in αERKO mice fed lab chow, whereas 87% fewer sperm/ml were observed in αERKO mice fed casein, suggesting an enhanced role for sperm production and concentration in a diet containing phytoestrogens. All sperm motility parameters were altered to some degree in αERKO mice fed lab chow. Alterations in sperm motility parameters were also detected, but were less dramatic in αERKO mice fed casein. These data suggest that the decrease in AQP expression in the efferent ductules of αERKO mice contributes in part to water retention in this tissue, eventually leading to backflow of water into the testis, with subsequent decreases in sperm concentration and motility. The data also suggest that phytoestrogens, which are present in regular lab chow, can influence the male reproductive tract with and without the presence of ERα, promoting efferent ductule and epididymal functions when ERα is

expressed, but inhibiting these same functions when ERα is missing. Taken together the data underscore the importance of estrogens and ERα in maintaining sperm maturation and preventing male infertility.

INTRODUCTION

Many mammalian tissues require rapid transport of water into and out of constituent cells. As a consequence, protein water channels, referred to as aquaporins (AQPs), have evolved to serve this purpose (Preston and Agre, 1991; Wintour, 1997; Verkman and Mitra, 2000; Schrier and Cadnapaphornchai, 2003). AQPs are homologous to the major intrinsic protein superfamily of integral membrane proteins and are assembled in plasma membranes as homotetramers. Each 30kD monomer consists of 6 membrane-spanning α-helical domains and has its own distinct pore to allow bi-directional water transport (King and Agre, 1996; Wintour, 1997; Brown et al., 1998; Verkman and Mitra, 2000). Currently, 11 AQPs (0-10) have been identified in various tissues and cells. They have been divided into 2 groups, based on their permeability properties: the water-selective AQPs and the aqua-glyceroporins, which, in addition to water, are also highly permeable to urea, glycerol and small uncharged molecules (Preston and Agre, 1991; Deen and van Os, 1998; Borgnia et al., 1999; Sansom and Law, 2001; Hatakeyama et al., 2001).

AQPs are expressed throughout the mammalian body and have been studied extensively (Verkman and Mitra, 2000; Nielson et al., 2002; Schrier and Cadnapaphornchai, 2003). Many are tissue-, region- and even cell-specific, but more than one AQP can be expressed in the same cell (King and Agre, 1996; Echevarria and Ilundain, 1998; Verkman and Mitra, 2000; Nielson et al., 2002). While hormones regulate some AQPs, others are constitutively expressed (Verkman and Mitra, 2000; Nielson et al., 2002; Schrier and Cadnapaphornchai, 2003). Alterations in the expression of AQPs have been shown to cause a variety of pathological states (King et al., 2000; Verkman and Mitra, 2000; Nielson et al., 2002; Schrier and Cadnapaphornchai, 2003).

Water plays an important role in the male reproductive tract. In seminiferous tubules of the testis, water is secreted into the lumen by Sertoli cells in order to create a fluid environment essential for maintaining spermatogenesis and transporting sperm out of the testis. In the efferent ductules, linking the rete testis to the epididymis, up to 90% of testicular luminal fluid is reabsorbed. This process appears to concentrate sperm as an initial step to promoting fertility and motility as sperm pass along the epididymis (Hess et

al., 2002). In the epididymis, water serves as a vehicle for sperm passing through this convoluted duct (Setchell et al., 1969; Setchell and Brooks, 1988).

The distributions of several members of the AQP family have been characterized in the male reproductive tract (Brown et al., 1998; Andonian and Hermo, 1999; Pastor-Soler et al., 2001; Nihei et al., 2001; Badran and Hermo, 2002; Hermo et al., 2004). In the testis, AQP-9 is localized to Leydig cells of the interstitial space and while AQP-7 is expressed in germ cells, AQP-8 is expressed in Sertoli cells of the seminiferous epithelium (Ishibashi et al., 1997; Nihei et al., 2001; Badran and Hermo, 2002). In the adult rat, AQP-1, -9 and -10 are localized to epithelial cells of efferent ductules, and while AQP-9 is expressed in principal cells of the epididymis in a region-specific manner, AQP-3 is expressed in basal cells (Fischer et al., 1998; Elkjaer et al., 2000; Pastor-Soler et al., 2001; Badran and Hermo, 2002; Hermo et al., 2004). AQP-1 is localized to endothelial cells of vascular channels throughout the efferent ductules and epididymis (Badran and Hermo, 2002).

Some studies on the hormonal regulation of AQPs in the male reproductive tract have been reported. In efferent ductules, expression of AQP-1 over the microvilli of nonciliated cells is not influenced by testosterone, whereas expression of AQP-9 in principal cells in some epididymal regions is dependent on both testosterone and other unidentified luminal testicular factors (Badran and Hermo, 2002) and in efferent ductule nonciliated cells are regulated by estrogen (Oliveira et al., 2005). Recent studies have suggested an important role for estrogen in regulating water transport in the efferent ductules (Hess et al., 2001b; Hess et al., 2002; Hess, 2003).

In males, estrogen is present in low concentrations in blood, but it can be extraordinarily high in semen and rete testis fluids (Ganjam and Amann, 1976; Free and Jaffe, 1979). Estrogen in the rete testis fluid is derived mainly from the conversion of testosterone to estradiol by P450 aromatase present in germ cells and cytoplasmic droplets of sperm traversing the lumen of the efferent ductules, as well as in Leydig and Sertoli cells of the testis (Payne et al., 1976; Carreau et al., 1999; Hess et al., 2001a). It is well known that male reproductive tissues express estrogen receptors (ERs) (Schleicher et al., 1984; West and Brenner, 1990; Cooke et al., 1991; Greco et al., 1993), and in particular, the efferent ductules, where ERα is abundant and where much of the fluid

coming from the testis is reabsorbed (Fisher et al., 1997; Hess, 2000; Zhou et al., 2002). Use of a mouse model that lacks ERα expression, known as the αERKO mouse, has revealed that water is retained at the level of the efferent ductules in these animals resulting in "back flooding" of seminiferous tubules. This dilutes sperm counts in the epididymis and contributes to infertility seen in these animals (Eddy et al., 1996; Hess, 2000; Hess et al., 2001b; Zhou et al., 2001, Hess et al., 2002; Cho et al., 2003; Oliveira et al., 2001; 2002). Although it has been shown that AQP-1 protein, but not mRNA, expression is partially regulated by ERα, AQP-1 null mice are fertile (Zhou et al., 2001) suggestive that more than one factor contributes to water retention observed in the αERKO mouse and dependent on morphology of the microvillar border (Oliveira et al., 2001; 2002). The specific effects of absence of ERα on AQP-1, as well as on AQP-9 and AQP-10, expression along the entire length of the efferent ductules that may contribute in part to the infertility of αERKO mice have not been fully documented. Furthermore, while sperm counts and motility of sperm in αERKO and antiestrogen treated mice are known to be reduced (Eddy et al., 1996; Cho et al., 2003), detailed studies on motility behavior of sperm in the αERKO mice compared to wild type mice are presently lacking.

In assessing the role(s) that estrogens play in water dynamics of the efferent ducts, the presence of estrogen-mimicking substances in the food is a factor that must also be taken into consideration. A typical rodent lab chow diet consists of mixtures of alfalfa and soybeans (Gaido et al., 1997). These plants are known to contain significant levels of phytoestrogens, which exhibit weak estrogenic activity *in vitro* and *in vivo* (Makela et al., 1995; Whitten and Naftolin, 1998). Soybean meal, for example, has moderate estrogenic content at 0.039 – 0.125 μg/g estradiol, due to genistein (Santell et al., 1997; Nishihara et al., 2000; Kato et al., 2004). Hence, the goals of this study were not only to characterize AQP expression and sperm concentrations and motility behavior in wild type and αERKO mice, but also to determine what happens in mice fed lab chow versus an alternative diet such as purified casein, which is phytoestrogen-free.

MATERIALS AND METHODS

Animals

A total of 20 wild type and 20 αERKO (Lubahn et al., 1993) mice (C57bl/6j) at 3-4 months of age were utilized. Both wild type and αERKO mice were randomly assigned to subgroups based on two diets (diet type). Regular lab chow diet (Nestlé Purina, St. Louis, MO) was administered after weaning to one group of ten wild type and ten αERKO mice, while a diet free of soybean and rich in casein, but otherwise containing all the ingredients of lab chow diet (AIN 93G), was administered to a duplicate group of mice. All mice were housed at controlled temperature, had free access to food and water and were maintained on 12 hr light-dark cycles. Experiments involving these mice were approved by the Animal Care Committees of the different universities involved in this project.

Immunocytochemistry

Mice (5 per treatment type and diet type) were anesthetized with sodium pentobarbital and the efferent ductules were removed and fixed by immersion in Bouin's solution. After several minutes, the efferent ductules were removed and prepared such that subsequent sections would include the entire length extending from the rete testis to the initial segment. The tissues were left in fixative for 72 hours, after which they were dehydrated and embedded in paraffin.

The Santa Cruz protocol was used for all immunocytochemical procedures (ImmunoCruz Staining System, Santa Cruz Biotechnology, Santa Cruz, CA). The anti-AQP-1, -9 and -10 antibodies were purchased from Alpha Diagnostics International (San Antonio, TX). Sections at 5 μm thickness were deparaffinized in Histoclear (Diamed Lab Supplies Inc, Mississauga, ON, Canada) and hydrated in a series of graded ethanol solutions. During hydration, residual picric acid was neutralized in 70% ethanol containing 1% lithium carbonate, and endogenous peroxidase activity was abolished in 70% ethanol containing 1% (vol/vol) H_2O_2. Once hydrated, the tissue sections were washed in distilled water containing glycine to block free aldehyde groups. Antigen

retrieval was performed using a sodium citrate buffer-microwave method (Santa Cruz Biotechnology, Santa Cruz, CA).

Before staining, the tissues were blocked for 1 hour with a solution containing 1% goat serum albumin. The tissues were incubated with primary antibody at 1:100 dilution in blocking buffer overnight at 4°C. Each slide was then incubated with biotinylated secondary antibody followed by Streptavidin-horseradish peroxidase for amplification. The sections were finally incubated with 0.05% diaminobenzidine tetrahydrochloride (Bio Fx Laboratories, Owings-Mills, MD), rinsed with tap water for 5 min, and counterstained with 0.1% methylene blue. Negative controls were prepared by incubating additional sections in all solutions except primary antibody.

Sperm Motility Analyses

Mice (5 per treatment type and diet type) were weighed and lightly anesthetized with isofurane and killed by cervical dislocation. The left cauda epididymides were removed and frozen (-20°C) for subsequent sperm count analyses. The right cauda epididymides were clamped proximally and distally prior to excision, rinsed in pre-warmed M199 medium (GIBCO) and placed in a Petri dish containing M199+0.5% BSA, preheated to 37°C. Each cauda was poked with the tip of a scalpel blade to permit the release of the sperm into the media. The sperm-media suspension was incubated at 37°C for five minutes, after which 100 µl aliquots were diluted with medium and transferred into each of two compartments on a glass cannula for Computer Assisted Sperm Analysis (CASA) using the Integrated Visual Optical System (IVOS) Motility Analyzer (Hamilton Thorne Research Inc, Beverly, MA). For each sample, 3-5 slides, with 5-10 scans per slide were analyzed. Fourteen of 15 measurement parameters (variables) available through software were analyzed (see footnote 1 in Table 1).

Correlation and statistical analyses and power tests of motility data were done using Version 7.0 of the Statistica Data Miner for Windows (Statsoft Inc., Tulsa, OK). Initial analyses indicated that there were some outliers present in the dataset and these were removed using the Grubb's test. Raw data for some parameters also did not follow normal distributions and these were obtained by doing $\log_{10}$ transformations on "regular" (continuous) variables (e.g. VAP) or arcsine of the square root transformations for ratio

variables (e.g. STR) as required. In subsequent Univariate Factorial ANOVA test and Post-hoc unequal N HSD *t*-tests for continuous variables and Fisher's exact tests for ratio variables, p values < 0.05 were considered significant.

Sperm Counts

The frozen left cauda epididymides from each animal used for sperm motility were thawed on ice and homogenized in a 50 ml conical tube containing 20 ml of distilled water. Aliquots (100 µl) of the resulting homogenate were diluted with 100 µl of distilled water in 1.5 ml microcentrifuge tubes coated with "IDENT fluorescent dye" (Hamilton-Thorne Biosciences) and incubated at room temperature for 2 minutes. The solution was mixed and a 5 µl aliquot was placed on a 20 µm sperm analysis chamber (2X Cel; Hamilton-Thorne Biosciences) and quantified with the IVOS semen analyzer under ultraviolet light. Data for sperm counts were analyzed as concentration (10_6/ml). As with motility data, sperm counts in αERKO and wild type mice were not normally distributed and $\log_{10}$ transformations of raw values were done prior to carrying out t-tests assuming unequal variances; p values < 0.05 were considered significant.

RESULTS

Immunocytochemical localization of AQP-1, -9 and -10 in the efferent ductules of adult wild type and αERKO mice

In the efferent ductules of wild type mice immunostained for anti-AQP-1 antibody, an intense immunoperoxidase reaction was noted over the microvilli of all nonciliated cells, as well as the cilia of the ciliated cells (Fig. 1A). The basolateral plasma membranes between adjacent epithelial cells were also intensely reactive (Fig. 1A). In contrast, in αERKO mice, there was a noticeable absence of reaction over the microvilli and cilia of some nonciliated and ciliated cells, respectively (Fig. 1B). In addition, staining of the basolateral plasma membranes was markedly reduced compared to localizations obtained in wild type mice (Fig. 1B). The finding of alternating strips of reactive versus unreactive epithelial cells in αERKO mice was more prominent in distal than proximal regions of the efferent ductules (not shown).

With the anti-AQP-9 antibody, an intense reaction was observed over the microvilli of nonciliated cells of the efferent ductules in wild type mice (Fig. 1C). Ciliated cells were unreactive, as were the basolateral plasma membranes between adjacent epithelial cells (Fig. 1C). In the αERKO mice, microvillar staining was maintained over some nonciliated cells, whereas others were unreactive, giving the epithelium a patchy appearance of strips of reactivity versus unreactivity (Fig. 1D). Distal efferent ductules were more affected than the proximal ductules (not shown).

In wild type mice, staining for anti-AQP-10 antibody occurred exclusively over the cilia of ciliated cells, with no reaction being observed over nonciliated cells (Fig. 1E). In comparison, in αERKO mice, the reaction over cilia was dramatically reduced over some ciliated cells (Fig. 1F). In addition, a patchy intense reaction appeared over the microvilli of a few nonciliated cells (Fig. 1F), unlike the absence of reaction over these cells in wild type mice (Fig. 1E). As for AQP-1 and -9, differences in staining patterns were more apparent in distal than proximal efferent ductules (not shown). While differences in staining patterns and intensities were noted between wild type and αERKO mice, no major differences in the staining pattern of AQP-1, -9 and -10 were evident by LM immunocytochemistry relative to the diet these animals consumed. Control sections

in which the primary antibody was eliminated demonstrated an absence of reaction over the epithelium, luminal contents or intertubular spaces (not shown).

Sperm Counts and Motility Analyses: Lab Chow Diet

Sperm counts from the cauda epididymidis were 62% lower in αERKO mice compared to wild type mice fed the lab chow diet (Table 1, Lab Chow, top). CASA measurements indicated that both the raw numbers and relative percentages of sperm subclassified as Motile, Progressive, Rapid, and Medium were noticeably lower, whereas sperm subclassified as Slow and especially Static were much higher, in the αERKO mice (Table 1, Lab Chow, top; raw counts and percentages; Fig. 2). The data further indicated that the movement velocities of sperm (VAP, VSL, and VCL), the linearity of their motion (LIN; ratio of VSL/VCL), and the amplitudes of their lateral head displacements (ALH) were all greatly reduced in the αERKO mice (Table 1, Lab Chow, top). The beat cross frequency (BCF) of sperm in αERKO mice, in contrast, was much greater than in controls (Table 1, Lab Chow, top). Both the head elongation ratios of sperm (Elong) and straightness of sperm movement paths (STR; ratio of VSL/VAP) showed no significant differences in mean values between αERKO mice and controls (Table 1, Lab Chow, top). There was a trend, however, for increasingly more negative correlations of the parameter Elong to occur in αERKO mice compared to wild type mice fed the lab chow diet (Fig. 2).

Sperm Counts and Motility Analyses: Casein Diet

Similar trends for reduced motility values in αERKO mice compared to wild type mice were observed for sperm sampled from animals fed the casein diet (Table 1, Casein, bottom). As well, sperm showed increases in BCF and in the relative number of sperm that moved slowly or were static in the αERKO mice (Table 1, Casein, bottom; BCF, %Slow, %Static). Features of sperm behaviour that were noticeably distinct included the findings that: (1) the degree of changes between αERKO and control mice for the other motility parameters were generally less dramatic for animals fed casein as compared to

those fed lab chow (Table 1, other parameters, top group versus bottom group; Fig. 3), and (2) raw sperm counts in the Medium, Slow, and Static categories and their expressions as percentages did not show exactly the same trends or the same proportional amount of change between αERKO and control mice fed casein versus the lab chow diets (Table 1, Medium-%Medium, Slow-%Slow, Static-%Static, top group versus bottom group; compare Figs. 2 and 3). In addition, sperm counts were much lower between αERKO mice and controls in animals fed the casein diet compared to those fed lab chow diet (Table 1, Sperm counts, top group versus bottom group).

Sperm Counts and Motility Analyses: Wild Type and αERKO Responses by Diet

Table 2 shows results of comparisons by diet within the wild type and αERKO groups based on mean values given in Table 1 (results in Table 2 are "vertical" comparisons across groups as opposed to the "horizontal" comparisons made in Table 1). It is evident from Table 2 that diet affected sperm counts and sperm motility in both cases, although more dramatically in the case of αERKO mice than in the case of wild type mice (Table 2, bottom group versus top group; Fig. 4). In broad terms, the lab chow diet for wild type mice appeared mildly stimulatory resulting in higher sperm counts, higher velocities of sperm movement, greater amplitude of lateral sperm head movement (ALH), as well as increases in the numbers of sperm subclassified as Motile, Prog, Rapid, and Medium without significant changes in their relative proportions (%Motile, %Prog, %Rapid etc. same for both diets) (Table 2, top group; Fig. 4 A,B). αERKO mice fed the lab chow diet showed substantially higher sperm counts relative to αERKO mice fed casein, but the lab chow diet otherwise appeared inhibitory both in terms of sperm movement velocities and in terms of the numbers of sperm subclassified as Medium, Slow, and especially Static (Table 2, bottom group; Fig. 4 C). In relative terms, the %Motile, %Prog, and %Rapid sperm in these mice were greatly reduced, whereas the %Static sperm showed a huge increase (Table 2, bottom group; Fig. 4D).

DISCUSSION

AQP expression in wild type and αERKO mice and effect of diet

In the present study, AQP-1 expression in αERKO mice showed patches of reactive versus unreactive epithelial cells in the efferent ductules as compared to the uniform homogeneous staining seen in wild type mice (Table 3). Thus estrogen appears to regulate expression of AQP-1 protein, either directly or indirectly, on microvilli and cilia of some but not all nonciliated and ciliated cells, respectively, suggesting the dependence of these cells on ERα activation for AQP-1 expression. Although ERβ is present in the efferent ductule epithelium (Zhou et al., 2002), it is unlikely that estrogen action through ERβ would maintain AQP-1 expression, as other data have shown that ICI 182,780, which blocks both receptors, had little effect on AQP-1 expression (Zhou et al., 2001). Alternatively, a factor other than estrogen may regulate AQP-1 expression in the remaining reactive cells of αERKO mice. A recent study suggests that AQP-1 is expressed constitutively in both the efferent ductules and initial segment of the epididymis (Oliveira et al., 2005), similar to its expression in the kidney (Nielsen, 1993; Borgnia et al., 1999). The sporadic loss of this water channel in the apical region of the efferent ductule epithelium in αERKO and ICI 182,780 treated mice (Zhou et al., 2001; Oliveira et al., 2005) could be an indirect effect due to the sporadic loss of the microvillus border in these animal models (Hess et al., 1997; Zhou et al., 2001; Oliveira et al., 2002; Hess, 2003; Cho et al., 2003). In addition, targeting of AQP-1 to the basolateral membrane was dramatically reduced throughout the epithelium of αERKO mice, suggesting the requirement of ERα for maintaining expression on this particular membrane domain (Table 3). Staining for AQP-9 on the microvilli of nonciliated cells was also reduced in αERKO mice (Table 3), and with ICI 182,780 treatment, AQP-9 disappears from the efferent ductule epithelium, even while microvilli were still intact (Oliveira et al., 2005). Thus AQP-9 expression in some nonciliated cells appears to be closely regulated by estrogen activation of ERα; however, this effect appears to be region specific, as the antiestrogen showed no effect on AQP-9 in the initial segment epididymal epithelium (Oliveira et al., 2005).

In the case of AQP-1 and -9, a more dramatic reduction of staining was noted in distal rather than proximal regions of the efferent ductules in αERKO mice, suggesting a more prominent role for ERα in distal regions. Regulation of other proteins expressed by the epithelial cells of the efferent ductules on a regional basis has not been carefully analyzed, although it has been well described that the nonciliated cells of proximal regions differ morphologically from those of distal regions (Robaire and Hermo, 1988; Ilio and Hess, 2002; Hess, 2002). On the other hand, region-specific regulation of proteins expressed by a given epithelial cell type in the epididymis has been documented by several investigators (Hinton et al., 1998; Robaire and Viger, 1995; Hermo and Robaire, 2002; Cornwall et al., 2002).

Cilia of ciliated cells reactive for AQP-10 in wild type mice showed a marked reduction in staining in αERKO mice (Table 3). However, the microvilli of some nonciliated cells, which in wild type mice never reacted positively, became reactive in αERKO mice (Table 3). Such data suggest regulation at the transcriptional or translational level for AQP-10 expression in nonciliated cells. Why this occurs is unknown, but it is possible that the reduction of AQP-1 and -9 associated with the microvilli of these cells in αERKO mice invokes a compensatory role for AQP-10 expression. While we do not know what evokes expression of AQP-10 in nonciliated cells in αERKO mice, we hypothesize that the presence of active ERα in wild type mice inhibits the factor, to be identified, that regulates AQP-10 expression in nonciliated cells of wild type mice. Interestingly, while expression of AQP-1 and -10 was reduced over the cilia of ciliated cells in αERKO mice, there was no obvious compensatory mechanism of expression of AQP-9 in the ciliated cells, which in wild type mice is not normally expressed. However, there may have been a compensatory shift toward microvillar activity in the ciliated cells, as the number of cilia per cell was reduced nearly 50% in αERKO ductules (Hess et al., 2000). Overall, these data suggest that nonciliated cells serve a more important role in water reabsorption than the ciliated cells.

Mice fed either lab chow or the phytoestrogen-free casein diet showed no major differences in the pattern of staining for AQP-1, -9 or -10. This suggests that the small traces of estrogenic compounds present in the lab chow diet exert minimal effects on AQP expression in the epithelium of the efferent ducts of either wild type or αERKO

mice. This is in sharp contrast to the major changes observed for sperm counts and sperm motility as a consequence of diet (discussed below).

Sperm Counts

The concentration of sperm in the cauda epididymidis was markedly lower in αERKO mice (Table 1), which confirms previous reports (Eddy et al., 1996; Cho et al., 2003). In αERKO mice, a dramatic reduction of water reabsorption, occurring at the level of the efferent ductules, results in a highly diluted sperm. In addition, the accumulation of water leads to a backflow into the lumen of seminiferous tubules, and disruption of the integrity of the developing germ cell population (Eddy et al., 1996; Lee et al., 2000; Hess et al., 2002). Thus, two mechanisms are involved in the reduction of sperm counts in the epididymis in the αERKO mouse, one which appears to be directly due to the failure of efferent ductules to reabsorb luminal water (Hess et al., 1997). From this study, it is also noteworthy that sperm counts differed in both wild type and αERKO animals depending upon the diet (Table 1). In both animal groups, there was a marked reduction in sperm counts when fed casein rather than lab chow. This suggests that despite their small amounts, phytoestrogens in the lab chow diet enhance either germ cell production or epididymal function, or both mechanisms, in both wild type and αERKO mice (Table 1).

A possible explanation for the dietary effects could be that phytoestrogens in the lab chow diet augment water reabsorption in the efferent ductules, possibly reducing the backflow of water. Furthermore, while AQP expression was diminished between wild type and αERKO mice, no apparent changes in immunostaining was observed due to the different diets. Taking these points into account, it is suggested that in αERKO mice, phytoestrogens in the lab chow diet may stimulate ERβ, which is expressed constitutively in the male (Oliveira et al., 2004) or elicit a non-genomic effect that indirectly increases the concentration of sperm in the epididymis. The differences in sperm counts in wild type mice between the different diets could be due to a combined effect of the phytoestrogens on both ERα and ERβ and their effects on Sertoli, germ and Leydig cells, respectively, as well as augmentation of efferent ductule functions. Hence, although we

cannot rule out the possible effects of phytoestrogens on sperm production at the level of the testis, we cannot overlook the concentrating function of the efferent ductules.

Sperm Motility

While sperm counts were improved in αERKO mice fed lab chow, the results of this study also clearly indicate that this increased concentration of sperm consists of a lower quality sperm (Table 1). The effects of diet were more dramatic in the case of αERKO mice than in wild type mice (Table 2; Fig. 4). Taken together with results from sperm counts, these data suggest that the effects on αERKO mice fed the different diets cannot be explained simply as the consequence of water retention at the level of efferent ducts. Rather it is suggested that altered sperm motilities in αERKO mice are a reflection of diminished epididymal functions, as the epithelial cells of this tissue play a major role in producing motile and fertile sperm (Robaire and Hermo, 1988; Orgebin-Crist, 1996; Cornwall et al., 2002). Furthermore, because sperm motility parameters in αERKO mice that were fed lab chow diet were diminished compared to those fed the casein diet, the data suggest that phytoestrogens in the absence of an ERα may be inhibitory through ERβ activity and adversely influence epididymal function and sperm maturation. However, wild type mice fed lab chow showed significantly improved sperm motility compared to those fed casein, suggesting that phytoestrogens in the presence of ERα may be stimulatory through ERβ activity, enhancing epididymal function and hence sperm motility. In the uterus, ERβ appears to play a role in modulation of ERα functions (Weihua et al., 2000), but whether ERα and ERβ modulate each other's function in the male reproductive tract is not known, despite the fact that both ERs are expressed along the epididymis in mice (Fisher et al., 1997; Couse et al., 2001; Zhou et al., 2002).

The epididymis is regarded as a tissue that plays a role in sperm maturation, whereby sperm acquire their proper motility characteristics (Robaire and Hermo, 1988; Cooper, 1995; Cornwall et al., 2002). The coordinated activities of the epithelial cells of the epididymis monitor the luminal environment by secretion and endocytosis of various substances including proteins, water and ions that leads to sperm maturation (Robaire and Hermo, 1988; Cooper, 1995; Turner, 2002). In the present study, multiple motility

parameters were altered in αERKO mice as compared to wild type mice and alterations were noted in both cases depending on the diet fed to the animals. This would suggest that different functions of one or more than one epithelial cell type must play a role in the ultimate production of sperm with diverse motility characteristics, and that the composition of the diet can also affect their functions related to motility. However, despite these findings, we cannot at this time ascribe which specific function(s) of the different epithelial cells are altered that lead to the varying sperm motility parameters noted in αERKO mice and wild type mice fed on different diets. The data would suggest, however, that the diverse motility features gained by sperm as they traverse the epididymis are governed by a host of varying epithelial cell functions. In summary, the present data reveal a role for ERα on expression of AQP-1, -9 and -10 in the efferent ductules. In addition, αERKO mice show reduced sperm counts and motility as compared to wild type mice. A role for the presence of phytoestrogens in the diet is also established for sperm counts and motility in the presence or absence of ERα.

FIGURE LEGENDS

Figure 1A-F: Immunolocalization of AQP-1 (A, B), AQP-9 (C, D) and AQP-10 (E, F) in the efferent ductules of wild type (A, C, E) and αERKO (B, D, F) mice. In (A), AQP-1 expression is uniformly distributed over microvilli (thick arrows) and cilia (arrowheads) of nonciliated and ciliated cells, respectively. Basolateral staining (thin arrows) is also evident over the epithelium. In (B), there is absence of reaction over microvilli and cilia of many, but not all nonciliated and ciliated cells (curved arrows), and basolateral staining is dramatically reduced. In (C), AQP-9 expression is uniformly distributed over the microvilli (thick arrows) of nonciliated cells, but no staining of ciliated cells (arrowheads). In (D), there is absence of reaction over the microvilli of some nonciliated cells (curved arrows). In (E), AQP-10 expression is observed exclusively over the cilia of ciliated cells (arrowheads) and there is no reaction over microvilli of nonciliated cells. In (F), there is absence of reaction over cilia of some ciliated cells (arrowheads). However, a distinct reaction appears over the microvilli of some nonciliated cells (arrows). Note the lumen (Lu) of αERKO mice (B, D, F) is enlarged compared to wild type mice (A, C, E). IT intertubular space. 420X.

Figure 2: Scatter plots summarizing changes in the motility behavior of sperm from αERKO mice compared to Wild Type controls for animals fed lab chow. In Panels A and B, differences in means determined for each of the 14 motility parameters analyzed by CASA are plotted as percentages along the abscissa (from column 4 of Lab Chow group in Table 1; Mean $_{\alpha ERKO}$ – Mean$_{WILD\ TYPE}$ / Mean$_{WILD\ TYPE}$ X 100%), and differences between the sums of correlation coefficients computed for each parameter are plotted along the ordinate (‡" *Pearson r* for Parameter$_A$ across Parameters$_{A\text{-}N}$ in αERKO mice – ‡" *Pearson r* for Parameter$_A$ across Parameters$_{A\text{-}N}$ in wild type mice for lab chow). Panel A shows results for correlation coefficients computed from raw sperm counts (Table 1, Raw Values, Lab Chow) whereas Panel B shows counts show a single cluster of 9 mildly altered parameters and 5 additional parameters residing at more outlying positions representing (1) slight decrease (Medium) or increase (Slow) in mean value and correlations much more strongly positive overall in αERKO mice, (2) no change of mean

(Elong) and correlations more highly negative overall in αERKO mice, (3) two-fold increase in mean value (BCF) and correlations slightly more positive overall in αERKO mice, and (4) five-fold increase in mean value (Static) and correlations slightly more negative overall in αERKO mice. : motility parameters computed as percentages when plotted show PANEL B clustering and outlier distribution very similar to Panel A. This indicates that changes in motility behavior in mice fed lab chow are uniform and affect sperm equally in all categories. Taken together these graphs provide a visual "fingerprint" of changes in sperm numbers and behavior that characterize the αERKO condition in mice fed lab chow that contains phytoestrogens. : motility analyses based on raw results for correlation coefficients computed from motility data expressed as percentages of total sperm cell counts (Table 1, Percentages, Lab Chow). If there were no differences in the motility behavior of sperm from αERKO mice and wild type mice then all points should plot near the "0" x-axis and "0" y-axis position (which they do not). PANEL A

Figure 3: Scatter plots summarizing changes in the motility behavior of sperm from αERKO mice compared to Wild Type controls for animals fed casein. In Panels A and B, differences in means determined for each of the 14 motility parameters analyzed by CASA are plotted as percentages along the abscissa (from column 4 for Casein group in Table 1), and differences between the sums of correlation coefficients computed for each parameter are plotted along the ordinate (see legend of Fig. 2 for additional details). Panel A shows results for correlation coefficients computed from raw sperm counts (Table 1, Raw Values, Casein) whereas Panel B shows results for correlation coefficients computed from motility data expressed as percentages of total sperm cell counts (Table 1, Percentages, Casein). PANEL A: motility analyses based on raw counts show a much different distribution pattern for parameters compared to the lab chow diet (compare Fig. 3 to Fig. 2). With casein, two main parameter clusters are evident. One cluster contains motility descriptors (Motile, Prog, Rapid, Medium) (means much less and correlations more positive overall in αERKO mice), and the second cluster contains the remaining motility descriptors (Slow, Static), velocity descriptors (VAP, VSL, VCL), and a single sperm feature descriptor (Elong) (means less and correlations slightly more negative,

more positive or unchanged in αERKO mice). There are also 4 additional parameters residing at more outlying positions representing (1) two directional descriptors (LIN, STR) (means slightly less and correlations more positive overall in αERKO mice) and (2) two additional sperm descriptors (ALH, BCF) altered in different ways (mean more positive and correlations more negative in αERKO mice (BCF); mean more negative and correlations considerably more positive in αERKO mice (ALH)). PANEL B: motility parameters computed as percentages when plotted show clustering and outlier distribution that is similar to Panel A for 6 parameters (%Motile, %Prog, %Rapid, VCL, ALH, BCF) and much different for the remaining 8 parameters. This indicates that changes in motility behavior in mice fed casein are not uniform and affect sperm in multiple ways.

Figure 4: Scatter plots summarizing changes in the motility behavior of sperm comparing Wild Type against Wild Type mice (Panels A, B) and αERKO against αERKO mice (Panels C, D) for animals fed Lab Chow versus Casein diet. In all Panels, only those parameters which showed a significant difference between means by diet (see column 2 of Table 2) are plotted as percentages along the abscissa (from column 1 in Table 2 for Wild Type (Panels A, B) or for αERKO (Panels C, D) groups), and differences between the sums of correlation coefficients computed for each of these parameters are plotted along the ordinate (see legend of Fig. 2 for additional details). Panels A and C show results for correlation coefficients computed from raw sperm cell counts (Table 2, Raw Values, Wild Type and αERKO) whereas Panel B and D show results for correlation coefficients computed from motility data expressed as percentages of total sperm cell counts (Table 2, Percentages, Wild Type and αERKO). PANELS A and B: the lab chow diet, containing phytoestrogens, is mildly stimulatory in Wild Type mice; sperm velocities are greater and more sperm are motile and progressive and travel at a rapid or medium speed. This diet also results in more positive correlations for all parameters except ALH, which is more negatively correlated across other parameters with the lab chow diet. In terms of relative motility, velocity descriptors and ALH increase but collectively have more negative inter correlations. PANELS C and D: the lab chow diet is mildly stimulatory to two motility descriptors (numbers of Motile and

Medium) and one sperm descriptor (BCF), and strongly stimulatory to two measures of sluggish sperm (numbers of Slow and Static) in αERKO mice. Sperm velocities are also slightly depressed in αERKO mice fed lab chow. In relative terms, the percentage of sperm that are motile and progressive and moving rapidly is less and the percentage of sperm that are static is many times greater in αERKO mice fed lab chow. Panels C and D validate that a component in the diet (phytoestrogens) is responsible for very large changes in parameters BCF and Static observed between αERKO and wild type mice maintained on a lab chow diet (compare to Fig. 2).

Table 1. Sperm Counts & Motility Changes Comparing αERKO to Wild Type Mice

Parameter[1]	Wild Type Mice (Num Obs)[2]	αERKO Mice (Num Obs)[2]	Change[3]	p values[4]		Power[5]
DIET = Lab Chow	Mean ± SD	Mean ± SD				
	(80)	(123)				
Sperm Counts	35.0 ± 15.0	13.4 ± 8.9	-62%	0.0000		1.0000
Raw Values:	(300)	(252)				
VAP	125.5 ± 26.9	68.1 ± 24.5	-46%	0.0000		1.0000
VSL	98.8 ± 23.7	48.1 ± 19.7	-51%	0.0000		1.0000
VCL	196.1 ± 38.1	134.5 ± 40.6	-31%	0.0000		1.0000
ALH	6.1 ± 1.0	4.8 ± 2.3	-21%	0.0000		1.0000
BCF	1.5 ± 1.6	4.1 ± 4.1	173%	0.0000		1.0000
Motile	44.0 ± 15.3	22.4 ± 19.2	-49%	0.0000		1.0000
Prog(ressive)	17.4 ± 6.9	5.2 ± 10.5	-70%	0.0000		1.0000
Rapid	30.5 ± 10.3	10.5 ± 9.9	-66%	0.0000		1.0000
Medium	13.5 ± 8.3	11.9 ± 10.6	-12%	0.0481		0.5050
Slow	1.7 ± 1.8	2.7 ± 3.4	59%	0.0000		0.9902
Static	11.1 ± 9.3	68.8 ± 76.0	520%	0.0000		1.0000
Ratios:						
STR	76.3 ± 4.5	69.1 ± 9.2	-9%	0.0582	NS	0.4810
LIN	50.6 ± 5.6	36.7 ± 8.2	-27%	0.0011		0.9086
Elong(ation)	49.5 ± 4.1	50.4 ± 6.8	2%	0.8332	NS	0.0551
Percentages:						
%Motile	78.9 ± 10.1	35.1 ± 23.3	-56%	0.0000		1.0000
%Prog(ressive)	31.5 ± 9.5	8.1 ± 8.0	-74%	0.0000		1.0000
%Rapid	55.3 ± 11.6	15.9 ± 13.1	-71%	0.0000		1.0000
%Medium	23.7 ± 9.9	19.2 ± 15.2	-19%	0.2015	NS	0.2494
%Slow	2.9 ± 2.8	4.0 ± 4.6	38%	0.4779	NS	0.1128
%Static	18.2 ± 10.2	60.9 ± 25.1	235%	0.0000		1.0000
DIET = Casein						
	(109)	(37)				
Sperm Counts	25.5 ± 8.8	3.4 ± 2.4	-87%	0.0000		1.0000
Raw Values:	(193)	(107)				
VAP	118.5 ± 22.1	91.2 ± 28.5	-23%	0.0000		1.0000
VSL	92.8 ± 19.6	69.5 ± 24.8	-25%	0.0000		1.0000
VCL	188.1 ± 32.2	154.0 ± 41.7	-18%	0.0000		1.0000
ALH	5.8 ± 1.2	4.4 ± 1.6	-24%	0.0000		1.0000
BCF	1.7 ± 2.0	2.6 ± 3.5	53%	0.0038		0.8197
Motile	37.7 ± 16.4	16.7 ± 9.9	-56%	0.0000		1.0000
Prog(ressive)	15.5 ± 8.4	6.1 ± 4.4	-61%	0.0000		1.0000
Rapid	26.7 ± 12.7	10.2 ± 6.5	-62%	0.0000		1.0000
Medium	11.0 ± 5.9	6.4 ± 4.7	-42%	0.0000		1.0000
Slow	1.4 ± 1.5	1.1 ± 1.5	-21%	0.0291		0.5827
Static	10.1 ± 8.7	8.6 ± 8.8	-15%	0.1703	NS	0.2773
Ratios:						
STR	76.2 ± 4.9	74.2 ± 7.6	-3%	0.7007	NS	0.0694
LIN	49.7 ± 5.8	46.6 ± 8.7	-6%	0.6072	NS	0.0816
Elong(ation)	46.0 ± 4.6	45.3 ± 6.7	-2%	0.9073	NS	0.0515
Percentages:						
%Motile	77.9 ± 11.2	65.1 ± 19.6	-16%	0.0169		0.6659
%Prog(ressive)	31.8 ± 9.9	23.4 ± 12.6	-26%	0.1249	NS	0.3342
%Rapid	54.9 ± 11.4	40.3 ± 17.0	-27%	0.0160		0.6769
%Medium	23.1 ± 8.6	24.7 ± 13.2	7%	0.7550	NS	0.0625
%Slow	3.2 ± 3.2	4.4 ± 6.1	38%	0.5948	NS	0.0918
%Static	18.9 ± 11.3	30.6 ± 20.6	62%	0.0219		0.6240

[1]Explanation of parameters

Sperm Counts (millions/ml)
VAP: Smoothed Path Velocity (μm/sec)
VCL: Track Velocity (μm/sec)
VSL: Straight Line Velocity (μm/sec)
ALH: Amplitude of Lateral Head Displacement (μm)
BCF: Beat Cross Frequency (hertz)
Number (in millions/ml) or Percent of:
Motile, Progressively Motile (Prog), Rapid, Medium, Slow and Static Cells
STR: Straightness (ratio of VSL/VAP)
LIN: Linearity (ratio of VSL/VCL)
Elongation: head shape (ratio of minor to major axis of sperm head)

[2]Total number of observations (measurements) made from a pool of 5 mice per group

[3]For αERKO mice compared to wild type mice

[4]p values < 0.05 are considered significantly different (NS, Not Significant). A Fisher's Exact Test was used to compare differences between means for "Ratios" and "Percentages"

[5]This is the power associated with rejecting the null hypothesis the two means are equal. The Z-test for comparing two proportions was used in power calculations for variables listed under "Ratios" and "Percentages"

Table 2. Effect of Diet on Sperm Counts & Sperm Motility Comparing Mice Fed Lab Chow To Those Fed Casein[1]

Parameter	Change[2]	p values[3]		Power[4]
GROUP = Wild Type				
Sperm Counts	37%	0.0000		0.9990
Raw Values:				
VAP	6%	0.0025		0.8534
VSL	6%	0.0033		0.8315
VCL	4%	0.0162		0.6684
ALH	5%	0.0012		0.8946
BCF	-12%	0.2223	NS	0.2301
Motile	17%	0.0000		0.9897
Prog(ressive)	12%	0.0062		0.7782
Rapid	14%	0.0003		0.9482
Medium	23%	0.0003		0.9521
Slow	21%	0.1384	NS	0.3157
Static	10%	0.2275	NS	0.2261
Ratios:				
STR	0%	0.9594	NS	0.0503
LIN	2%	0.8454	NS	0.0544
Elong(ation)	8%	0.4353	NS	0.1198
Percentages:				
%Motile	1%	0.7920	NS	0.0588
%Prog(ressive)	-1%	0.9443	NS	0.0506
%Rapid	1%	0.9306	NS	0.0508
%Medium	3%	0.8781	NS	0.0521
%Slow	-9%	0.5171	NS	0.0566
%Static	-4%	0.8380	NS	0.0555
GROUP = αERKO				
Sperm Counts	294%	0.0000		1.0000
Raw Values:				
VAP	-25%	0.0000		1.0000
VSL	-31%	0.0000		1.0000
VCL	-13%	0.0000		0.9807
ALH	9%	0.0678	NS	0.4448
BCF	58%	0.0012		0.8945
Motile	34%	0.0038		0.8202
Prog(ressive)	-15%	0.1281	NS	0.3296
Rapid	3%	0.7999	NS	0.0574
Medium	86%	0.0000		0.9985
Slow	145%	0.0000		0.9949
Static	700%	0.0000		1.0000
Ratios:				
STR	-7%	0.3325	NS	0.1555
LIN	-21%	0.0815	NS	0.4167
Elong(ation)	11%	0.3772	NS	0.1432
Percentages:				
%Motile	-46%	0.0000		0.9997
%Prog(ressive)	-65%	0.0001		0.9602
%Rapid	-61%	0.0000		0.9976
%Medium	-22%	0.2009	NS	0.2267
%Slow	-9%	0.8724	NS	0.0570
%Static	99%	0.0000		0.9998

[1]Means for each parameter are explained and listed in Table 1. Comparisons in this table (Table 2) are made vertically across the DIET categories of Table 1 (e.g., sperm counts in Table 1 for Wild Type compares 25.5 ± 8.8 to 35.0 ± 15.0 and for αERKO compares 3.4 ± 2.4 to 13.4 ± 8.9, and so on)

[2]For mice fed regular lab chow compared to mice fed casein

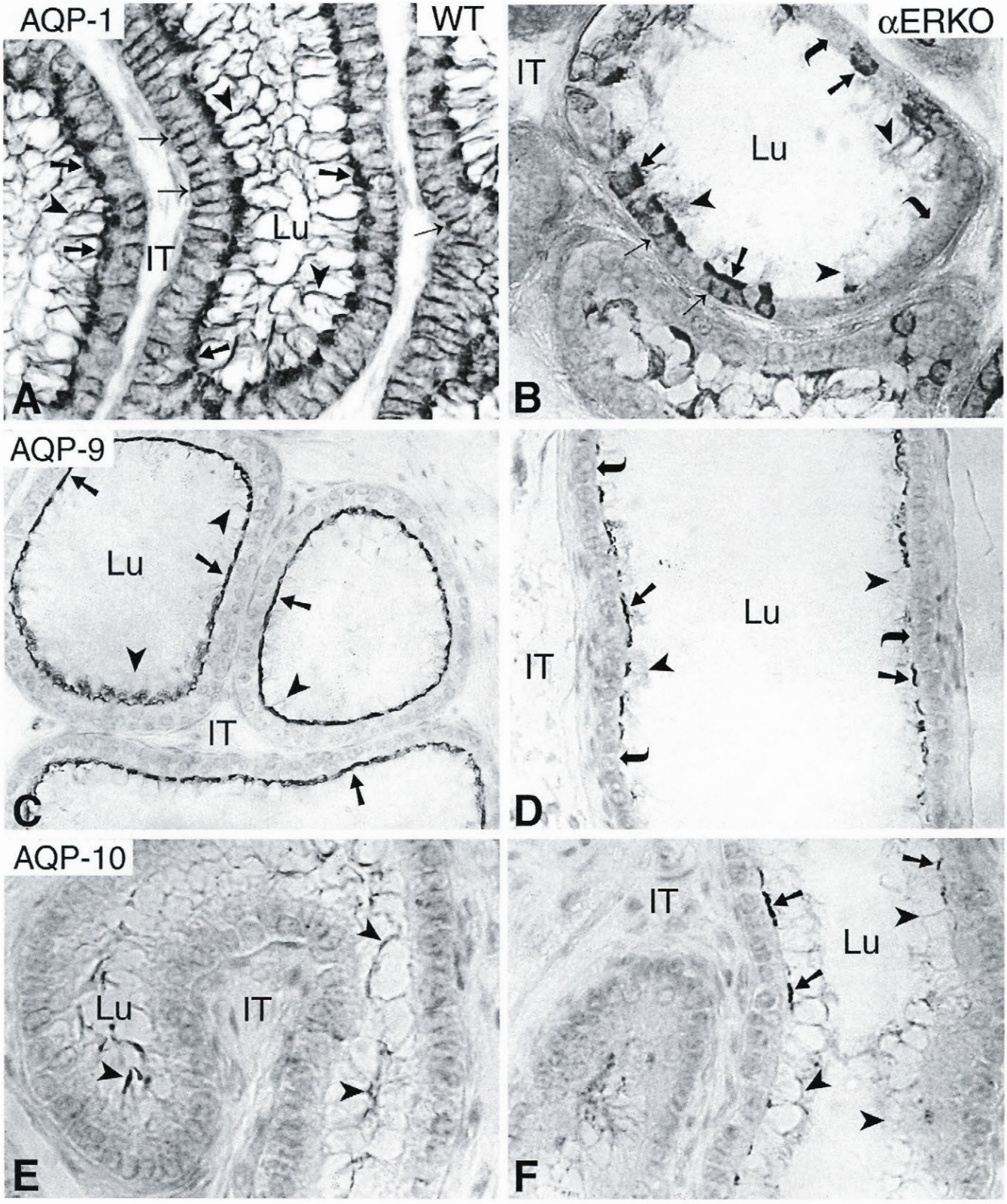
[3]p values < 0.05 are considered significantly different (NS, Not Significant). A Fisher's Exact Test was used to compare differences between means for "Ratios" and "Percentages"

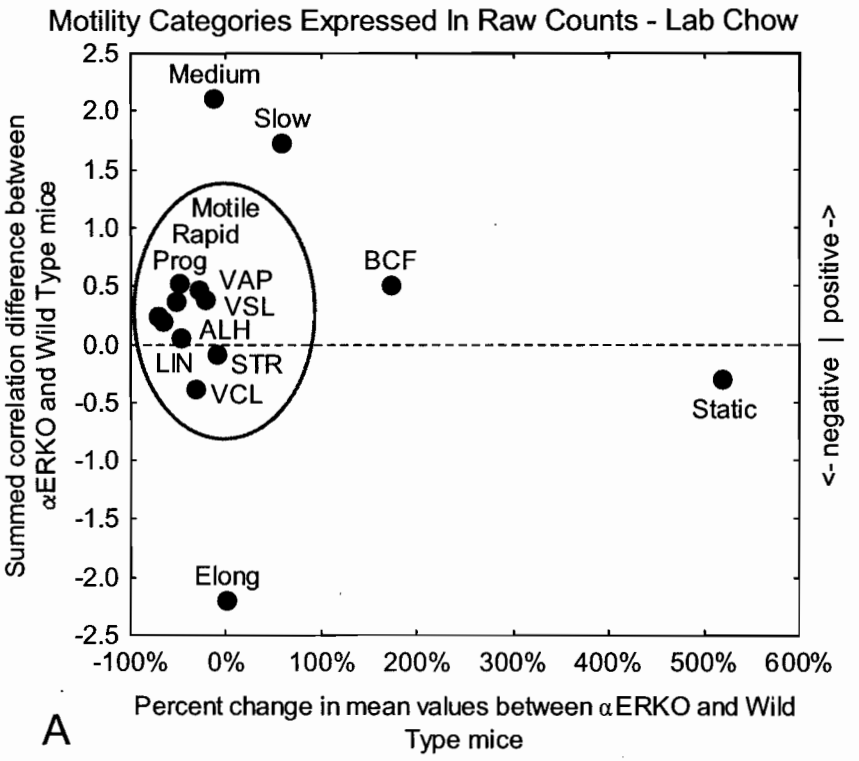
[4]This is the power associated with rejecting the null hypothesis the two means are equal. The Z-test for comparing two proportions was used in power calculations for variables listed under "Ratios" and "Percentages"

Table 3: Expression Of Aquaporins (Aqps)-1, -9 And -10 In The Efferent Ducts Of Wild Type And αERKO Adult Mice Fed On Lab Chow Or Casein Diets

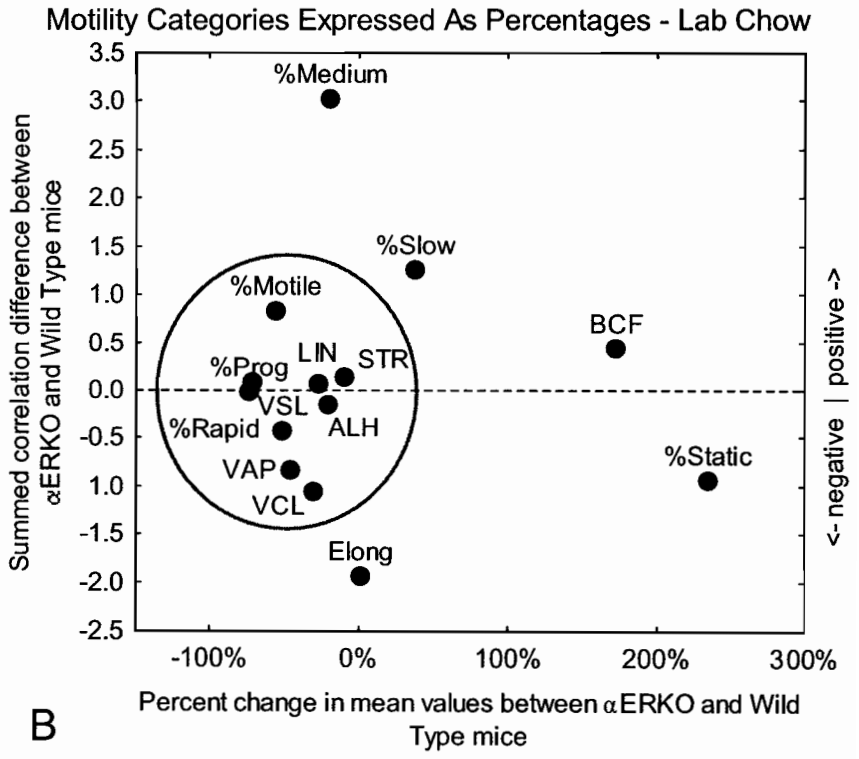
	Nonciliated Cells (Microvilli)		Nonciliated Cells (Basolateral)		Ciliated Cells (Cilia)	
	Wild Type	αERKO	Wild Type	αERKO	Wild Type	αERKO
AQP-1 [4]	+ [1]	+/- [2]	+	+/-	+	+/-
AQP-9 [4]	+	+/-	-	-	-	-
AQP-10	- [3]	+/-	-	-	+	+/-

[1] + indicates reactivity in all cells
[2] +/- indicates that reactivity is observed in some but not all cells
[3] - indicates absence of reaction
[4] The effect in KO mice is more pronounced in distal than proximal regions of the efferent ducts


AQP-1
WT
IT
Lu
A
αERKO
IT
Lu
B
AQP-9
Lu
IT
C
Lu
IT
D
AQP-10
Lu
IT
E
IT
Lu
F


Motility Categories Expressed In Raw Counts - Lab Chow
Medium
Slow
Motile
Rapid
Prog
VAP
VSL
ALH
LIN
STR
VCL
BCF
Static
Elong
Summed correlation difference between αERKO and Wild Type mice
<- negative | positive ->
Percent change in mean values between αERKO and Wild Type mice
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
-100%
0%
100%
200%
300%
400%
500%
600%

A


Motility Categories Expressed As Percentages - Lab Chow
%Medium
%Slow
%Motile
%Prog
LIN
STR
VSL
%Rapid
ALH
VAP
VCL
BCF
%Static
Elong
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Percent change in mean values between αERKO and Wild Type mice
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3.0
2.5
2.0
1.5
1.0
0.5
0.0
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1.0
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-2.5
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0%
100%
200%
300%

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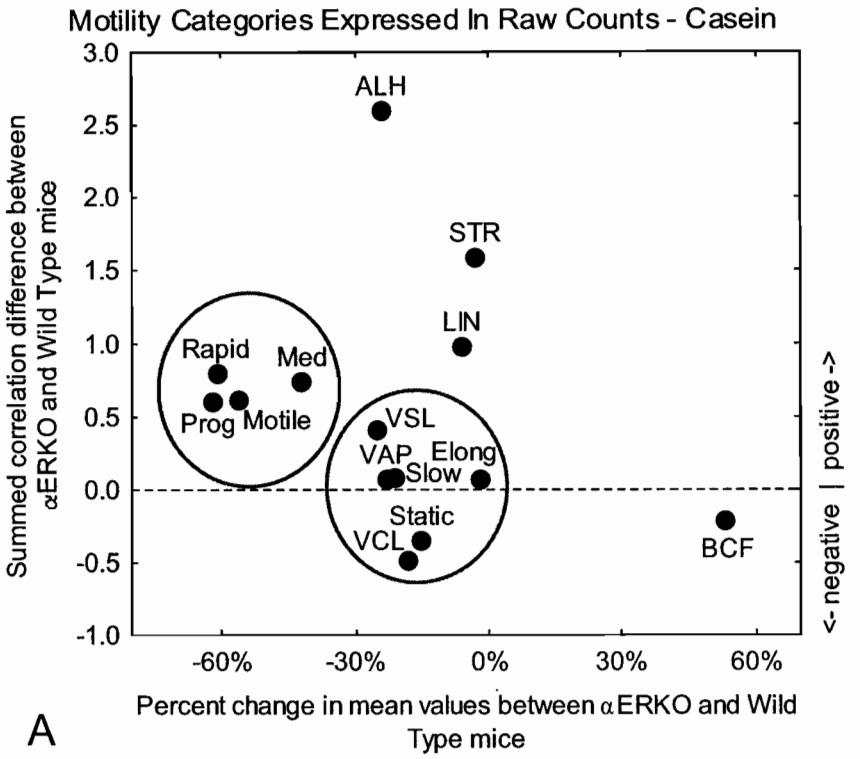
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STR
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Med
Prog
Motile
VSL
VAP
Elong
Slow
Static
VCL
BCF
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2.5
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30%
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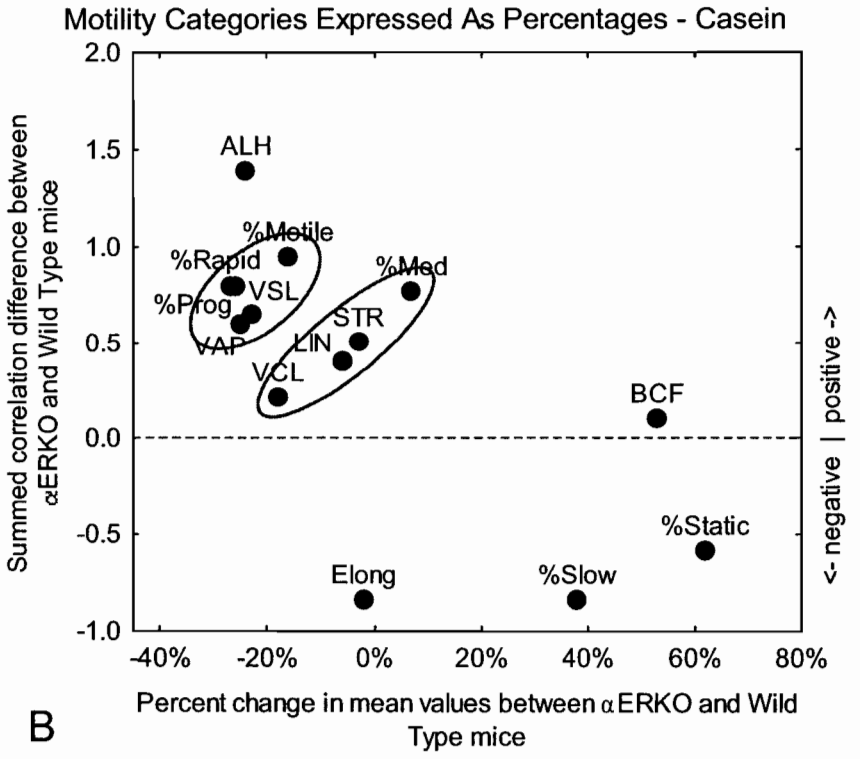
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%Prog
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STR
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BCF
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1.5
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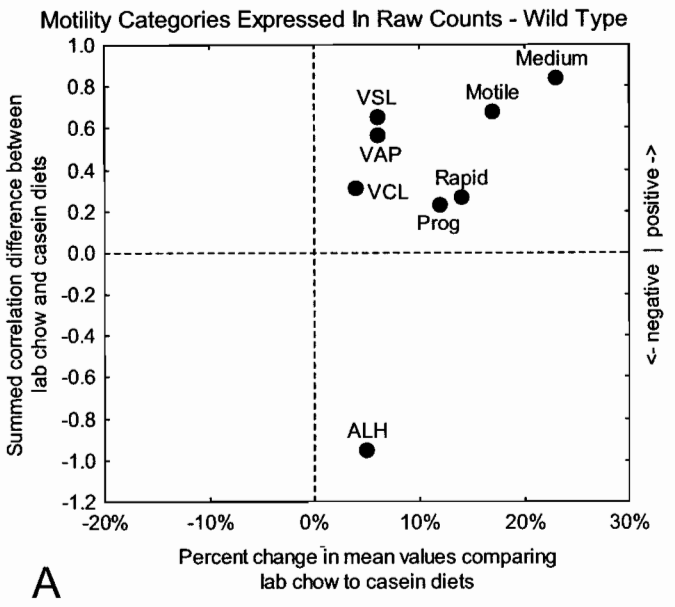
60%
80%

B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Prog
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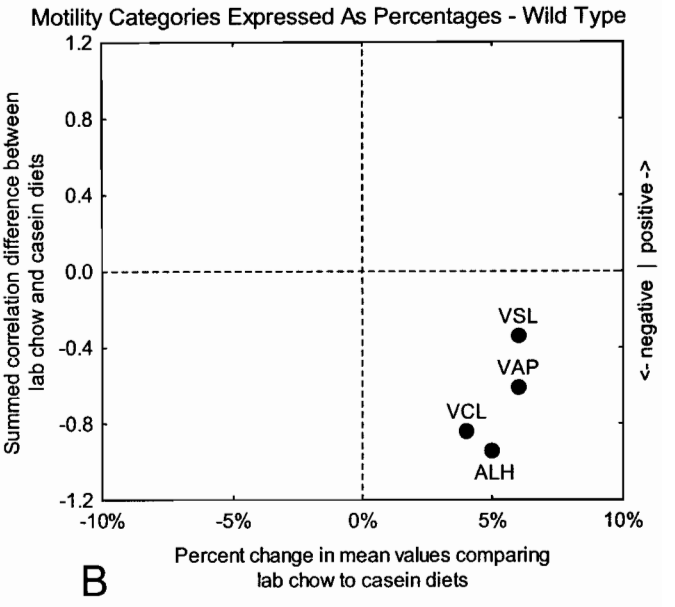
Percent change in mean values comparing lab chow to casein diets
A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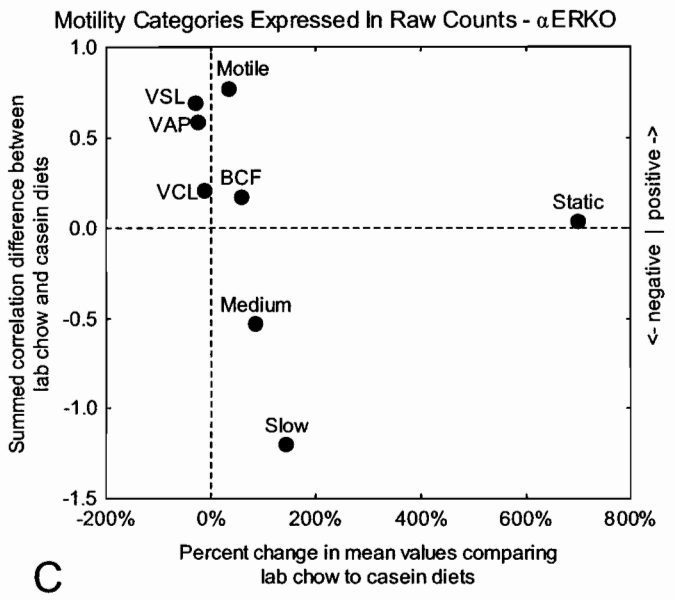
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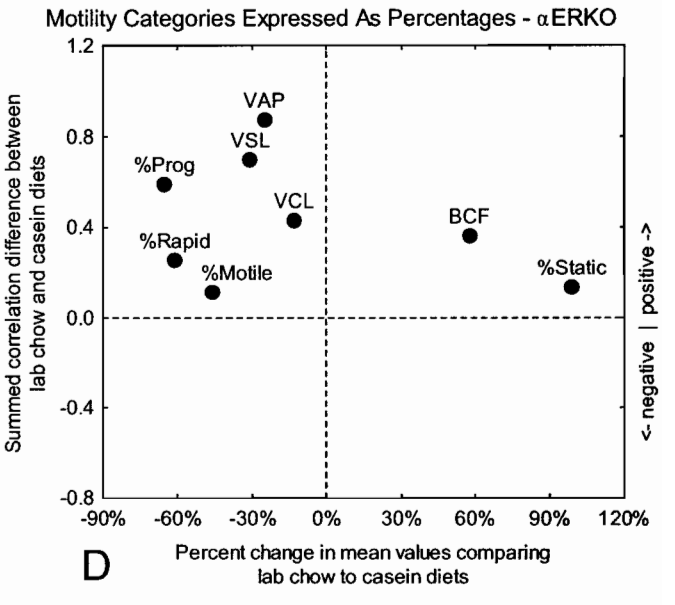
Medium
Slow
Summed correlation difference between lab chow and casein diets
<- negative | positive ->
Percent change in mean values comparing lab chow to casein diets
C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Percent change in mean values comparing lab chow to casein diets
D

CHAPTER 4

Immunolocalization and regulation of cystic fibrosis transmembrane conductance regulator (CFTR) in the adult rat epididymis

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ABSTRACT

Cystic fibrosis (CF) is the most common serious autosomal recessive condition in Caucasians and more than 95 % of CF men are infertile. The cystic fibrosis transmembrane conductance regulator (CFTR), a cAMP-regulated chloride channel has been localized in the efferent duct, however, its cell and region specific expression and regulation in the epididymis by testicular factors have not been examined. In the present study, these parameters were examined immunocytochemically with the light microscope with an anti-CFTR antibody in Bouin-fixed, paraffin embedded control adult rat epididymidis and both orchidectomized (O) adult rats with or without testosterone (T) supplementation and efferent duct ligated rats (EDL) sacrificed at different time points. In control animals, a dense band of immunoperoxidase reaction product was visualized over the apical plasma membrane of principal cells, but not their microvilli, along with a diffuse weak apical cytoplasmic reaction. The apical band was prominent only in the corpus and cauda regions and no reaction was evident in clear or basal cells. Examination of O rats with or without T supplementation revealed that CFTR was no longer expressed as a dense band on the apical plasma membrane of principal cells of the corpus and cauda regions. However, under these conditions, an intense apical/supranuclear reaction was noted in the form of small vesicular structures. This data would suggest that the synthesis of small CFTR-containing vesicles from the Golgi apparatus is maintained and not regulated by testicular factors, but that their targeting to the apical plasma membrane is inhibited, resulting in their accumulation in the cytoplasm. Nevertheless, as EDL also revealed similar results, these data, taken together, suggest that a luminal factor derived from the testis regulates the targeting of CFTR-containing vesicles to the apical plasma membrane of principal cells. Thus, CFTR expression in principal cells of the epididymis appears to be region specific, and its targeting to the apical plasma membrane from de novo Golgi derived CFTR-vesicles appears to be influenced by a luminal testicular factor.

INTRODUCTION

Sperm passage through the epididymis is crucial for their maturation, as is the formation of a suitable luminal environment as the major role of the epithelial cells lining the entire duct (Orgebin-Crist, 1967; Bedford, 1967). To this end, the epithelial cells resorb and secrete various electrolytes and transport fluid to concentrate or bathe the sperm as they traverse the duct (Wong, 1986; Wong et al., 2002). Although resorption (lumen to blood) is an element of fluid flow, secretion (blood to lumen) also plays an important role in the epididymis. Absorption driven by active sodium import and secretion driven by active anion export influence water movement inwards and outwards, respectively, across the epithelium (Wong et al., 2002). Indeed, the epithelium of the epididymis is highly permeable to water due to expression of aquaporin water channels in their membranes (Badran and Hermo, 2002).

Water flows down the osmotic gradient created by active ion transport according to the standing gradient model of Diamond and Wright (1969). Secretion may act as a counterbalance to absorption thereby exerting a fine control over the net movement of water across the epididymal epithelium. Secretion is mediated by a Na^+-K^+-$2Cl^-$ symport, a Na^+-H^+ and a Cl^--HCO_3^- exchanger located in the basolateral membrane, which serve to accumulate Cl^- and HCO_3^- inside the cell, while anion channels on the apical plasma membrane result in the secretion of these two ions, followed by passage of water into the epididymal lumen (Wong, 1990; Wong and Huang, 1989).

Several anion channels with different characteristics have been described in the epididymis (Chan et al., 1993, 1994) However, the cystic fibrosis transmembrane conductance regulator (CFTR), a small conductance cAMP-activated chloride channel, has the greatest relevance to transepithelial secretion of electrolytes and fluid (Pollard et al., 1991; Wong et al., 1992; Tizzano et al., 1994). The CFTR channel plays an improtant role in the secretion of electrolytes and fluid and may also be a regulator of other transport proteins that together determine the fluidity of the microenvironment in which sperm undergo maturation (Gray et al. 1993, Morris et al. 1993, Riordan 1993; Wong, 1998)

The CFTR gene encodes a 168-kDa protein designated as the cystic fibrosis transmembrane conductance regulator (CFTR) (Riordan et al., 1989; Fiedler et al., 1992).

CFTR, a membrane transport protein, is composed of five domains: two membrane-spanning domains, two nucleotide-binding domains, and a regulatory domain (Sheppard, 1999). CFTR is known to function as a cAMP-dependent protein kinase-activated Cl^- channel and is located primarily in the apical membrane of polarized epithelial cells (Denning et al., 1992; Crawford et al., 1991). However, similarities in sequence and domain organization between CFTR and the ABC superfamily of ATP-linked transporters suggest that CFTR may also act as a complex regulator of other membrane or membrane-associated proteins (Hyde et al., 1990; Greger, 1999; Kunzelmann et al., 1997; Mall et al., 1996; McNicholas et al., 1994; Stutts et al., 1995).

The importance of CFTR has been borne out by the genetic disease cystic fibrosis. CF is caused by mutation of a membrane protein called cystic fibrosis transmembrane conductance regulator (CFTR) normally present in epithelial cells. Several hundred forms of mutation of the CFTR gene have been identified, leading to a variety of diseases. From the most severe forms of mutations to the least, it is apparent that the male reproductive system is highly dependent on CFTR for normal function. While the most severe form of CFTR mutation (□508) results in obstruction or agenesis of the vas deferens, even moderate loss of CFTR functions is associated with congenital bilateral absence of the vas deferens (Anquiano et al., 1992; Patrizio et al., 1993; Oates and Amos, 1994; Chillon et al., 1995; Rave-Harel et al., 1997). In fact, even the least severe form of CFTR function, results in poor sperm quality of otherwise apparently normal men (Van der Ven et al., 1996).

Previous studies have shown that CFTR is expressed on the apical domains of the nonciliated epithelial cells of the efferent ducts (Leung et al. 2001). By effecting fluid secretion, CFTR acts counter to fluid reabsorption and serves to fine-tune the fluidity of the epididymal microenvironment (Wong et al., 2002). It has been demonstrated that the luminal milieu of the epididymis is actively maintained and that this plays a crucial role in the maturation process of spermatozoa (Robaire and Hermo, 1988; Wong 1978). While CFTR has been documented to be functional in the epididymis, immunocytochemical studies have not been performed, nor have studies examining its regulation by testicular factors, whether gaining access to the epididymis via the circulation or lumen of the duct.

In the present study, the expression of CFTR in the epididymis was examined on Bouin-fixed adult rat epididymidis using an anti-CFTR antibody. In addition, the regulation of CFTR was examined in adult orchidectomized and efferent duct ligated rats to determine the role of testicular factors on its expression. The data reveal that in the epididymis, CFTR is distributed in a cell and region specific manner, and that although its synthesis is not regulated by testicular factors, a luminal testicular factor is required for targeting Golgi derived CFTR-vesicles to the apical plasma membrane.

MATERIALS AND METHODS

Animals and experimental protocols:

Adult male Sprague Dawley rats (350-450g, 3-4 months of age) were obtained from Charles River Laboratory Ltd. (St. Constant, Quebec) and were subdivided into six groups. The first group consisted of four adult control animals. Bilateral ligation of the efferent ducts constituted the second group. After an intraperitoneal injection of sodium pentobarbital (Somnitol, MTC Pharmaceuticals, Hamilton, Ontario), the testes and epididymides of adult rats were exposed through an incision of the anterior abdominal wall. Using a dissecting microscope, a ligature was placed around both right and left efferent ducts at a site close to and further removed from the rete testis, with care being taken to avoid interference of the blood vessels entering the testis. The interval between the two ligatures was then excised to ensure that no sperm or fluids would enter the epididymis from the testis. The animals (four per interval) were sacrificed at 3, 7, 14 and 21 days following surgery. Bilateral orchidectomy constituted the third group. After anaesthesia, both testes of each rat were removed after a ligature was placed around the efferent ducts and testicular blood vessels. The animals (four per interval) were sacrificed at 3, 7, 14 and 21 days after surgery. Bilaterally orchidectomized rats that received three 6.2 cm testosterone-filled implants constituted the fourth group. Testosterone-filled polydimethyl-siloxane (silastic) implants were prepared according to the method of Stratton et al. (1973) and have well-characterized steroid release rates (Brawer et al. 1983). Subsequent to anaesthesia, both testes were removed from each rat and the implants placed subcutaneously immediately after orchidectomy. The rats (four per interval) were sacrificed at 3, 7, 14 and 21 days after surgery. The fifth group consisted of four sham-operated animals, two of which received three empty 6.2 cm-long implants, with all rats being sacrificed 14 days after initiation of the experiment. All experimentation was carried out with minimal stress and discomfort being placed on the animals both during and after surgery as set up by the guidelines and approval of the McGill University Animal Care Committee.

Tissue preparation for light microscope immunocytochemistry:

At the end of each experiment, the epididymides of each rat were fixed by perfusion with Bouin's fixative via the abdominal aorta for 10 minutes. Following perfusion, the epididymides were removed and cut so that given sections would include all of the major regions of the epididymis, i.e. the initial segment, intermediate zone, caput, corpus and cauda (Hermo et al. 1991). The tissue was then immersed in Bouin's fixative for 72 hours, after which it was dehydrated and embedded in paraffin.

Immunoperoxidase staining of sections was carried out according to the procedure of Oko and Clermont (1989). Monoclonal, affinity-purified anti-CFTR antibody was used at different dilutions in Tris-buffered saline (TBS), pH 7.4. The anti-CFTR antibody was obtained from NeoMarkers (Fremont, CA). The antibody has been well characterized and was found to be specific for its respective peptide. The anti-CFTR antibody was raised against a 10 amino acid synthetic peptide within the carboxy terminus of the protein (Kartner et al., 1992). The antibodies were purified from ascites fluid by Protein G chromatography and were found to be specific to its target. It was supplied as a 200 □g/mL solution in phosphate-buffered saline (PBS), pH 7.4, with 0.2% bovine serum albumin as stabilizer. The antibody solution also contained 15 mM sodium azide as a preservative. Paraffin sections, 5□m thick, were deparaffinized in Histoclear (Diamed Lab Supplies Inc, Mississauga, ON, Canada) and hydrated in a series of graded ethanol solutions. During hydration, residual picric acid was neutralized in 70% ethanol containing 1% lithium carbonate, and endogenous peroxidase activity was abolished in 70% ethanol containing 1% (vol/vol) H_2O_2. Once hydrated, the tissue sections were washed in distilled water containing glycine to block free aldehyde groups.

Antigen was retrieved by treatment in 100□L of 0.025% trypsin solution for 9 minutes. Sections were then rinsed three times in 1X PBS. After rinsing with pure water and PBS, sections were incubated in normal blocking serum (Vectastain Elite ABC kit, Vector K-6101; Vector Laboratories, Burlingame, CA) for 30 min and then with the monoclonal anti-CFTR antibodies (NeoMarkers, Fremont, CA), diluted to 1:100 with diluting buffer (1X PBS). Sections were then washed three times with PBS and incubated with biotinylated secondary antibody (ABC kit) for 30 min. After washing three times with PBS, sections were incubated with the ABC reagent for 30 min and finally washed

again three times with PBS. Visualization of the stain was achieved by incubating sections with 0.05% diaminobenzidine tetrahydrochloride (Sigma) until desired staining intensity developed. Slides were rinsed with pure water for 5 min, counterstained with 0.1% methylene blue. Passing them through a graded ethanol series dehydrated the tissues. Thereafter, the tissue sections were mounted on glass slides with Permount for observation. Negative controls were obtained by omission of primary antibodies.

RESULTS

Expression of CFTR in the efferent ducts and epididymis of normal adult rats

In the efferent ducts of normal adult rats, with an anti-CFTR antibody, a thick band of intense immunoperoxidase reaction product was noted on the apical plasma membrane of the nonciliated epithelial cells, in addition to a weak diffuse homogeneous reaction immediately subjacent to it. There was, however, no reaction in the supranuclear region of the cell cytoplasm, nor was any reaction visible on their microvilli. Ciliated cell also showed an apical reaction, but no reaction over their cilia.

In the initial segment of the epididymis, principal cells revealed a weak diffuse reaction over their apical cytoplasm, but no discrete reaction over their apical plasma membrane. In the caput epididymidis, a diffuse apical reaction was also evident over principal cells (Figure 1a). In contrast to these two epididymal regions, principal cells of the corpus and cauda epididymidis revealed an intense thick band of reaction product over their apical plasma membrane, while a moderate diffuse reaction, at times granular in appearance, was noted apically (Figure 1b and 1d). There was no staining of the microvilli of principal cells, and clear cells of all epididymal regions were consistently unreactive (Figure 1c and 1e). The peritubular cells enveloping epididymal tubules of all regions demonstrated moderate levels of reaction product. Sperm in the lumen were unreactive.

CFTR immunolocalization in orchidectomized and efferent duct ligated adult rats

After efferent duct ligation at the 3, 7, 14 and 21-day intervals, there was a dramatic redistribution in expression of CFTR in the epididymis. In the initial segment (not shown) and in the caput region (Figure 2a), there was an absence of reaction over the apical cytoplasm of principal cells, albeit, this region was not prominently reactive even in normal animals. However, in the corpus and cauda epididymidis, there was an absence of the thick band of reaction product over the apical plasma membrane of principal cells (not shown). Noteworthy, however, was the conspicuous apical/supranuclear cytoplasmic reaction in these cells, which consisted of numerous small intensely reactive vesicular profiles. Such vesicular profiles were not apparent in principal cells of normal animals.

After orchidectomy at the 3, 7, 14 and 21-day intervals, the same type of staining pattern was noted in principal cells as seen for efferent duct ligated animals. There was no change in CFTR expression in principal cells of the initial segment and caput epididymidis; however, the prominent thick band of reaction over the apical plasma membrane of principal cells of the corpus and cauda regions was absent. In its place, were numerous small reactive vesicular profiles in the apical and supranuclear cytoplasm of these cells (Figure 2b). As was noted for normal animals, the microvilli of principal cells and clear cells were consistently unreactive, while the peritubular cells of the tubular epididymal wall maintained their reactivity. The same staining pattern in the various epididymal regions was observed for rats supplemented with testosterone for 14 days following orchidectomy (Figures 2c, d and e). Thus these data suggest that the absence of testicular factors, including androgens, does not inhibit the synthesis of CFTR. On the other hand, the targeting of CFTR to the apical plasma membrane, where it is prominent in the apical plasma membrane of principal cells of the corpus and cauda regions is disrupted in the absence of testicular factors, as also evidenced by the accumulation of numerous small CFTR-positive vesicles in the cytoplasm of these cells. Since androgens did not restore its expression to the apical plasma membrane, and since its presence on the apical cell surface was also absent after efferent duct ligation, it is concluded that a luminal factor emanating from the testis is involved in regulating CFTR expression to the apical plasma membrane of principal cells, the identity of which still needs to be determined.

DISCUSSION

Expression of CFTR in the epididymis of normal adult rats

The presence of CFTR in the epididymis is not novel, as a functional role for CFTR has been well described for several years (Wong et al., 2002). However, in the present study, we reveal that CFTR shows cell type-specific expression as well as region specificity. Indeed, only principal cells show a prominent reaction over their apical plasma membrane and this is restricted to those of the corpus and cauda regions only. In the initial segment and caput epididymidis, the reaction over principal cells was never intense and seen only as a weak, diffuse, apically located reaction. Furthermore, there was no evidence of CFTR expression in clear or basal cells of the entire epididymis.

The presence of weak expression of CFTR in the initial segment would support that finding that water in this region is driven mainly from the lumen to the intertubular space. This has been suggested to be of importance for concentrating sperm in the small luminal diameter of the initial segment so as to better facilitate interactions of the sperm surface with the secretion products of the epithelial cells lining this region of the duct (Hess et al., 1997, 2002 (a,b)). In fact, in the initial segment, aquaporin-9 has been localized to the microvilli of principal cells which may aid passage of water from the lumen to the underlying intertubular space (Badran and Hermo, 2002).

In the corpus epididymidis, where sperm begin to accumulate, and in the cauda region where they are stored, we noted maximal expression of CFTR. It has been noted that under basal conditions, there is a net reabsorption of Na+ and water from the lumen of the epididymis. However, various transporters located in the basolateral membrane of the epididymal epithelium appear to take up chloride into their cytoplasm so that intracellular chloride activity is held above its electrochemical equilibrium. When cells are stimulated there is an increase in intracellular cAMP, which activates CFTR, allowing chloride efflux into the lumen (Wong, 1988; Chan et al., 1995). Under such stimulated conditions, the net secretory flux is increased to a level that exceeds the absorptive flux, resulting in a net secretion of water into the lumen. Thus, sperm in the corpus and cauda regions, while encountering their immobilizing protein immobilin, which gradually fills the lumen from the caput to the cauda regions to eventually engulf them (Hermo et al.,

1992), are nevertheless maintained in a fluid environment created by the input and output of water, the fine tuning of which is exerted by various transporter proteins, including CFTR (Wong et al., 2002).

The cAMP-mediated activation of CFTR has also been shown to activate aquaporin water membrane channels (AQP-3) in Xenopus oocytes, and the water permeability of respiratory cells is enhanced with CFTR activation (Schreiber et al., 1997, 1999). In the epididymis, we have shown that aquaporin-9 expression gradually increases in staining intensity on the microvilli and cell surface of principal cells from the caput to the cauda region of adult rats (Badran and Hermo, 2002). Aquaporins allow the rapid passage of water across an epithelium at low energy cost to cells. The finding of CFTR and AQP-9 on the surface of principal cells of the corpus and cauda regions suggests that these two proteins may functionally interact with one another, in some as yet undetermined manner. In this way, the fluidity of the epididymal lumen can be fine tuned resulting in a microenvironment that is conducive not only for sperm maturation, but for their passage down the duct and storage in the cauda epididymidis.

Regulation of CFTR in orchidectomized and efferent duct ligated adult rats

In the present study, there was a dramatic alteration in the staining pattern of CFTR in the epididymis following orchidectomy, and one, which was not restored with testosterone administration. The alteration was seen as a complete absence of the dense band of reaction over the apical plasma membrane of principal cells of the corpus and cauda epididymidis. However, under treatments, numerous small CFTR-positive vesicles appeared in the apical and supranuclear cytoplasm of principal cells. Such vesicles were not evident in control animals. It is suggested, therefore, that the synthesis of Golgi-derived CFTR-positive secretion vesicles is ongoing and unaffected after orchidectomy, but that these vesicles in the absence of testicular factors are not targeted to the apical plasma membrane. The administration of testosterone to orchidectomized animals also failed to restore staining to the apical plasma membrane, excluding the possibility that androgens are responsible for the apical targeting of CFTR-positive vesicles. Furthermore, as efferent duct ligation also revealed similar results, it is suggested that luminal factors emanating from the testis are not essential in regulating synthesis of

CFTR and its presence in cytoplasmic vesicles, but that these factors are normally responsible for targeting CFTR to the apical membrane of principal cells, explained by the prevention of CFTR-positive vesicles from fusing with the apical membrane in their absence.

Although it is well established that many epididymal functions are under the control of androgens (Robaire and Hermo, 1988; Orgebin-Crist, 1996; Ezer and Robaire, 2003; Cornwall et al., 2001), several proteins have been shown to be unaffected in their protein or mRNA expression by androgen withdrawal, such as SGP-1, cystatin c, SGP-2, cathepsins A and D (Hermo et al., 2000; Luedtke et al., 2000; Hermo and Andonian, 2003; Wassler et al., 2002). However, in addition to the regulation mediated by androgens, factors emanating from the testis that enter the epididymis via the lumen of the duct, defined as lumicrine factors, also play a role in regulating epididymal functions (Hinton et al., 1998, Cornwall et al., 2002; Ezer and Robaire, 2003). Lumicrine factors derived from the testis have been shown to regulate several proteins synthesized by the epididymis. Indeed, ligation of the efferent ducts induces changes in epididymal gene and protein expression (Cornwall et al., 2002). The luminal testicular factors that regulate gene expression in the initial segment include ions, solutes, proteins, steroids and even germ cells. These factors can up- or down-regulate gene expression after efferent duct ligation (Brooks, 1983). Expression of cystatin-related protein, lipocalin, and proenkephalin are amongst the proteins that are regulated by lumicrine factors (Garrett et al., 1990; Cornwall et al., 2002; Lareyre et al., 2001). Some proteins expressed by the same cell type, but in different regions can be regulated by different factors (Cyr et al., 1992; Hermo and Andonian, 2003). Interestingly, at the mRNA level, gamma glutamyl-transpeptidases, which show multiple transcripts, are differentially regulated by androgens and/or lumicrine factors in the different epididymal regions (Palladino and Hinton, 1994), as is their secretion and activity (Agrawal and Vanha-Perttula, 1988). In the present study, CFTR synthesis by principal cells is not regulated by testicular factors, but its targeting to the apical plasma membrane, where it is normally found, appears to be regulated by a lumicrine factor, the identity of which remains to be determined.

An alternative possibility to the targeting and fusion of de novo Golgi-derived CFTR vesicles to the apical membrane, may be the inhibition of CFTR-vesicles recycling

from endosomes to the apical plasma membrane. However, several findings do not support this hypothesis. Namely, recycling occurs near the apex of the cell, and numerous supranuclear CFTR-positive vesicles are evident; recycling usually occurs via tubular structures and not vesicles; recycling of CFTR vesicles should be associated with other recycling plasma membrane proteins, such as AQPs and osteopontin, since immunostaining in both cases is uniform along the entire apical plasma membrane of principal cells and in fact, never seen as an accumulation of AQP-recycling vesicles in the principal cells after orchidectomy or efferent duct ligation (Luedtke et al., 2001; Badran and Hermo, 2002).

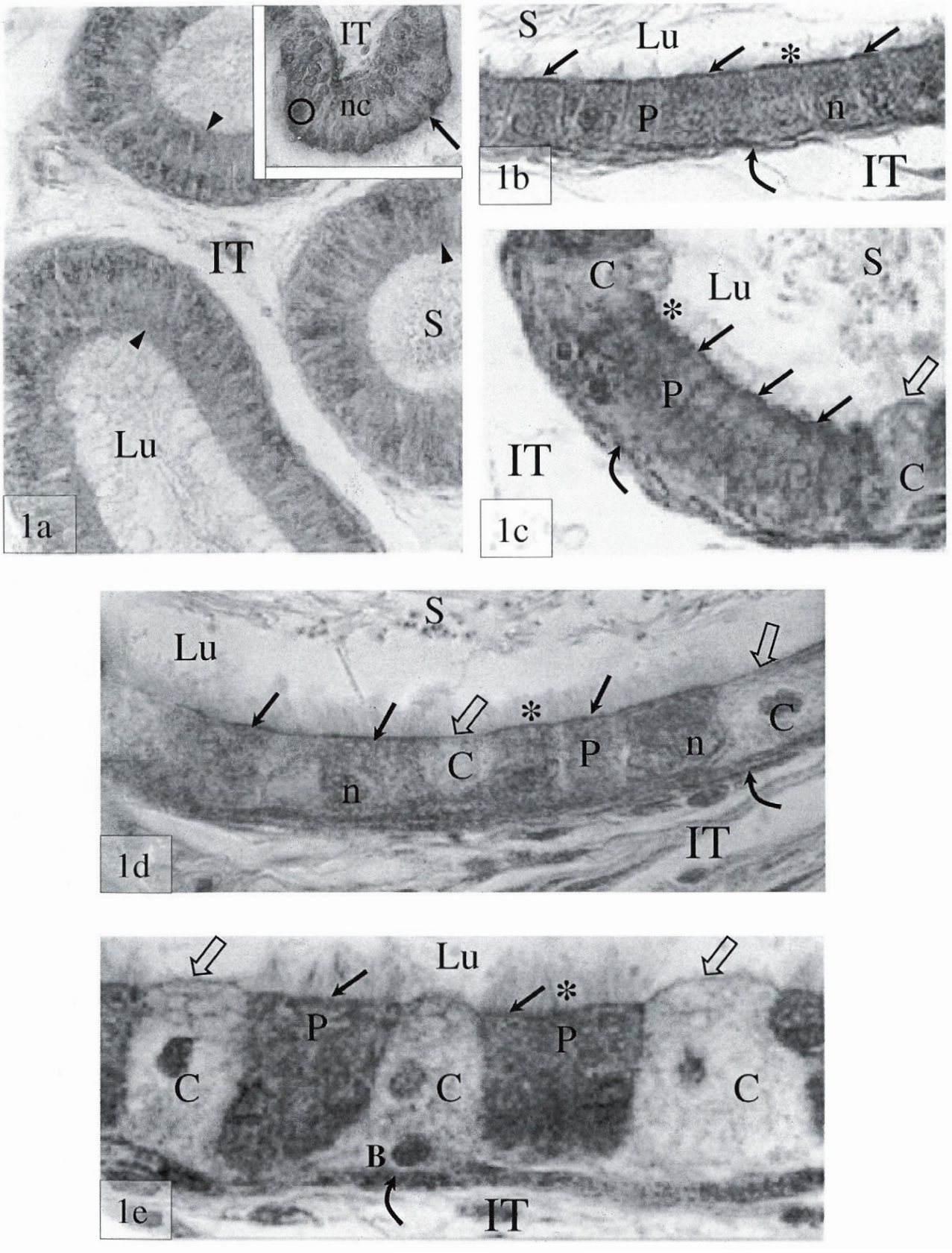
It has been documented that multiple regulatory pathways control secretion of electrolytes and water transport across the epididymal epithelium, such as neurotransmitters, prostaglandins, bradykinins and other peptide hormones (Wong et al., 1990, 1998, 1999). Prostaglandins (PGE2) synthesized in basal cells diffuse out and act on prostaglandin receptors on the basolateral membrane of principal cells. This causes an increase in intracellular cAMP through receptor-G protein-coupled adenylate cyclase, which then activates CFTR resulting in the secretion of anions and water (Wong et al., 2002). The present study further suggests that in vivo, a lumicrine factor in some as yet undetermined way regulates the fusion of CFTR-Golgi derived vesicles with the apical plasma membrane and that in its absence such vesicles are maintained in the cytoplasm.

Figures 1 a-e:

Caput (a), corpus (b, c) and cauda (d, e) of the epididymis at low (a) and high (b-e) magnification of a 90-day old adult rat immunostained with an anti-CFTR antibody. In (a), a moderate homogeneous, at times granular, reaction product is evident over the apical cytoplasm of the epithelial principal cells, but none over the supranuclear region of these cells. In (b-e), an intense thick band of reaction product appears over the apical plasma membrane of principal cells, which is not evident in those of the caput epididymidis (a). Clear cells of all epididymal regions appear unreactive and display only background levels of staining. No reaction is visible over the microvilli of principal cells, other than background levels of reaction. S, sperm; L, lumen; n, nuclei. X320 (a, b); X512 (c, d); X1280 (e)

Figures 2 a-e:

Caput (a), corpus (b-d) and cauda (e) epididymis of a 3-day efferent duct ligated rat (a), 21-day orchidectomized rat without testosterone restoration (b), 7-day efferent duct ligated (c), 14-day efferent duct ligated (d) and 14-day orchidectomized rat with testosterone supplementation (e); all tissues immunostained with an anti-CFTR antibody. While no apparent reaction is noted over principal cells in (a), as also noted for normal animals, an intense diffuse granular reaction product is prominent over the apical and supranuclear regions of principal cells of the corpus and cauda epididymidis (b-e), suggesting the presence of numerous small CFTR-vesicular structures. Note that clear cells continue to be unreactive (e). S, sperm; L, lumen; n, nuclei. X320 (a); X512 (b-e)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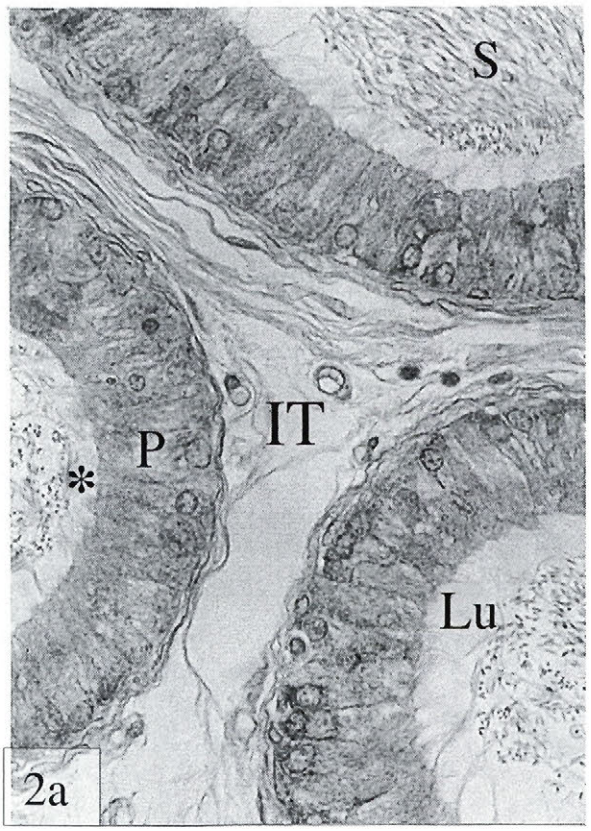
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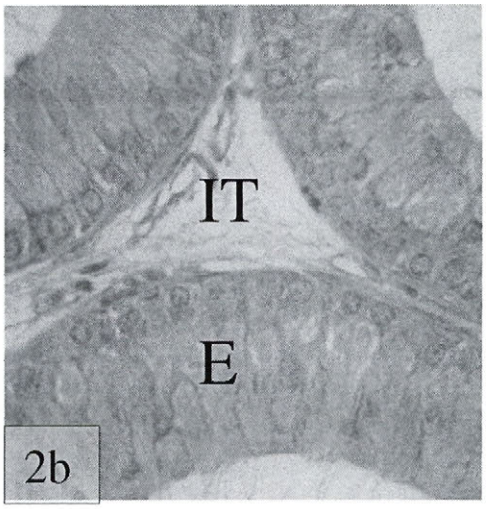
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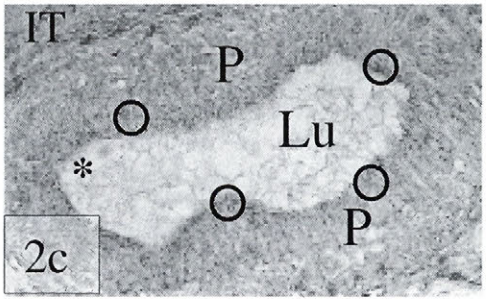
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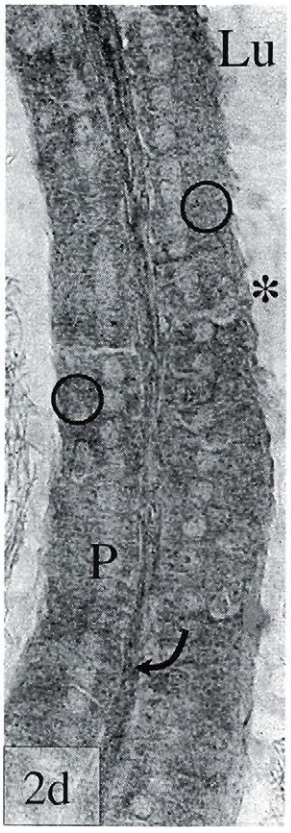
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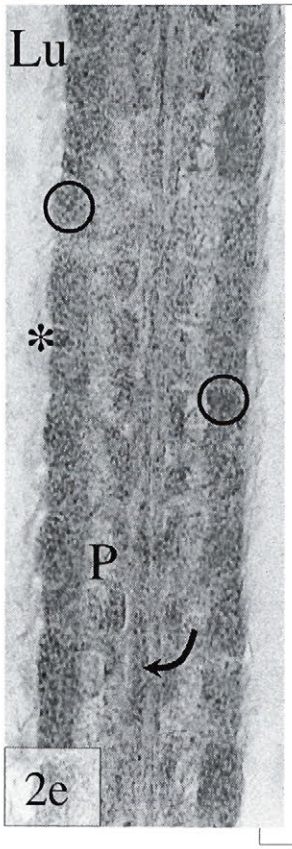
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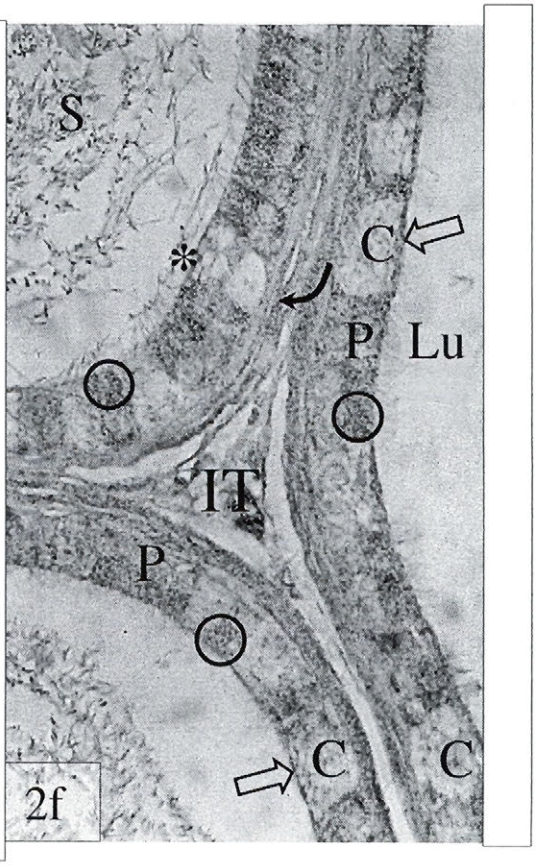
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SUMMARY

Aquaporins (AQPs) are transmembrane protein channels that allow the rapid passage of water across an epithelium at low energy requirement, though they also transport glycerol, urea and solutes of various sizes. At present 11 members of the AQP family of proteins have been described in mammals, with several being localized to the testis (AQP-7, 8), efferent ducts (AQP-1, 9) and epididymis (AQP-1, 9) of adult rats. With the discovery of expression of multiple AQPs in different tissues, we undertook a systematic analysis of several other members of the AQP family on Bouin-fixed tissues of the male reproductive tract employing light microscope immunocytochemistry. In the testis, AQP-0 expression in the seminiferous epithelium was restricted to Sertoli cells and to Leydig cells of the interstitial space; no reaction was observed in the efferent ducts or epididymis. In the case of Sertoli cells, a semicircular or pie-shaped pattern of staining was noted with only a quarter or half of the Sertoli cells of a given tubule showing a reaction product. Furthermore, while Sertoli cells at stages VI-VIII of the cycle showed intense staining, those at stages IX-XIV were least reactive, with Sertoli cells at stages I-V revealing intermediate levels of reaction product. The epithelial expression of AQP-10 was restricted to the microvilli of the nonciliated cells and cilia of ciliated cells of the efferent ducts, however, the endothelial cells of vascular channels of the efferent ducts and epididymis were also intensely reactive. AQP-3 expression was localized exclusively to the epididymis, where intense staining was noted exclusively over basal cells. Examination of orchidectomized rats revealed that AQP-3 expression was abolished over basal cells, and that it was greatly diminished after efferent duct ligation. As the reaction was not fully restored in orchidectomized animals supplemented with high levels of testosterone, it is suggested that AQP-3 expression in basal cells is regulated in part by testosterone, in addition to a luminal factor emanating from the testis. Taken together the data indicate a cell type and tissue specific expression for AQP-0, -3 and -10 in the testis, efferent ducts and epididymis, as well as differential regulating factors for the expression of AQP-3 in basal cells.

Estrogens play an important role in the male reproductive tract especially in the efferent ducts (EDs), where α-estrogen receptors (αER) have been localized. Mice

deficient in this receptor are infertile, and the effect appears to be due in part to retention of water at the level of the EDs. In the present study, we examined the role of the αER on immunolocalizations of aquaporins (AQPs) in the EDs and on epididymal sperm counts and sperm motility in adult wild type (WT) and α-estrogen receptor knock out (αERKO) mice. In addition, these studies were carried out with mice fed a regular lab chow diet, which contains phytoestrogens, versus a casein diet, which does not. Epithelial basolateral plasma membrane staining for AQP-1, prominent in WT mice, was dramatically reduced in αERKO mice. In addition, unlike the uniform homogeneous reactions seen on the microvilli of nonciliated cells of WT mice, staining for AQP-1 and -9 appeared patchy and uneven in αERKO mice, with distal areas of the EDs being more affected than proximal areas. Staining for AQP-10, present on the cilia of ciliated cells in WT mice, was reduced in αERKO, concomitant with patches of reactive microvilli of nonciliated cells, which were never reactive in WT mice, suggesting a compensatory role for AQP-10 due to the dramatic reduction in AQP-1 and -9 expression in the nonciliated cells. No additional changes in immunolocalizations were noted as a consequence of diet. Computer-assisted sperm analyses indicated that 62% fewer sperm were present in the cauda epididymidis of αERKO mice compared to WT mice fed a lab chow diet whereas 87% fewer sperm were found in αERKO mice fed casein. All sperm motility parameters were altered in αERKO mice fed lab chow including 31-51% decreases in the velocities of sperm movement, 2.7-fold increases in their beat cross frequencies, and 3.3-fold increases in the percentage of sperm that were static (unmoving). Alterations in sperm motility parameters were also present but were less dramatically different in αERKO mice compared to WT mice fed casein. This suggests that phytoestrogens, which are present in regular lab chow and can bind to βER, may be functioning to promote efferent duct and epididymal functions in the presence αER but to inhibit these functions in their absence, as happens in the case of αERKO mice fed lab chow. This underscores the importance of αER in preventing infertility.

Cystic fibrosis (CF) is the most common serious autosomal recessive condition in Caucasians and more than 95 % of CF men are infertile. The cystic fibrosis transmembrane conductance regulator (CFTR), a cAMP-regulated chloride channel has been localized in the efferent duct, however, its cell and region specific expression and

regulation in the epididymis by testicular factors have not been examined. In the present study, these parameters were examined immunocytochemically with the light microscope with an anti-CFTR antibody in Bouin-fixed, paraffin embedded control adult rat epididymidis and both orchidectomized (O) adult rats with or without testosterone (T) supplementation and efferent duct ligated rats (EDL) sacrificed at different time points. In control animals, a dense band of immunoperoxidase reaction product was visualized over the apical plasma membrane of principal cells, but not their microvilli, along with a diffuse weak apical cytoplasmic reaction. The apical band was prominent only in the corpus and cauda regions and no reaction was evident in clear or basal cells. Examination of O rats with or without T supplementation revealed that CFTR was no longer expressed as a dense band on the apical plasma membrane of principal cells of the corpus and cauda regions. However, under these conditions, an intense apical/supranuclear reaction was noted in the form of small vesicular structures. This data would suggest that the synthesis of small CFTR-containing vesicles from the Golgi apparatus is maintained and not regulated by testicular factors, but that their targeting to the apical plasma membrane is inhibited, resulting in their accumulation in the cytoplasm. Nevertheless, as EDL also revealed similar results, these data, taken together, suggest that a luminal factor derived from the testis regulates the targeting of CFTR-containing vesicles to the apical plasma membrane of principal cells. Thus, CFTR expression in principal cells of the epididymis appears to be region specific, and its targeting to the apical plasma membrane from de novo Golgi derived CFTR-vesicles appears to be influenced by a luminal testicular factor.

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