Immunolocalization, regulation and postnatal expression of aquaporins 1, 8 and 9 in the testis, efferent ducts and epididymis of adult rats.

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To my mother…

"How can I not love you when my
heart has seen you before my eyes."

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ACKNOWLEDGEMENTS

"It takes a village to raise a child" –African proverb.

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Contribution of Authors

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ABSTRACT

Aquaporins (AQPs) are membrane protein channels that allow rapid passage of water through an epithelium containing tight junctions. In the present study, light and electron microscope immunocytochemistry were utilized to localize several members of the aquaporin family in the testis, efferent ducts and epididymis of adult animals, animals at different postnatal ages and efferent duct ligated and orchidectomized animals with or without testosterone supplementation. In adult animals, AQP-8 expression was noted exclusively in the testis where it outlined Sertoli cells of the seminiferous epithelium. AQP-9 expression in the testis was seen to outline the periphery of Leydig cells but not cells of the seminiferous epithelium. AQP-9 was also mainly expressed in the apical cytoplasm of the nonciliated cells of the efferent ducts corresponding in the electron microscope to labelling of early endocytic structures. In the epididymis, AQP-9 was localized to the microvilli of principal cells of all regions with more intense reaction being noted in the initial segment and cauda regions. Clear cells of the cauda region only also expressed AQP-9. In the case of AQP-1, no expression was noted in the testis or epithelium of the epididymis. However, anti-AQP-1 antibody was intensely reactive over microvilli of the nonciliated cells of the efferent ducts and over endothelial cells of vascular channels of the efferent ducts and epididymis. These results indicate cell, region and subcellular specificity with respect to the distribution of aquaporin expression in cells of the male reproductive tract. After efferent duct ligation or orchidectomy up to day 21, there was no change in expression of AQP-1 or AQP-9 in the case of the efferent ducts or vascular channels. In contrast, AQP-9 expression on the microvilli of principal cells of the initial segment only and expression in the cytoplasm of clear cells of the cauda region

of the epididymis was dramatically reduced after orchidectomy or efferent duct ligation and not maintained by testosterone replacement suggesting a luminal factor(s) derived from the testis regulated its expression in these regions. These data also suggest that androgens were not essential for AQP-1 or AQP-9 expression in the efferent ducts or epididymis, and that there is region specificity with respect to factors regulating AQP-9 expression on the microvilli of principal cells of the epididymis. Postnatal studies revealed that expression of AQP-8 on Sertoli cells and AQP-9 on Leydig cells appeared by day 21. However, while AQP-1 was expressed on nonciliated cells of the efferent ducts by day 7, that for AQP-9 appeared by day 21; endothelial cells of vascular channels were already reactive by day 7. AQP-9 was expressed on microvilli of principal cells of all regions as seen in control adult animals by day 21, except in the initial segment which occurred only by day 29 as was also the case for clear cells of the cauda. These data suggest cell and region specific factors regulating expression of AQP-1 and AQP-9 during postnatal development and that these factors appear between day 7 and 29, eliminating sperm and high androgen levels as possible regulating factors. Taken together, these data suggest cell, region and subcellular specificity with respect to expression of AQP-1, -8, and –9 in the testis, efferent ducts and epididymis, and that their expression is regulated by a variety of different factors.

RESUME

Les « aquaporins » (AQP) sont des protéines qui forment des canaux dans les membranes des cellules pour accélerer le passage de l'eau a travers l'épithelium. La présente étude explique l'expression de plusieurs membres de la famille des « aquaporins » dans le testicule, les ductuli efferentes et l'épididyme du rat adulte, des animaux de différents âges après la naissance, des animaux châtres avec ou sans remplacement de testosterone, ainsi que des animaux dont les ductuli efferentes ont été ligaturés. Dans le cas des animaux adultes, l'expression de AQP-8 était notée exclusivement dans le testicule là ou elle délineait les cellules de Sertoli de l'épithelium. L'expression de AQP-9 dans le testicule délineait le périmètre des cellules de Leydig mais non des cellules de l'épithelium. AQP-9 était aussi présent dans le cytoplasme des cellules non-ciliés des ductuli efferentes correspondant aux structures endocytiques observées au microscope électronique. Dans l'épididyme, AQP-9 était localizé sur les microvillis des cellules principales de touts les régions de cet organ mais d'une manière plus intense dans le segment initial et le cauda epididymidis. Les cellules claires du cauda epididymidis seulement exprimaient aussi l'AQP-9. Dans le cas de AQP-1, aucune expression n'était notée ni dans le testicule ni dans l'épididyme. Cependant, l'anticorps contre l'AQP-1 a réagit fortement sur les microvillis des cellules non-ciliés des ductuli efferentes et sur les cellules endotheliales des canaux vasculaires des ductuli efferentes et de l'épididyme. Ces résultats indiquait une distribution régionale, cellulaire et subcellulaire spécifique dans tous les tissues etudiés du système reproductif male. Jusqu'à 21 jours aprs la ligature des ductuli efferentes ou castration, il n'y a eu aucun changement dans l'expression de AQP-1 ou AQP-9 dans le cas des ductuli efferentes ou

des canaux vasculaires. Par contrast, l'expression de AQP-9 sur les microvillis des cellules principales du segment initial seulement et l'expression dans le cytoplasme des cellules claires de la région caudale de l'épididyme a été réduite considerablement après castration ou ligature des ductuli efferentes. L'expression n'a pas été maintenu par le remplacement du testosterone, suggérant ainsi la régulation de ces « aquaporins » par un ou des facteurs luminals venants des testicules. Ces données suggèrent aussi que les androgynes n'étaient pas essentiels pour l'expression de AQP-1 ou AQP-9 dans les ductuli efferentes ou l'épididyme et qu'il existe une spécificité régionale en ce qui concerne les facteurs qui reglementent l'expression de AQP-9 sur les microvillis des cellules principales de l'épididyme. Les études entreprises sur les spécimens de differents âges suivant la naissance révèlent que l'expression de AQP-8 et AQP-9 sur les cellules de Sertoli et les cellules de Leydig respectivement, apparaissait au 21^{e} jour. Parcontre, pendant que AQP-1 s'est exprimé dans les cellules non-ciliés des ductuli efferentes au 7^{e} jour, l'expression de AQP-9 s'est demontré seulement au 21^{e} jour. Les cellules endotheliales des canaux vasculaires réagissaient déjà au 7^{e} jour. L'expression de AQP-9 a été observée sur les microvillis des cellules principales de toutes les régions de l'épididyme à une intensité comparable aux animaux adultes, le 21^{e} jour venu, excepté dans le segment initial, où l'expression s'est faite observer seulement au 29^{e} jour, de même pour l'expression dans les cellules claires de la région caudale de l'épididyme. Ces données suggèrent une spécificité cellulaire et régionale en ce qui concerne les facteurs qui reglementent l'expression de AQP-1 et AQP-9 suivant la naissance et que ces facteurs apparaissent entre 7 et 29 jours suivant la naissance, ce qui élimine la présence de sperm ainsi que des taux elevés d'androgynes en tant que facteurs régulateurs

possibles. Ensemble, toutes les données suggèrent une spécificité régionale, cellulaire et subcellulaire en ce qui concerne l'expression de AQP-1, -8 et –9 dans le testicule, les ductuli efferentes et l'épididyme et que leur expression est régulé par differents facteurs.

CHAPTER I

Literature Review

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 at al., 1969; Clark, 1976). Also present in these spaces are Leydig cells, mast cells (Nistal et al., 1984; Hermo & 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 types of cells: germ cells that evolve into the gametes and the supporting cells, 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 & 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 is 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 & Clermont, 1952). The lateral cell membranes of adjacent Sertoli cells form occluding junctions with each other (Flickinger & Fawcett, 1967; Russell & 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, 1993) 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 & 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, 1978) 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 & Einer-Jensen, 1974; Free & 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 & 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 and then 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 & 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 & Hall, 1965; Christensen & 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 & 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 & 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 & Perey, 1957; Clermont & Leblond, 1953; Steinberger & 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 appear at days 16 to 19 after parturition, which is after the cessation of Sertoli cell division and the onset of spermatogenesis (Connell, 1980; Pelletier & 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 & 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 & Jackson, 1972; Guttroff et al., 1992; Jones & Jurd, 1987; Lewis-Jones et al., 1982; Reid & Cleland, 1957).

The epithelium of the efferent ducts is composed of ciliated and nonciliated cells (Byers et al., 1985; Hermo & Morales, 1984; Hermo et al., 1988; Hamilton, 1975; Hess & Bassily, 1988; Hoffer & Greenberg, 1978; Jones & Jurd, 1987; Ramos & 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 & 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 & Hermo, 1988). Occasionally, these apical tubules are seen connected to endosomes in the apical cytoplasm of the cells (Wrobel, 1972; Robaire & Hermo, 1988). Below these endosomes, multivesicular bodies are present and in the supranuclear region, lysosomes can be seen (Robaire & 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 & 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 & Jones, 1983; Howards et al., 1975; Jones, 1981; Jones & Jurd, 1987; Levine & 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., 1973; Flickinger et al., 1978). Others however propose that movement of fluid across the epithelium is coupled to the active transport of electrolytes (Hamilton, 1975; Hohlbrugger, 1980; Jones & Jurd, 1987). 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 energy-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 on 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 & 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 & Kormano, 1975; Olson & 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 possibly 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 the 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 & Graves, 1977; Free & Jaffe, 1979) and estrogen receptors have been reported in the male reproductive tract of several species (Danzo & Eller, 1979; Murphy et al., 1980; Younes & Pierrepoint, 1981; Schleicher et al., 1984; Tekpetey & Amann, 1988; Toney & Danzo, 1988; West & 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 of 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 & Hermo, 1988).

Anatomy of the duct

The epididymis is a highly convoluted single 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 & 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 & 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 junctions between principal cells (Suzuki & Nagano, 1978; Greenberg & Forssmann, 1983; Hoffer & 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 & Gustafsson, 1964; Jenkins 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 & 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 & Cone, 1983; Turner & Giles, 1982; Carr & 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 & 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 & Jahct, 1978) and sperm motility (Turner & 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 & 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 the 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 & Graham, 1990). The sodium gradient created then draws in 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 produced 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 & Hoffer, 1979). These factors are probably secreted by the Sertoli cells (Robaire & Ewing, unpublished observations). Water resorption has been shown to be dependant not only on androgens (Wong & Yeung, 1977) but also aldosterone (Au et al., 1978; Turner & Cesarini, 1983) and affected by a number of diuretics (Jenkins et al., 1983; Wong & 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 segment 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).

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. 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, it 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 the aquaporin family possess 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 & Saier Jr, 1996; King and Agre, 1996) suggesting an ancient internal gene duplication (Park & Saier, 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 12q13 and AQPs 3 and 7 have been located at chromosome 9p13 (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 extensivley 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-spanning domains (King and Agre, 1996; Preston & 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 & 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 & 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 Å in diameter (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

independantly functional pores (Verkman et al, 1996) associate non-covalently in the membranes 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 & Goodenough, 1996).

Tissue distribution of the known members

So far, 10 aquaporins (AQP-0 to AQP-9) 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 there is thought to maintain the desiccation and transparency of the lens (Nielsen et al 1993). First identified from erythrocyte 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 choroid 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 & 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 thought 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 selectivity of the pores. All but 2 of the aquaporins (AQP-4 and AQP-7) are inhibited by mercurial compounds (King & 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 disease 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 & Agre, 1996; Shiels & 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 & Agre, 1996). These 5 (Colton-null) persons are virtually AQP-1 knockouts. Surprisingly, these individuals present no overt pathological phenotype (King & Agre, 1996). Whether other water channels compensate for the lack of AQP-1 is at present unknown. Interestingly, the 5 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 & Mitra, 2000).

Mutations in AQP-2 cause a severe 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 & Agre, 1996). AQP-3 null mice, as in AQP-1 knockouts also display difficulty in concentrating their urine (Verkman & Mitra, 2000). The lack of AQP-4 may be implicated in pseudotumor cerebri which is believed to result from abnormal CSF resorption (King & 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 & Mitra, 2000). Aquaporin activators or aquaporin gene replacement might be useful in glandular hypofunction such as in Sjogren's syndrome and in water diuretic states (Verkman & 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.

Present Study

Aquaporins have been localized in many cell types of the body. However, little has been done to characterize their localization and regulation in the male reproductive tract. The purpose of this study was to localize members of the aquaporin family in the testis, efferent ducts and epididymis of adult rats using immunocytochemistry for light

and electron microscopy. Regulation of these proteins in the different tissues as well as hormonal regulation were also studied. Finally, postnatal expression of the channels was determined to correlate their expression with known events taking place during development.

REFERENCES

Agre P, Preston GM, Smith BL, Jung JS, Raina S, Moon C, Guggino WB, Nielsen S. (1993) Aquaporin CHIP: the archetypal molecular water channel. *Am. J. Physiol.* 34: F463-F476.

Alumot E, Lensky Y, Schindler H. (1971) Separation of proteins in the epididymal fluid of the ram. *J. Reprod. Fertil.* 25: 349-353.

Amann RP, Johnson L, Pickett BW. (1977) Connection between the seminiferous tubules and efferent ducts in the stallion. *Am. J. Vet. Res.* 38: 1571-1579.

Andonian S, Hermo L. (1999) Principal cells of the vas deferens are involved in water transport and steroid synthesis in the adult rat. *J. Androl.* 20: 158-176.

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

Bai L, Fushimi K, Sasaki S, Marumo F. (1996) Structure of aquaporin-2 vasopressin warter channel. *J. Biol. Chem.* 271: 5171-5176.

Bardin CW, Cheng CY, Musto NA, Gunsalus GL. (1988) The Sertoli cell. *In*: The physiology of reproduction, edited by E Knobil & JD Neill, pp. 933-974. Raven Press, Ltd., New York.

Baumgarten R, van de Pol MH, Wetzels JF, van Os CH, Deen PM. (1998) Glycosylation is not essential for vasopressin-dependant routing of aquaporin-2 in transfected Madin-Darby canine kidney cells. *J. Am. Soc. Nephrol.* 9: 1553-1559.

Brown D, Katsura T, Gustafson CE. (1998) Cellular mechanisms of aquaporin trafficking. *Am. J. Physiol. Renal Physiol.* 44: F328-F331.

Cooper TG. (1995) Role of the epidiymis in mediating changes in the male gamete during maturation. *In*: Tissue Renin-Angiotensin Systems, edited by AK Mukhopadhyay and MK Raizada, pp. 87-101. Plenum Press, New York.

Crabo B (1965) Studies on the composition of epididymal content in bulls and boars. *Acta. Vet. Scand.* 6: 8-94.

Crabo B, Gustafsson B. (1964) Distribution of sodium & potassium and its relation to sperm concentration in the epididymal plasma of the bull. *J. Reprod. Fertil.* 7: 337-345.

Danzo BJ, Eller BC. (1979) The presence of a cytoplasmic estrogen receptor in sexually mature rabbit epididymides: comparison with the estrogen receptor in immature rabbit epididymal cytosol. *Endocrinol.* 105: 1128-1134.

Deen PMT, Croes H, van Aubel RAMH, Ginsel LA, van Os CH. (1995) Water channels encoded by mutant aquaporin-2 genes in nephrogenic diabetes insipidus are impaired in their cellular routing. *J. Clin. Invest.* 95: 2291-2296.

Deen PMT, van OS CH. (1998) Epithelial aquaporins. *Curr. Opin. Cell. Biol.* 10: 435-442.

DeJong FN, Hey AH, Van der Molen HJ. (1974) Estradiol & testosterone in rat testis tissue: localization and production in vitro. *J. endocrinol.* 60:409.

De Kretser DM, Kerr JB. (1988) The cytology of the testis. In: The physiology of reproduction, edited by E Knobil & JD Neill, pp. 837-932. Raven Press, Ltd., New York.

Diamond JM and Tormey McD. (1966) Studies on the structural basis of water transport across epithelial membranes. *Fed. Proc.* 25: 1458-1463.

DiGiovanni SR, Nielsen S, Christensen EI, Knepper MA. (1994) Regulation of collecting duct water channel expression by vasopressin in Brattleboro rat. *Proc. Natl. Acad. Sci. USA.* 91: 8984-8988.

Djakiew D, Jones RC. (1983) Sperm maturation, fluid transport, and secretion and absorption of protein in the epididymis of the echidna, *Tachyglossus aculeatus* and Wistar rat. *Aust. J. Zool.* 30: 865-875.

Dorrington JH, Armstrong DT. (1975) FSH stimulates estradiol-17ß synthesis in cultured Sertoli cells. *Proc. Natl. Acad. Sci. USA.* 72: 2677.

Dym M. (1973) The fine structure of the monkey (Mecaca) Sertoli cell and its role in maintaining the blook testis barrier. *Anat. Rec.* 175: 639-656.

Dym M, Fawcett DW. (1970) The blood-testis barrier in the rat and the physiological compartmentation of the seminiferous epithelium. *Biol. Reprod.* 3: 308-326.

Echevarria M, Ilundain AA. (1998) Aquaporins. *J. Physiol. Biochem.* 54: 107-118.

Echevarria M, Windhager EE, Frindt G. (1996) Selectivity of the renal collecting duct water channel aquaporin-3. *J. Biol. Chem.* 271: 25079-25082.

Echevarria M, Windhager EE, Tate SS, Frindt G. (1994) Cloning and expression of AQP3, a water channel from the medullary collecting duct of rat kidney. *Proc. Natl. Acad. Sci. USA.* 91: 10997-11001.

Eik-Nes KB, Hall PF. (1965) Secretion of steroid hormones in vivo. *Vitam. Horm.* 23:153.

Eiler H, Graves C. (1977) Oestrogen content of semen and the effect of exogenous estradiol-17α on the oestrogen and androgen concentration in semen and blood plasma of bulls. *J Reprod. Fertil.* 50: 17-21.

Fawcett DW. (1973) Observation on the organization of the interstitial tissue of the testis and on the occluding cell junctions in the seminiferous epithelium. *Adv Biosci.* 10: 83-99.

Fawcett DW. (1975) Ultrastructure and function of the Sertoli cell. *In:* Handbook of physiology, edited by DW Hamilton and RO Greep, Section 7, Endocrinology, Vol. 5, Male Reproductive System, pp. 21-55. William and Wilkins, Baltimore.

Fawcett DW. (1977) The ultrastructure and functions of the Sertoli cell. *In* Frontiers in reproduction and fertility control, edited by RO Green and MA Koblinsky, pp. 302-320. M.I.T. Press, Cambridge, Mass.

Fawcett DW, Heidger PJ, and Leak LV. (1969) The lymph vascular system of the interstitial tissue of the testis as revealed by electron microscopy. *J. Reprod. Fert.* 19: 109-119.

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

Fawcett DW, Leak LV, and Heidger PM. (1970) Electron microscopial observations on the structural components of the blood-testis barrier. *J. Reprod. Fert.* 10: 105-122.

Fisher JS, Turner KJ, Fraser HM, Saunders PTK, Brown D, Sharpe RM. (1998) Immunoexpression of aquaporin-1 in the efferent ducts of the rat and marmoset monkey

during development, its modulation by estrogens, and its possible role in fluid resorption. *Endocrinol.* 139: 3935-3945.

Flickinger CJ and Fawcett DW (1967) The junctional specializations of Sertoli cells in the seminiferous epithelium. *Anat. Rec.* 158: 207-222.

Flickinger JA, Howards SS, English HF. (1978) Ultrastructural differences in the efferent ducts and several regions of the epididymis of the hamster. *Am. J. Anat.* 152: 557-586.

Free MJ, Jaffee RA. (1979) Collection of rete testis fluid from rats without previous efferent duct ligation. *Biol. Reprod.* 20: 269-274.

Freeman DA, Ascoli M. (1983) The LDL pathway of cultured Leydig tumor cells. *Biochem. Biophys. Acta.* 754: 72.

Fushimi K, Uchida S, Hara Y, Hirata Y, Marumo F, Sasaki S. (1993) Cloning and expression of apical membrane water channel of rat kidney collecting tubule. *Nature.* 361: 549-552.

Goyal HO, Bartol FF, Wiley AA, Neff CW. (1997) Immunolocalization of receptors for androgen and estrogen in male caprine reproductive tissues: unique distribution of estrogen receptors in efferent ductile epithelium. *Biol. Reprod.* 56: 90-101.

Greenberg J, Forssmann WG. (1983) Studies of the guinea pig epididymis. II. Intercellular junctions of principal cells. *Anat. Embryol.* 168: 195-209.

Griswold MD. (1993) Protein secretion by Sertoli cells: General considerations. In: The Sertoli cell, edited by LD Russell & MD Griswold, pp. 195-200. Cache River Press, Clearwater, FL.

Guttroff RF, Cooke PS, Hess RA. (1992) Blind-ending tubules and branching patterns of the rat ductuli efferentes. *Anat. Rec.* 232: 423-431.

Hall PF. (1963) The effect of interstitial cell-stimulating hormone on the biosynthesis of testicular cholesterol from acetate-1-^{14}C. *Biochem.* 2: 1232.

Hall PF. (1970) Endocrinology of the testis. *In*: Testicular Physiology & biochemistry, edited by AD Johnson, WR Gomes, VL van Denmark, Vol. 2, p 1. Academic Press, New York.

Hall PF. (1979) Testicular hormones: synthesis & control. *In*: Endocrinology, edited by DeCroot et al., Vol. 3, p. 1511. Grure & Stratton, New York.

Hall PF. (1988) Testicular steroid synthesis: Organization and regulation. *In*: The physiology of reproduction, edited by E Knobil & JD Neill, pp. 975-998. Raven Press, Ltd., New York.

Hamilton DW. (1975) Structure and function of the epithelium lining the ductuli efferentes, ductus epididymidis, and ductus deferens in the rat. *In*: Handbook of physiology, section 7: Endocrinology, Vol. 5, pp. 259-300. The male reproductive system, edited by DW Hamilton and RO Greep. American Physiology Society. Washington.

Hamilton DW, Olson GE, Cooper TG. (1977) Regional variation in the surface morphology of the epithelium of the rat ductuli efferentes, ductus epididymidis and vas deferens. *Anat. Rec.* 188: 13-28.

Hemeida NA, Sack WO, McEntee K. (1978) Ductuli efferentes in the epididymis of boar, goat, ram, bull and stallion. *Am. J. Vet. Res.* 39: 1892-1900.

Hermo L, Barin K, Robaire B. (1992) Structural differentiation of the epithelial cells of the testicular excurrent duct system of rats during postnatal development. *Anat. Rec.* 233: 205-228.

Hermo L, Clermont Y, Morales C. (1985) Fluid-phase and adsorptive endocytosis in ciliated epithelial cells of the rat ductuli efferentes. *Anat. Rec.* 211: 285-294.

Hermo L and Lalli M. (1978) Moncytes and mast cells in the limiting membrane of human seminiferous tubules. *Biol. Reprod.* 19: 92-100.

Hermo L, Morales C. (1984) Endocytosis in nonciliated epithelial cells of the ductuli efferentes in the rat. *Am. J. Anat.* 171: 59-74.

Hermo L, Oko R, Morales C. (1994) Secretion and endocytosis in the male reproductive tract: A role in sperm maturation. *Int. Rev. Cyto.* 154: 105-189.

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

Hermo L, Spier N, Nadler NJ. (1988) Role of apical tubules in endocytosis in nonciliated cells of the ductuli efferentes of the rat: a kinetic analysis. *Am. J. Anat.* 182: 107-119.

Hess RA, Bassilly N. (1988) Structure of the ductuli efferentes in the dog. *J. Vet. Med. C.* 17: 85 (abstract).

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

Hess RA, Gist DH, Bunick D, Lubahn DB, Farrell A, Bahr J, Cooke PS, Greene GL. (1997) Estrogen receptor (α and β) expression in the excurrent ducts of the adult male rat reproductive tract. *J. Androl.* 18: 602-611.

Hinton BT, Palladino MA. (1995) Epididymal epithelium: Its contribution to the formation of a luminal fluid microenvironment. *Micros. Res. Tech.* 30: 67-81.

Hinton BT, Turner TT. (1988) Is the epididymis a kidney analogue? *News Physiol. Sci.* 3: 28-31.

Hoffer AP. (1972) the fine structure of the ductuli efferentes in mouse and rat. *Anat. Rec.* 172: 331-332.

Hoffer AP, Greenberg J. (1978) The structure of the epididymis, efferent ductules and ductus deferens of the guinea pig: A light microscope study. *Anat. Rec.* 190: 659-678.

Hoffer AP, Hamilton DW, Fawcett DW. (1973) The ultrastructural pathology of the rat epididymis after administration of α-chlorhydrin (U-5897). *Anat. Rec.* 175: 203-230.

Hoffer AP, Hinton BT. (1984) Morphological evidence for a blood-epididymis barrier and the effects of gossypol on its integrity. *Biol. Reprod.* 30: 991-1004.

Hohlbrugger G. (1980) A micropuncture study of transepithelial water reabsorption in the rat ductuli efferentes. *Fertil. Steril.* 34: 50-54.

Howards SS, Johnson A, Jessee S. (1975) Micropuncture and microanalytic studies of the rat testis and epididymis. *Fertil. Steril.* 26: 13-19.

Iguchi T, Uesugi Y, Sato T, Ohta Y, Takasugi N. (1991) Developmental pattern of estrogen receptor expression in male mouse genital organs. *Mol. Androl.* 6: 109-119.

Ilio KY, Hess RA. (1992) Localization and activity of NA^+, K^+-ATPase in the ductuli efferentes of the rat. *Anat. Rec.* 234: 190-200.

Ilio KY, Hess RA. (1994) Structure and function of the ductuli efferentes: A review. *Micros. Res. Tech.* 29: 432-467.

Inase N, Fushimi K, Ishibashi K, Uchida S, Ichioka M, Sasaki S, Marumo F. (1995) Isolation of human aquaporin 3 gene. *J. Biol. Chem.* 270: 17913-17916.

Ishibashi K, Kuwahara M, Gu Y, Kageyama Y, Tohsaka F, Sugaki F, Marumo F, Sasaki S. (1997) Cloning and functional expression of a new water channel abundantly expressed in the testis permeable to water, glycerol, and urea. *J. Biol. Chem.* 272: 20782-20786.

Ishibashi K, Kuwahara M, Gu Y, Tanaka Y, Marumo F, Sasaki S. (1998) Cloning and functional expression of a new aquaporin (AQP9) abundantly expressed in the peripheral leukocytes permeable to water and urea, but not to glycerol. *Biochem. Biophys. Res. Comm.* 244: 268-274.

Ishibashi K, Kuwahara M, Kageyama Y, Tohsaka A, Marumo F, Sasaki S. (1997) Cloning and functional expression of a second new aquaporin abundantly expressed in testis. *Biochem. Biophys. Res. Comm.* 237: 714-718.

Ishibashi K, Sasaki S, Fushimi K, Uchida S, Kuwahara M, Saito H, Furukawa T, Nakajima K, Yamaguchi Y, Gojobori T, Marumo F. (1994) Molecular cloning and expression of a member of the aquaporin family with permeability to glycerol and urea in addition to water expressed at the basolateral membrane of kidney collecting duct cells. *Proc. Natl. Acad. Sci. USA.* 91: 6269-6273.

Ishibashi K, Sasaki S, Saito F, Ikeuchi T, Marumo F. (1995) Structure and chromosomal localization of a human water channel (AQP3) gene. *Genomics.* 30: 633.

Ishibashi K, Yamauchi K, Kageyama Y, Saito-Ohara F, Ikeuchi T, Marumo F, Sasaki S. (1998) Molecular characterization of human aquaporin-7 gene and its chromosomal mapping. *Biochim. Biophys. Acta.* 1399: 62-66.

Jenkins AD, Lechne CP, Howards SS. (1980) Concentrations of seven elements in the intraluminal fluids of the rat seminiferous tubules, rete testis, and epididymis. *Biol. Reprod.* 23: 981-987.

Jenkins AD, Lechene CP, Howards SS. (1983) Effect of spironolactone on the elemental composition of the intraluminal fluids of the seminiferous tubules, rete testis and epididymis of rat. *J. Urol.* 129: 851-854.

Jiang JX, Goodenough DA. (1996) Heteromeric connexons in lens gap junction channels. *Proc. Natl. Acad. Sci. USA.* 93: 1287-1291.

Johnson AD. (1979) Testicular Lipids. *In*: The testis, edited by AD Johnson, WR Gomes, VL van Demark, Vol. 2, p. 193. Academic Press, New York.

Jones RC. (1987) Changes in protein composition of the luminal fluids along the epididymis of the tammar, *Macropus eugenii. J. Reprod. Fertil.* 89: 193-199.

Jones RC, Jurd KM. (1987) Structural differentiation and fluid reabsorption in the ductuli efferentes of the rat. *Aust. J. Biol. Sci.* 40: 79-90.

Jung JS, Preston GM, Smith BL, Guggino WB, Agre P. (1994) Molecular structure of the water channel through aquaporin CHIP. The hourglass model. *J. Biol. Chem.* 269: 14648-14654.

Katsura T, Ausiello DA, Brown D. (1996) Direct demonstration of aquaporin-2 water channel recycling in stably transfected LLC-PK_1 epithelial cells. *Am. J. Physiol.* 270: F548-F553.

Kerr JB, Rich KA, DeKrester DM. (1979) Alteration of the fine structure and androgen secretion of the interstitial cells in the experimentally cryptorchid rat testis. *Biol. Reprod.* 20: 409-422.

King LS, Agre P. (1996) Pathophysiology of the aquaporin water channels. *Ann. Rev. Physiol.* 58: 619-648.

Kirchoff C. (1999) Gene expression in the epididymis. *Int. Rev. Cytol.* 188:133-202.

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

Kuriyama H, Kawamoto S, Ishida N, Ohno I, Mita S, Matsuzawa Y, Matsubara K, Okubo K. (1997) Molecular cloning and expression of a novel human aquaporin from adipose tissue with glycerol permeability. *Biochem. Biophys. Res. Comm.* 241: 53-58.

Kuwahara M, Gu Y, Ishibashi K, Marumo F, Sasaki S. (1997) Mercury-sensitive residues and pore site in AQP3 water channel. *Biochem.* 36: 13973-13978.

Lanahan A, Williams JB, Landers LK, Nathans D. (1992) Growth factor-induced delayed early response genes. *Mol. Cell Biol.* 12: 3919-3929.

Leblond CP and Clermont Y. (1932) Definition of the stages of the cycle of the seminiferous epithelium in the rat. *Ann. NY Acad. Sci.* 55: 548-573.

Lee MD, Bhakta KY, Raina S, Yonescu R, Griffin CA, Copeland NG, Gilbert DJ, Jenkins NA, Preston GM, Agre P. (1996) The human *aquaporin-5* gene: Molecular characterization and chromosomal localization. *J. Biol. Chem.* 271: 8599-8604.

Lee MD, King LS, Agre P. (1997) The aquaporin family of water channel proteins in clinical medicine. *Medicine*. 76, 141-156.

Levine N, Marsh DJ. (1971) Micropuncture studies of the electrolyte aspects of fluid and electrolyte transport in individual seminiferous tubules, in the epididymis and the vas deferens in rats. *J. Physiol.* 213: 557-579.

Lewis-Jones DI, Harrison RG, MacMillan EW. (1982) A re-examination of rat ductuli efferentes. *Anat. Rec.* 203: 461-462.

Li H, Lee S, Jap BK. (1997) Molecular design of aquaporin-1 water channel as revealed by electron crystallography. *Nat. Struct. Biol.* 4: 263-265.

Lu M, Lee MD, Smith BL, Jung JS, Agre P, Verdijk MAJ, Merkx G, Rijss JPL, Deen PMT. (1996) The human *AQP4* gene: Definition of the locus encoding two water channel polypeptides in brain. *Proc. Natl. Acad. Sci. USA*. 93: 10908-10912.

Ma T, Frigeri A, Hasegawa H, Verkman AS. (1994) Cloning of a water channel homolog expressed in brain meningeal cells and kidney collecting duct that functions as a stilbene-sensitive glycerol transporter. *J. Biol. Chem.* 269: 21845-21849.

Ma T, Yang B, Kuo W-L, Verkman AS. (1996) cDNA cloning and gene structure of a novel water channel expressed exclusively in human kidney: Evidence for a gene cluster of aquaporins at chromosome locus 12q13. *Genomics*. 35: 543-550.

Mason KE, Shaver SL. (1952) Some functions of the caput epididymidis. *Ann. N.Y. Acad. Sci.* 55: 585-593.

Meinild AK, Klaerke DA, Zeuthen T. (1998) Bi-directional water fluxes and specificity for small hydrophilic molecules in aquaporins 0-5. *J. Biol. Chem.* 273: 32446-32451.

Miller SC, Bowman BM, and Roberts LK. (1984) Identification and characterization of mononuclear phagocytes isolated from rat testicular interstitial tissues. *J. Leucocyt. Biol.* 36: 679-687.

Mitra AK, van Hoek AN, Wiener MC, Verkman AS, Yeager M. (1995) The CHIP28 water channel visualized in ice by electron crystallography. *Nat. Struct. Biol.* 2: 726-729.

Montorzi NM, Labiano, SA. (1970) Uptake of water, sodium and glucose by the ductuli efferentes of the hamster in vitro. *Acta. Physiol. Norte Amer.* 20: 47-51.

Morales C, Hermo L. (1983) Demonstration of fluid-phase endocytosis in epithelial cells of the male reproductive system by means of horseradish peroxidase-colloidal gold complex. *Cell Tissue Res.* 230: 503-510.

Mulders SM, Olde Weghuis D, van Boxtel JAF, Guerst van kessel A, Echevarria M, van Os CH, Deen PMT. (1996) Localization of the human gene for aquaporin 3 (AQP3) to chromosome 9, region p21æp12, using fluorescent in situ hybridization. *Cytogenet. Cell Genet.* 72: 303-305.

Murphy JB, Emmott RC, Hicks LL, Watsh PC. (1980) Estrogen receptors in the human prostate, seminal vesicle, epididymis, testis, and genital skin: a marker for estrogen-resonsive tissues? *J. Clin. Endocrinol Metab.* 50: 938-948.

Nicorder L. (1967) An electron microscopial study of cell contacts in the seminiferous tubules of some animals. *Zeitsch fur Zellforsch.* 83: 375-397.

Nielsen S, Chou CL, Marples D, Christensen EI, Kishore BK, Knepper MA. (1995) Vasopressin increases water permeability of kidney collecting duct by inducing translocation of aquaporin-CD water channels to plasma membrane. *Proc. Natl. Acad. Sci. USA*. 92: 1013-1017.

Nielsen S, Pallone T, Smith BL, Christensen EL, Agre P, Maunsbach AB. (1995) Aquaporin-1 water channels in short and long loop descending thin limbs and in descending vasa recta in rat kidney. *Am. J. Physiol.* 268: F1023-F1037.

Nielsen S, Smith BL, Christensen EI, Agre P. (1993) Distribution of the aquaporin CHIP in secretory and resorptive epithelia and capillary endothelia. *Proc. Natl. Acad. Sci. USA*. 90: 7275-7279.

Niemi M, Sharpe RM and Brown WRA. (1986) Macrophages in the interstitial tissue of te rat testis. *Cell Tiss. Res.* 243: 337-344.

Nistal M, Paniagua R. (1984) Development of the male genital tract. *In*: Testicular and epididymal pathology, edited by M Nistal & R Paniagua, pp. 1-13. Thieme-Stratton, New York.

Nistal M, Santamaria L, and Paniagua R. (1984) Mast cells in the human testis and epididymis from birth to adulthood. *Acta Anat.* 119: 155-160.

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

Orgebin-Crist M-C. (1996) Androgens and epididymal function. *In*: Pharmacology, Biology, and Clinical Applications of Androgens, edited by S Bhasin et al., Wiley- Liss.

Orgebin-Crist M-C, Danzo BJ, Davies J. (1975) Endcrine control of the development and maintenance of sperm fertilizing ability in the epididymis. *In* Handbook of physiology, Section 7. Endocrinology, Vol. 5. pp. 319-338. The male reproductive system.edited by Hamilton DW, and Greep RO. American Physiological Society, Washington DC.

Orgebin-Crist M-C, Jahad N. (1978) The maturation of rabbit epididymal spermatozoa in organ culture: Inhibition by antiandrogens and inhibitors of ribonucleic acid and protein synthesis. *Endocrinol.* 103: 46-53.

Orgebin-Crist M-C, Olson GE. (1984) Epididymal sperm maturation. *In*: The male in farm animal reproduction, edited by M Courot, pp. 80-102. Martines Nijhoff, Amsterdam.

Parisi M, Ibarra C. (1996) Aquaporins and water transfer across epithelial barriers. *Braz. J. Med. Biol. Res.* 29: 933-939.

Park JH, Saier MH Jr. (1996) Phylogenetic characterization of the MIP family of transmembrane channel proteins. *J. Membrane Biol.* 153: 171-180.

Pelletier RM, Friend DS, and Aoki A. (1976) The Sertoli cell occluding junction and gap junction in mature and developing mammalian tstis. *Dev. Biol.* 50: 142-168.

Preston GM, Agre P. (1991) Molecular cloning of the red cell integral protein of Mr 28,000: A member of an ancient channel family. *Proc. Natl. Acad. Sci. USA.* 88: 11110-11114.

Preston GM, Jung JS, Guggino WB, Agre P. (1993) The mercury-sensitive residue at cysteine-189 in the CHIP28 water channel. *J. Biol. Chem.* 268: 17-20.

Preston GM, Jung JS, Guggino WB, Agre P. (1994) Membrane topology of aquaporin CHIP: analysis of function epitope-scanning mutants by a vectorial proteolysis. *J. Biol. Chem.* 269: 1668-1673.

Ramos AS Jr, Dym M. (1977) Ultrastructure of the ductuli efferentes in monkeys. *Biol. Reprod.* 17: 339-349.

Reid BL, Cleland KW. (1957) The structure and function of the epididymis. I. The histology of the rat epididymis. *Aust. J. Zool.* 5: 223-246.

Ren G, Melnyk P, Mitra AK. (1999) Structural analysis of the binding of a mercurial agent to AQP1 by electron cryocrystallography (abstract). *Biophys.* J. 76: 137.

Robaire B, Hermo L. (1988) Efferent ducts, epididymis, and vas deferens: Structure, functions, and their regulation. *In*: The physiology of reproduction, edited by E Knobil and J Meill, pp. 999-1080. Raven Press, New York.

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

Ross MH. (1976) The Sertoli cell junctional specialization during spermiogenesis and at spermiation. *Anat. Rec.* 186: 79-104.

Russell LD (1978) The blood-testis barrier and its formation relative to spermatocyte maturation in the adult rat: a lanthanum tracer study. *Anat. Rec.* 190: 99-112.

Russell LD, Peterson RN. (1985) Sertoli cell junctions: morphological & functional consideration. *Int. Dev. Cytol.* 94: 177-211.

Sasaki S, Fushimi K, Saito F et al. (1994) Cloning, characterization and chromosomal mapping of human aquaporin of collecting duct. *J. Clin. Invest.* 93: 1250-1256.

Schleicher G, Drews U, Stumpf WE, Sar M. (1984) Differential distribution of dihydrotestosterone and estradiol binding sites in the epididymis of the mouse. *Histochem.* 81: 139-147.

Schulze C. (1974) On the morphlogy of the human Sertoli cell. *Cell Tiss. Res.* 153: 339-355.

Setchell BP. (1970) Testicular blood supply, lymphatic drainage, and secretion of fluid. *In* The Testis. (Johnson AD, Gomes WR, and Vandemark NL, editors.) Vol. 1. New York: Academic Press. 101-239.

Setchell BP, Brooks DE. (1988) Anatomy, vasculature, innervation, and fluids of the male reproductive tract. *In*: The physiology of reproduction, edited by E Knobil & JD Neill, pp. 753-836. Raven Press, Ltd., New York.

Setchell, BP, Scott TW, Volgemayr JK, and Waits GMH. (1969) Characteristics of testicular spermatozoa and the fluid which transports them into the epididymis. *Biol. Reprod. Suppl.* 1: 40-66.

Setchell BP, Waites GMH. (1975) The blood-testis barrier. *In* Handbook of Physiology, edited by DW Hamilton and RO Greep, Section 7, Vol. 5, pp.143-172. William and Wlikins, Baltimore.

Shi LB, Skach WR, Ma T, Verkman AS. (1995) Distinct biogenesis mechanisms for water channels MIWC and CHIP28 at the endoplasmic reticulum. *Biochem.* 34: 8250-8256.

Shi LB, Verkman AS. (1996) Selected cysteine point mutations confer mercurial sensitivity to the mercurial-insensitive water channel MIWC. *Biochem.* 35: 538-544.

Shiels A, Basnetts S. (1996) Mutations in the founder of the MIP gene family underlie cataract development in the mouse. *Nature Genetics.* 12: 212-215.

Steinberger A and Steinberger E. (1977) The Sertoli Cell. *In* The Testis, edited by AD Johnson and WK Gomes, Vol. 4, pp. 371-399. Academic Press, New York.

Suzuki F, Nagano T. (1978) Regional differentiation of cell generation in the excurrent duct epithelium of the rat testis as revealed by freeze-fracture. *Anat. Rec.* 191: 503-520.

Tcholakian RK, Steinberger A. (1979) In vitro metabolism of testosterone by Sertoli cells & interstitial cells. *In*: Testicular development; structure & function, edited by A Steinberger & E Steinberger, p. 177, Raven Press, New York.

Tekpetey FR, Amann RP. (1988) Regional and seasonal differences in concentrations of androgen and estrogen receptors in ram epididymal tissue. *Biol. Reprod.* 38: 1051-1060.

Tindall DJ, Vitale R, Means AR. (1975) Androgen binding protein as a biochemical marker for formation of the blood-testis barrier. *Endocrinol.* 97: 636-648.

Toney TW, Danzo BJ. (1988) Developmental changes in and hormonal regulation of estrogen and androgen receptors present in the rabbit epididymis. *Biol. Reprod.* 39: 818-828.

Tsukaguchi H, Shayakul C, Berger UV, Mackenzie B, Devidas S, Guggino WB, van Hoek AN, Hediger MA. (1998) Molecular characterization of a broad selectivity neutral solute channel. *J. Biol. Chem.* 273: 24737-24743.

Tsukaguchi H, Weremowicz S, Morton CC, Hediger MA. (1999) Functional and molecular characterization of the human neutral solute channel aquaporin-9. *Am. J. Physiol.* 277: F685-F696.

Turner T. (1979) On the epididymis and its function. *Invest. Urol.* 16: 311-321.

Turner T. (1984) Resorption versus secretion in the epididymis. *J. Reprod. Fertil.* 72: 509-514.

Turner T, Cesarini DM (1983) The ability of the rat epididymis to concentrate spermatozoa. Responsiveness to aldosterone. *J. Androl.* 4: 197-202.

Turner TT, Giles RD. (1982) Sperm motility-inhiniting factor in rat epididymis. *Am. J. Physiol.* 242: R199-R203.

Turner TT, Jones CE, Howards SS, Ewing LL, Zegeye B, Gonsales GL. (1984) On the androgen microenvironment of maturing spermatozoa. *Endocrinol.* 115: 1925-1932.

Turner TT, Plesums JL, Cabot CL. (1979) Luminal fluid proteins of the male reproductive tract. *Biol Reprod.* 21: 883-890.

Uchida S, Sasaki S, Fushimi K, Marumo F. (1994) Isolation of human aquaporin-CD gene. *J. Biol. Chem.* 269: 23451-23455.

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

Van Hoek AN, Wiener MC, Bicknese S, Miercke L, Biwersi J, Verkman AS. (1993) Secondary structure analysis of purified CHIP28 water channels by CD and FTIR spectroscopy. *Biochem.* 32: 11847-11856.

Van Hoek AN, Wiener MC, Verbavatz J-M, Brown D, Townsend RR, Lipniunas PH, Verkman AS. (1995) Purification and structure-function analysis of PNGase F- and endo-β-galactosidase-treated CHIP28 water channels. *Biochem.* 34: 2212-2219.

Van Lieberg AF, Knoers NVAM, Deen PMT. (1995) Discovery of aquaporins: A breakthrough in research on renal water transport. *Pediatr. Nephrol.* 9: 228-234.

Verbavatz JM, Brown D, Sabolic I, Valenti G, Ausiello DA, van Hoek AN, Ma T, Verkman AS. (1993) Tetrameric assembly of CHIP28 water channels in liposomes and cell membranes: A freeze-fracture study. *J. Cell Biol.* 123: 605-618.

Verbavatz JM, Ma T, Gobin R, Verkman AS. (1997) Absence of orthogonal arrays in kidney, brain and muscle from transgenic knock-out mice lacking water channel aquaporin-4. *J. Cell Sci.* 110: 2855-2860.

Verkman AS, Mitra AK. (2000) Structure and function of aquaporin water channels. *Am. J. Physiol. Renal. Physiol.* 278: F13-F28.

Verkman AS, Van Hoek AN, Ma T, Frigeri A, Skach WR, Mitra A, Tamarappoo BK, Farinas J. (1996) Water transport across mammalian cell membranes. *Am. J. Physiol.* 270: C12-C30.

Vitale R, Fawcett DW, and Dym M. (1973) The normal development of the blood-testis barrier and the effects of clomiphere and estrogen treatment. *Anat. Rec.* 176: 333-344.

Vogl AW, Linck RW, Dym M. (1983) Colchicine-induced changes in the cytoskeleton of the golden mantled ground squirrel (spermophilus lateralis) Sertoli cell. *Am. J. Anat.* 168: 99-108

Voglmayer JK, Larsen LH, White IG. (1970) Metabolism of spermatozoa and composition of fluid collected from the rete testis of living bulls. *J. Reprod. Fertil.* 21: 449-460.

Waites GMH, Einer-Jensen N. (1974) Collection and analysis of rete testis fluid from macaque monkeys. *J. Reprod. Fertil.* 41: 505-508.

Walz T, Hirai T, Murata K, Haymann JB, Mitsuoka K, Fujiyoshi Y, Smith BL, Agre P, Engel A. (1997) The three-dimensional structure of aquaporin-1. *Nature.* 387: 624-627.

Walz T, Smith BL, Zeidel ML, Engel A, Agre P. (1994) Biologically active two-dimensional crystals of aquaporin CHIP. *J. Biol. Chem.* 269: 1583-1586.

West NB, Brenner RM. (1990) Estrogen receptor in the ductuli efferentes, epididymis, and testis of rhesus and cynomolgus macaques. *Biol. Reprod.* 42: 533-538.

Wintour EM. (1997) Water channels and urea transporters. *Clin. Exp. Pharmaco. Physiol.* 24: 1-9.

Wong PYD. (1990) Abnormal fluid transport by the epididymis as a cause of obstructive azospermia. *Reprod. Fertil. Dev.* 2: 115-127.

Wong PYD, Au CL, Ngai HK. (1978) Absorptive and secretory functions of the perfused rat cauda epididymidis. *J. Physiol.* 275: 13-26.

Wong PYD, Lee WM. (1982) Effects of spironolactone (aldosterone antagonist) on electrolyte and water content of the cauda epididymidis and fertility of male rats. *Biol. Reprod.* 27: 771-777.

Wong PYD, Yeung CH. (1977) Hormonal regulation of fluid resorption in isolated rat cauda epididymidis. *Endocrinol.* 101: 1391-1397.

Wong PYD, Yeung CH. (1977) Fluid reabsorption in the isolated duct of the rat cauda epididymidis. *J. Reprod. Fertil.* 49: 77-81.

Wong PYD, Yeung CH. (1978) Absorptive and secretory functions of the perfused rat cauda epididymidis. *J. Physiol.* 275: 13-26.

Wong V, Russell LD. (1983) Three-dimensional reconstruction of a rat stage V Sertoli cell: I. Methods, basic configuration and dimensions. *Am. J. Anat.* 167: 143-161.

Wrobil K-H. (1972) Zur morphologie der ductuli efferentes des bullen. *Z. Zellforsch.* 135: 129-148.

Yang B, Brown D, Verkman AS. (1996) The mercurial insensitive water channel (AQP4) forms orthogonal arrays in stably transfected Chinese Hamster ovary cells. *J. Biol. Chem.* 271(9): 4577-4580.

Younes MA, Pierrepoint CG. (1981) Estrogen steroid-receptor binding in the canine epididymis. *Andrologia.* 13: 562-572.

Zhang R, Skach W, Hasegawa H, van Hoek AN, Verkman AS. (1993) Cloning, functional analysis and cell localization of a kidney proximal tubule water transporter homologous to CHIP28. *J. Cell Biol.* 120: 359-369.

CHAPTER II

Aquaporins

Immunolocalization, regulation and postnatal expression of aquaporins 1, 8 and 9 in the testis, efferent ducts and epididymis of adult rats.

ABSTRACT

Aquaporins (AQPs) are membrane protein channels that allow rapid passage of water through an epithelium containing tight junctions. In the present study, light and electron microscope immunocytochemistry were utilized to localize several members of the aquaporin family in the testis, efferent ducts and epididymis of adult animals, animals at different postnatal ages and efferent duct ligated and orchidectomized animals with or without testosterone supplementation. In adult animals, AQP-8 expression was noted exclusively in the testis where it outlined Sertoli cells of the seminiferous epithelium. AQP-9 expression in the testis was seen to outline the periphery of Leydig cells but not cells of the seminiferous epithelium. AQP-9 was also mainly expressed in the apical cytoplasm of the nonciliated cells of the efferent ducts corresponding in the electron microscope to labelling of early endocytic structures. In the epididymis, AQP-9 was localized to the microvilli of principal cells of all regions with more intense reaction being noted in the initial segment and cauda regions. Clear cells of the cauda region only also expressed AQP-9. In the case of AQP-1, no expression was noted in the testis or epithelium of the epididymis. However, anti-AQP-1 antibody was intensely reactive over microvilli of the nonciliated cells of the efferent ducts and over endothelial cells of vascular channels of the efferent ducts and epididymis. These results indicate cell, region and subcellular specificity with respect to the distribution of aquaporin expression in cells of the male reproductive tract. After efferent duct ligation or orchidectomy up to day 21, there was no change in expression of AQP-1 or AQP-9 in the case of the efferent ducts or vascular channels. In contrast, AQP-9 expression on the microvilli of principal cells of the initial segment only and expression in the cytoplasm of clear cells of the cauda region

of the epididymis was dramatically reduced after orchidectomy or efferent duct ligation and not maintained by testosterone replacement suggesting a luminal factor(s) derived from the testis regulated its expression in these regions. These data also suggest that androgens were not essential for AQP-1 or AQP-9 expression in the efferent ducts or epididymis, and that there is region specificity with respect to factors regulating AQP-9 expression on the microvilli of principal cells of the epididymis. Postnatal studies revealed that expression of AQP-8 on Sertoli cells and AQP-9 on Leydig cells appeared by day 21. However, while AQP-1 was expressed on nonciliated cells of the efferent ducts by day 7, that for AQP-9 appeared by day 21; endothelial cells of vascular channels were already reactive by day 7. AQP-9 was expressed on microvilli of principal cells of all regions as seen in control adult animals by day 21, except in the initial segment which occurred only by day 29 as was also the case for clear cells of the cauda. These data suggest cell and region specific factors regulating expression of AQP-1 and AQP-9 during postnatal development and that these factors appear between day 7 and 29, eliminating sperm and high androgen levels as possible regulating factors. Taken together, these data suggest cell, region and subcellular specificity with respect to expression of AQP-1, -8, and –9 in the testis, efferent ducts and epididymis, and that their expression is regulated by a variety of different factors.

INTRODUCTION

Water is an important component of many physiological processes of biological systems. While water has the ability to simply diffuse through the membrane lipid bilayer, this process is costly both in time and energy. 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, it does so at a fast rate and with low energy cost, only if the cell membrane contains an adequate number of specific membrane water channels collectively termed the aquaporins (Wintour, 1997).

Structure and function

The membrane water channels of which there are 10 members so far identified belong to the Major Intrinsic Protein family of proteins and share an NPA (Asp-Pro-Ala) motif. Their molecular weight ranges from 25 Kda to 30 Kda (in their non-glycosylated state) and they contain 6 membrane-spanning domains (Wintour, 1997; King & Agre, 1996; Preston & Agre, 1991). Internal homology was noted between the first and second halves of the channels (Park & Saier, 1996; King & Agre, 1996) suggesting an ancient gene duplication (King & Agre, 1996). AQP-1, the "archetypal water channel" is a non-covalently associated homotetramer, comprised of about 50% non-glycosylated subunits and 50% glycosylated subunit. The four subunits are believed to act as independent pores (Verkman et al., 1996; King & Agre, 1996). The pores of AQP-1 are thought to be constitutively open and allow the passage of water along an osmotic gradient created by ion pumps (Agre et al., 1993). Regulation of what passes through the channel is thought

to depend on the size of the pore. Phosphorylation of the channels is also believed to play a role in the regulation of some members of the aquaporin family (King & Agre, 1996).

Tissue distribution

So far, 10 aquaporins (AQP-0 to AQP-9) have been identified and localized in a variety of different tissues of the body. AQPs show cell and tissue specific distributions but some cell types express several aquaporins. They have been localized in tissues such as the eye lens epithelium, kidney, GI tract, lungs, CNS, muscle and glands. They have been shown to be regulated by various factors. For example, AQP-1 has been shown to be under the influence of estrogens in the efferent ducts of rats whereas vasopressin induces an upregulation of AQP-2 in collecting duct cells in the kidney.

Male reproductive tract

In the male reproductive tract, the Sertoli cells of the testis continuously produce fluid in which the developing germ cells are bathed. Water moves from the interstitial space to the lumen and is important in creating the fluid environment for passage of sperm towards the rete testis and eventually to the epididymis (Voglmayer et al., 1967; 1970; Waites & Einer-Jensen, 1974).

The efferent ducts have been shown to reabsorb between 50-90% of the fluid entering its lumen from the seminiferous tubules (Crabo, 1965). In addition the epididymis, an area where spermatozoa attain maturity and fertilizing ability, performs this function by creating a luminal microenvironment in which the sperm mature. For

this, the epithelial cells are actively involved in secretion and absorption of a variety of different substances. It has been suggested that sodium ions are passively transported from the luminal fluid at the luminal surface of the epididymal epithelial cells and actively transported at the serosal surface to establish a standing osmotic gradient which draws water and chloride ions from the luminal fluid (Setchell & Brooks, 1988). This mechanism of water reabsorption is reflected in an increase of the spermatocrit, and decrease in the concentration of the sodium and chloride ions in the lumen, from the seminiferous tubules to the vas deferens (Setchell & Brooks, 1988).

The tissue distriution of AQPs has not been examined in detail in the male reproductive tract. First identified from erythrocyte membranes as CHIP-28 (channel forming integral membrane protein of 28 Kda), AQP-1 has been localized in many tissues of the body (Ishibashi et al., 1997; Nielsen et al., 1993; Brown et al., 1993; Wintour, 1997). AQP-1 has been localized in the efferent ducts and shown to be regulated by estrogens (Fisher et al., 1998). 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 & Hermo (1999) using Bouin-fixed, paraffin-embedded material and diluted anti-AQP-1 antibody, reported expression of AQP-1 over the endothelium of vascular channels and capillaries in the lamina propria of the vas deferens. AQP-8 is abundant in testis where it was postulated to be expressed in germ cells (Ishibashi et al., 1997).

The purpose of the present study was to localize several members of the aquaporin family of membrane water channels in the testis and epididymis using Bouin-fixed, paraffin-embedded material for light microscope imuunocytochemistry mainly

with partial substantiation of the results at the electron microscope level using frozen ultrathin sections for electron microscope immunocytochemistry. In particular the cell, subcellular and tissue specific distribution of AQPs 1, 8 and 9 was examined in various regions of the adult male reproductive tract. In addition, the postnatal developmental pattern of expression of these AQPs as well as their regulation by testicular factors were examined.

Materials and Methods

Light Microscope Immunohistochemistry

Tissue Preparation : Four adult male Sprague Dawley rats (350-450g) obtained from Charles River Laboratories (St. Constant, PQ) were anesthetized with an intraperitoneal injection of sodium pentobarbital (Somnitol, MTC Pharmaceuticals, Hamilton, ON), and their reproductive tract fixed by perfusion through the abdominal aorta with Bouin's fixative for 10 minutes. After perfusion, the tissues were removed and immersed in Bouin's fixative for another 24 hours. After fixation, the tissue was dehydrated and eventually embedded in paraffin.

Immunoperoxidase Staining : Immunoperoxidase staining of sections was carried out according to the procedure of Oko and Clermont (1989). Polyclonal, affinity purified anti-aquaporin antibodies were used at different dilutions in Tris-Buffered saline (TBS), pH 7.4. The anti-aquaporin antibodies were obtained from Alpha Diagnostics Int. (San Antonio, TX). The antibodies have been well characterized and were found to be specific to their respective peptides. The affinity-purified antibodies were purified over a peptide-Sepharose column and supplied as 1mg/ml solution in PBS, pH 7.4 and 0.1% BSA as stabilizer. The antibodies also contained 0.1% sodium azide as preservative.

Paraffin sections, 5µm thick, were deparaffinized in Histoclear (Diamed Lab Supplies Inc.) 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% (v/v) H_2O_2. Once hydrated, the tissue sections were washed in distilled water containing glycine to block free aldehyde groups.

Before immunostaining, the sections were blocked for 15 minutes with 10% goat serum in TBS. This and subsequent treatments were accomplished by placing 100µl of a solution onto a coverslip and overturning the tissue face of the slide onto the drop, thus ensuring that the entire tissue was treated with minimal fluid (Oko and Clermont, 1989). Coverslips were removed by dipping the slides in TBS containing 1% Tween-20 (TWBS). Sections were then incubated in a 37°C humidified incubation chamber for 1.5 hours with the primary antibody at a dilution of 1:100 (protein concentration of 0.01 mg/ml). After three 2 minute washes in TWBS, sections were once again blocked with 10% goat serum in TBS. They were then incubated for 30 minutes (at 37°C) with goat anti-rabbit IgG conjugated to horseradish peroxidase (Sigma; St. Louis, MO) diluted 1:250 in TBS. This was followed by three 2 minute washes in TWBS.

The final product was achieved by incubating the sections for 10 minutes in 500 ml of TBS containing 0.03% H_2O_2, 0.1M imidazole, and 0.05% diaminobenzidine tetrahydrochloride (Sigma), pH 7.4. Slides were then washed in distilled water and counterstained with 0.1% methylene blue. The tissue was dehydrated by passing slides through a graded ethanol series, after which the sections were immersed in Histoclear and mounted with Permount. Specificity of the immunolabeling was confirmed in tissues by incubation without the primary antibody and using normal rabbit serum.

Electron Microscope immunocytochemistry

Tissue preparation: Four adult male Sprague-Dawley rats (350-450 g) were anaesthetized with sodium pentobarbital and their testes and epididymides fixed by perfusion through the abdominal aorta with a fixative containing 0.1-0.5% glutaraldehyde

and 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Following their removal, the tissues were trimmed into small pieces (0.5 mm^3), immersed for 2 hours in the above fixative at 4°C, followed by washing 2-3 times in 0.15 M phosphate-buffered saline (PBS), pH 7.4, and then treated with PBS containing 1.5 M sucrose. The tissues were then frozen in liquid nitrogen until sectioned.

Immunogold labeling of frozen sections: Ultrathin frozen sections of adult rat testes and epididymides were mounted on 200 mesh, formvar-coated copper grids. Each grid was blocked for 15 minutes with 10% goat serum/TBS solution, then incubated for 1 hour on drops of primary antibody diluted in TBS at a ratio of 1:1 (protein concentration of 0.5 mg/ml) for AQP-1 and a ration of 1:10 (protein concentration of 0.1 mg/ml) for AQP-9. Grids were washed three times for 5 minutes each in TWBS. Labeled grids were blocked again in 10% goat serum/TBS solution before being incubated for 1 hour on goat anti-rabbit antibodies conjugated to 10 nm colloidal gold. The sections were then washed three times for 5 minutes each in TWBS, followed by 5 minute washes in distilled water. In order to enhance membrane morphology, grids were stained with uranyl acetate in 30% ethanol for 2 minutes followed by 1 drop of lead citrate for 30 seconds. Electron micrographs were taken on a Philips 400 electron microscope.

Regulation

Animals: Adult male Sprague-Dawley rats (350-450 g) were obtained from Charles River Laboratory Ltd. (St Constant, Quebec, Canada). The animals were subsequently subdivided into five groups. The first group consisted of normal untreated 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 four rats were exposed through an incision of the anterior abdominal wall. A ligature was placed around both right and left efferent ducts at a site close to the rete testis. The animals were sacrificed at 3, 7, 14 and 21 days following surgery. Bilateral orchidectomy constituted the third group. After anesthesia, both testes of four rats were removed after a ligature was placed around the efferent ducts and testicular blood vessels. The animals were sacrificed at 3, 7, 14 and 21 days after surgery. Bilaterally orchidectomized rats which 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 anesthesia, both testes were removed from four rats, and the implants placed subcutaneously immediately after orchidectomy. The rats 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 University Animal Care Committee.

Postnatal development

Timed pregnant, female Sprague-Dawley rats were obtained from Charles River Laboratory Ltd. (St Constant, Quebec, Canada). Forty-eight male pups were chosen from a number of litters and were maintained on a 14-hour dark, 10-hour light cycle. They were provided with food and water *ad libitum.* After birth, the normal development of the male pups was monitored by assessing body weight gain and by palpating their testes and epididymides. Only those pups showing normal trends in development, as reported by Hermo et al (1992a), were used. At each of the following days after birth, 7, 15, 21, 28, 39, 49, 56 and 90, six rats were selected. At each interval, two rats were used to obtain their body weights and the weights of their testes and epididymides, while the other four rats were used to prepare the tissue for light microscope immunocytochemical analysis using anti-AQP antibodies as described above for adult animals.

RESULTS

Expression of AQPs in normal adult animals

In the testis, the anti-AQP-9 antibody showed an intense immunoperoxidase reaction product only in the interstitial space of the testis (Fig. 1) where it appeared as a lacy network outlining the periphery of Leydig cells. No reaction was seen in the seminiferous epithelium. With the anti-AQP-8 antibody, the reaction was seen in the seminiferous epithelium of the testis but not in the interstitial space (Figs. 2, 3). The reaction outlined the stellate-shaped Sertoli cells. In addition, the distribution of reaction product varied according to the stages of the cycle. At all stages except VII-VIII, the reaction appeared as a filamentous network extending from the base of the epithelium to the lumen. At stages VII-VIII, the reaction was mainly adluminal with some streaks extending to the base of the epithelium but weaker in intensity. There was no staining in the testis with the anti-AQP-1 antibody.

In the efferent ducts, anti-AQP-1 antibody revealed an intense immunoperoxidase reaction over the microvilli of the nonciliated cells of the efferent ducts (Fig. 4). Reaction was also seen in the apical rim of cytoplasm of these cells, some of which appeared as subsurface vesicular elements (Fig. 4). The basolateral plasma membranes of the nonciliated cells showed AQP-1 expression but weaker than microvillar reaction (Fig 4). The ciliated cells did not express AQP-1 (not shown). AQP-1 was also expressed on endothelial cells of vascular channels present in the intertubular spaces (Fig. 4). Anti-AQP-9 antibody was expressed in nonciliated cells and was most intense in the apical rim of their cytoplasm with the microvilli being weakly reactive (Fig. 5).

In the epididymis, expression of AQPs was often cell and region specific. In the initial segment of the epididymis, intense reactivity for anti-AQP-9 antibody was seen on the microvilli of principal cells (Fig. 6) as well as in the apical cytoplasm of some cells in the epithelium (Fig. 6). However, the caput and corpus regions of the epididymis displayed only moderate reaction product on the microvilli of the principal cells and none on clear cells (Fig. 7). In the cauda region, on the other hand, reaction product was noted on the microvilli of principal cells and these cells also contained reactive apical subsurface vesicles in their cytoplasm (Fig. 8). Furthermore, the clear cells of the cauda epididymidis showed an intense cytoplasmic reaction (Fig. 8). Throughout the epididymis, anti-AQP-1 antibody was expressed in endothelial cells of vascular channels present in the intertubular spaces but not in the epithelial cells of the epididymis (Fig. 9). There was no expression of AQP-8 in the efferent ducts or epididymis.

Regulation of AQPs in the efferent ducts and epididymis

At all days after orchidectomy, the expression of AQP-1 in the efferent ducts and epididymis remained the same as control animals (not shown). There was also no change in the pattern or intensity of expression of AQP-9 after orchidectomy in the efferent ducts (Fig. 10).

However, 3 days after orchidectomy and all later time points, the initial segment of the epididymis displayed neither the intense reaction on the microvilli of the principal cells nor the reaction in the apical cytoplasm of some of the cells with anti-AQP-9 antibody (Fig. 11). In contrast, the principal cells of the caput, corpus and cauda regions showed the same pattern of reactivity at all time points after orchidectomy as control

animals. On the other hand, the cytoplasmic reaction of clear cells of the cauda region was diminished but only at 14 and 21 days after orchidectomy (not shown). The administration of testosterone to orchidectomized animals did not maintain expression at control levels neither in the case of principal cells in the initial segment (Fig. 12) nor clear cells of the cauda region (not shown).

Ligation of the efferent ducts did not affect expression in the efferent ducts at any time point examined with anti-AQP-1 antibody. In the epididymis, AQP-1 expression was also unaffected by ligation (not shown). On the other hand, ligation of the efferent ducts at 3 days and all later time points abolished expression on the microvilli of the principal cells of the initial segment of the epididymis (not shown) and diminished the cytoplasmic reaction in clear cells of the cauda epididymidis (Fig. 13).

Postnatal expression of AQPs

Expression of AQP-1 was already noted in the efferent ducts by postnatal day 7 (Fig. 14a) and continued at day 21 (Fig. 14b) and into adulthood. The endothelial cells of the vascular channels also displayed reaction as early as postnatal day 7 (Fig. 15b) with the expression continuing into adulthood.

At postnatal day 7, there was no expression in the seminiferous epithelium of the testis with anti-AQP-8 antibody (not shown). At postnatal day 21, a weak reaction was localized near the lumen of the tubules, which by day 29 began to extend down to the base of the epithelium (not shown). By postnatal day 39, the seminiferous epithelium displayed a pattern of expression similar to that seen in adult animals (not shown).

At postnatal day 7, the testis, efferent ducts (Fig. 16a) and epididymis did not show expression of AQP-9. However by postnatal day 21, Leydig cells began to display a lacy weblike reaction on their plasma membrane which was maintained into adulthood (not shown). Efferent ducts at postnatal day 21 displayed intense reactivity in the apical cytoplasm of the nonciliated cells with the anti-AQP-9 antibody (Fig. 16b) which continued into adulthood. The initial segment at day 21 began to show a weak reaction product on the microvilli of the principal cells (Fig. 17a) which by day 29 was intense and comparable to adult animals (Fig. 17b). Principal cells of the caput and corpus regions of the epididymis displayed the reactivity of the adult animal by day 21 (not shown) but there was no expression in clear cells of this region. The cauda region at postnatal day 21 displayed intense reaction product on the microvilli of principal cells similar to adult animals (Fig. 18a), while clear cells of the cauda showed intense reactivity only by day 29 (Fig. 18b).

Immunocytochemistry for electron microscopy was performed on the efferent ducts with the anti-AQP-1 (Fig. 19) and anti-AQP-9 (Fig. 20) antibodies and verified the localization of these two proteins in subcellular organelles of the nonciliated cells.

Control slides where the primary antibody was omitted did not show any immunoreactivity for all antibodies studied (not shown).

DISCUSSION

Water transport is an activity that is shared by many cells of the human body. It is now believed that water will cross a non-fenestrated endothelium or an epithelium with tight junctions rapidly and at low energy cost only if an adequate supply of water channels is present on their plasma membranes (Wintour 1997). Ten membrane water channels have been discovered thus far and have been collectively termed the "aquaporins" (AQPs). AQPs have been localized to many tissues of the body that require the rapid transport of water, such as kidney and brain. AQPs are often expressed in a cell and tissue specific manner. In addition, it has been shown that some of the aquaporins (AQPs 3, 7 and 9) do not permeate water exclusively, they may form pores for urea and glycerol as well (Kuriyama et al., 1997; Ishibashi et al., 1997; 1998).

Expression of aquaporins in the testis, efferent ducts and epididymis of normal untreated animals.

The present study revealed a cell specific distribution of aquaporins in the testis (Table. 1), while Sertoli cells expressed AQP-8, Leydig cells expressed AQP-9, and there was no expression of AQP-1 in the testis. The expression of AQP-8 was ascribed to Sertoli cells as the reaction product in most stages of the cycle was seen to extend from the base of the seminiferous epithelium to the lumen and resembled in part expression of SGP-2 and SGP-1, well known secretory products of these cells (Hermo et al., 1991; 1992). It appeared as a filamentous network outlining the plasma membrane of Sertoli cells being closely apposed to that of germ cells. However, in many cases the entire periphery of germ cells, especially those close to the lumen was not immunoreactive.

This data suggested along with the absence of reaction product over spermatogonia that AQP-8 was expressed in Sertoli cells. In addition, at stages VII-VIII of the cycle, the reaction was noted predominantly at the lumenal front of the epithelium with moderate reaction over the basal compartment further attesting to the expression of AQP-8 in Sertoli cells. The latter are well known to vary in function and express stage specific proteins during the cycle of the seminiferous epithelium (Griswold, 1993; Parvinen, 1993). The varying distribution of AQP-8 in the epithelium during the cycle is thus consistent with Sertoli cell functions.

In the testis, Sertoli cells continuously produce fluid in which the developing germ cells are bathed and which serves as a medium to allow sperm to exit seminiferous tubules. In keeping with the standing osmotic gradient model of Diamond and Tormey (1966) which states that pumps would transport potassium into the cell, the ion would then be pumped again into the intercellular space to create a hypertonic region, and then diffuse down its concentration gradient through the lateral space. Because of the osmotic gradient, water would cross the basolateral membrane and enter the lateral spaces. Indeed, Na^+/K^+ ATPase has been localized to Sertoli cells (Barham et al., 1976; Gravis et al., 1976) as well as the lymphatic endothelium (Gravis et al., 1976). AQP-8 in Sertoli cells may be involved in transport of water across the seminiferous epithelium into the lumen especially as those cells span the entire width of the epithelium. The differential expression of this channel in the seminiferous epithelium at different stages of the cycle suggests that AQP-8 plays different roles at different stages. While water may pass through the cell at all stages, at stages VII-VIII of the cycle water may exit step 19 spermatids via their tubulobulbar complexes which are prominent at these stages and

which have been reported to have a watery reappearance (Russell, 1979). Thus AQP-8 expression in Sertoli cells could move water from the interstitial space to the tubular lumen but also serve to remove water from step 19 spermatids prior to their release into the lumen.

The distribution of AQP-9 was seen as an extensive lacy network in the interstitial space. In the rat testis, lymphatic channels do not exist (Fawcett et al., 1973). Rather the interstitial space is considered as a lymphatic sinusoid in which Leydig cells and macrophages bathe in the lymph contained therein. Thus the expression of AQP-9 would appear to be related to the cell surface of Leydig cells, the major cell type of the interstitial space (Wing & Christensen, 1982). However, why Leydig cells express AQP-9 is not clear. Aquaporin expression may maintain water equilibrium within the cell. Another possibility may be the passage of steroids out of the cell as a major function of Leydig cells is the production of testosterone.

In the efferent ducts, aquaporin expression was not only cell specific but subcellular and even tissue specific (Table. 1). The nonciliated cells expressed AQP-1 and AQP-9 but neither was noted in ciliated cells. In addition, AQP-1 was intensely expressed on the microvilli but only weakly so for AQP-9. AQP-1 expression was also noted along the basolateral plasma membranes of nonciliated cells but not so for AQP-9. Furthermore, AQP-9 was prominent in the apical rim of cytoplasm of nonciliated cells but not so for AQP-1. Thus while these two aquaporins were noted in nonciliated cells, each showed a specific subcellular distribution. In contrast, AQP-1 was also noted in the efferent ducts but its expression was restricted to endothelial cells of vascular channels of the intertubular space indicating a wide diversity in aquaporin expression in this tissue.

AQP-1 had already been documented to reside in the epithelium of the efferent ducts but its precise localization was not described (Fischer et al., 1998).

The efferent ducts are well recognized as a major site of reabsorption of water entering the lumen from the seminiferous tubules. In fact Crabo (1965) reported that 50-90% of fluid is removed from the lumen of the efferent ducts. A Na^+/K^+ ATPase pump has also been reported in the efferent ducts and other ion channels that would serve to create a gradient to allow passage of water (Ilio & Hess, 1992). The expression of AQP-1 on microvilli and basolateral plasma membrane of nonciliated cells would suggests its importance in transport of water from the lumen through and out the cell. In the intertubular space, AQP-1 expression in vascular channels would serve to remove incoming water from this site and thus maintain water equilibrium in this tissue. The removal of water from the efferent duct lumen would serve to concentrate sperm in the initial segment of the epididymis to provide for better interactions with the secretory products of its epithelial cells, especially as this is the region where sperm begin to acquire their maturation properties (Cooper, 1995). As noted in the EM, AQP-9 was mainly localized over structures in the apical cytoplasm of nonciliated cells and not over microvilli of these cells as seen for AQP-1. Gold particles appeared over the endocytic organelles of these cells such as coated pits and endosomes. The latter presumably fluid-filled and need to remove water as they gradually evolve into multi-vesicular bodies and eventually lysosomes (Hermo et al., 1988; 1994). AQP-1 expression related to these structures may remove water to allow for their eventual reported reduction in size and concentration of their content as they evolve to become smaller dense lysosomes (Hermo et al., 1994).

The epididymis is an area where spermatozoa attain their fertilizing ability (Orgebin-Crist & Olson, 1984). The epithelium is involved in this function by creating a suitable luminal microenvironment allowing for the maturation of sperm. For this, the epithelium is actively involved in secretion and absorption of numerous substances. It has been suggested that sodium ions are passively transported from the luminal fluid at the luminal surface of the epithelial cells, and actively transported at the serosal surface by Na^+-K^+ pumps located on the basolateral aspects of the cells (Byers and Graham, 1990) to establish a standing osmotic gradient which draws water and chloride ions from the luminal fluid (Setchell & Brooks, 1988). This mechanism of water reabsorption is reflected in an increase of the spermatocrit, and a decrease of the concentration of the sodium and chloride ions in the lumen moving from the seminiferous tubules to the vas deferens (Setchell & Brooks, 1988). In the present study, AQP-9 was expressed on the microvilli of principal cells of the entire epididymis (Table. 3) but expression was most intense in the initial segment and cauda regions suggesting more active water transport in these regions. In the initial segment, expression was also noted in the apical region of some cells. The nuclei of these cells were not apically located eliminating the possibility that they corresponded to narrow or apical cells (Adamali & Hermo, 1996). In addition, in the initial segment and cauda region, distinct subsurface vesicles of principal cells were also reactive suggestive of expression in endosomes, and for reasons as already outlined for nonciliated cells. However, a similar reaction was not noted in principal cells of the caput and corpus regions and the reason for this is at present unclear.

Clear cells of the cauda region also expressed AQP-9 with reaction being uniform throughout the cytoplasm. However, clear cells of the caput and corpus regions

were unreactive suggesting region specificity for AQP-9 in the case of these cells. As in the efferent ducts, AQP-1 was expressed in vascular channels of the intertubular space of the entire epididymis. Thus in the epididymis water may be transported from the lumen via AQP-9 expressed on principal cells and be removed in the intertubular space via AQP-1 expressed on vascular channels. Clear cells also express AQP-9 but only in the cauda where they may also transport water out of the lumen. The removal of water from the lumen of the cauda would allow for more efficient space for immobilin, a protein secreted by principal cells to immobilize sperm while they are stored in this region (Hermo et al., 1992). The cytoplasmic reaction of clear cells which is common for many lysosomal proteins expressed by these cells - such as ß-Hexosaminidase A, cathepsins D and A - suggests in the absence of EM localization that the plethora of endosomes and lysosomes of these cells known to fully occupy their cytoplasm express AQP-9 to remove water as these organelles concentrate the material contained therein to evolve into smaller dense lysosomes. Thus the present data on aquaporin expression in normal adult animals reveal a cell, region and tissue specific distribution for the different aquaporins as well as subcellular specific distribution (Tables 1 & 2).

Regulation of AQP expression in the efferent ducts and epididymis

It is well established that many epididymal functions including expression of different proteins are regulated by testicular factors (Cornwall & Hann, 1995; Orgebin-Crist, 1996; Kirchhoff, 1999). These factors include androgens synthesized by Leydig cells and entering the epididymis via the circulation or lumenal factors derived from Sertoli cells and entering from seminiferous tubules via the efferent ducts. The expression

of some proteins may be regulated in different epididymal regions by different factors (Robaire & Viger, 1995). The regulation of water transport in many tissues has been extensively studied. In the kidney collecting duct cells expressing AQP-2, water permeability is tightly regulated by the anti-diuretic homone arginine vasopressin (AVP). Upon an AVP signal, fuctional AQP-2 channels are inserted into the plasma membrane in a cAMP vesicular trafficking mechanism (Brown et al., 1998). Other aquaporins appear to be constitutively expressed at the plasma membrane of the cells where they are found. In the present study we examined the regulation of AQP-1 and AQP-9 in the efferent ducts and epididymis. At all time points examined after orchidectomy or efferent duct ligation there was no noticeable change to the expression of AQP-1 or AQP-9 in the efferent ducts. This included reaction over nonciliated cells as well as vascular channels. Thus these two aquaporins are not regulated by testicular factors in this tissue. In a recent study, AQP-1 expression in efferent ducts was stated to be regulated by estrogens (Fischer et al., 1998) that have been found in relatively high concentrations in the male reproductive tract (Claus et al., 1987; 1992). Estrogen receptor has been localized in many tissues of the male duct (West & Brenner, 1990; Iguchi et al., 1991; Goyal et al., 1997) at levels sometimes exceeding those found in female reproductive organs such as the uterus (Hess et al., 1997).

AQP-1 expression in vascular channels of the epididymis was also unaffected by efferent duct ligation or orchidectomy. In contrast however, AQP-9 expression in the epididymis was affected by both experimental conditions. Three days after orchidectomy or efferent duct ligation, the initial segment displayed neither the intense reaction on microvilli of principal cells nor reaction in the apical cytoplasm of some cells (Table. 3).

Testosterone replacement did not fully maintain expression suggesting that androgens were not the only factor affecting expression of AQP-9 in this region. These data thus suggest that AQP-9 expression in the initial segment is regulated by testicular factors derived from the lumen.

After orchidectomy or efferent duct ligation, expression of AQP-9 in principal cells of the caput, corpus and cauda was unaffected and remained similar to that seen in control animals at all time points examined (Table. 3). This was not the case however for clear cells of the cauda which at 14 days after orchidectomy or efferent duct ligation showed a diminished expression of AQP-9 (Table. 4). Testosterone administration to orchidectomized animals at a dose representative of high levels in the epididymis did not maintain expression at control levels. Together these data suggest that AQP-9 expression in clear cells of the cauda is regulated by luminal factors derived from the testis.

Postnatal expression of AQP-1, -8 and –9

It is well documented that expression of the same protein during postnatal development varies for a given cell type depending on the region where it is located leading to a complex multitude of factors regulating its expression and this pattern changes for different proteins (Hermo & Papp, 1996; Hermo et al., 1994; 1999). This also appears to be the case for aquaporins in the present study (Tables 5 & 6).

Expression of AQP-1 was already seen in the efferent ducts at postnatal day 7 and continued at day 21 and into adulthood. The endothelial cells of the vascular channels of the efferent ducts and epididymis also displayed reaction as early as postnatal day 7 with expression continuing into adulthood. It would seem that the expression of AQP-1 in the

male reproductive tract depends on neither the presence of high levels of androgens which appear only by day 39 (Robaire & Sheer, 1980), nor the presence of luminal testicular factors which would appear by about day 18 (Tindall et al., 1975). This is verified by the orchidectomy and ligation experiments that demonstrated that AQP-1 was not regulated in the efferent ducts or epididymis by testicular factors.

At postnatal day 7, AQP-8 was not expressed in the seminiferous epithelium of the testis. At postnatal day 21, shortly after the formation of a lumen in the tubules, AQP-8 began to be expressed near the lumen in the seminiferous epithelium. By postnatal day 39, the seminiferous epithelium displayed a pattern of expression as seen in adult animals. This suggests that the expression of AQP-8 in the seminiferous epithelium may depend on the presence of high androgen levels appearing by day 39 (Robaire & Sheer, 1980).

At postnatal day 7, the testis, efferent ducts and epididymis did not express AQP-9. However by postnatal day 21, Leydig cells displayed a lacy weblike reaction on their plasma membrane similar to that seen in adult animals. Efferent ducts at postnatal day 21 also displayed intense reactivity as seen in adult animals. At the same age, the initial segment showed a weak reaction product on the microvilli of the principal cell, while those of the caput, corpus and cauda regions of the epididymis displayed the reactivity of the adult animal. At postnatal day 29, intense reaction appeared on the microvilli of the principal cells of the initial segment. Clear cells of the cauda were unreactive until day 29 when they showed adult-like expression. Together these data suggest that AQP-1 and AQP-9 expression appearing between days 7 and 29 in the different regions of the duct are influenced by different factors but not by androgens.

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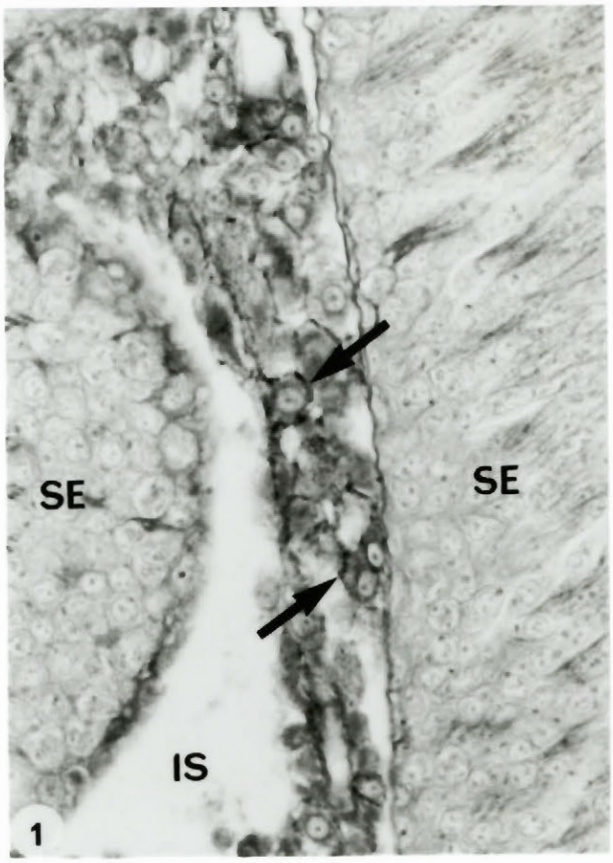
Fig 1: Testis immunostained with anti-AQP-9 antibody. A lacy reaction (arrows) that outlines the Leydig cells appears in the interstitial space (IS). The seminiferous epithelium (SE) is unreactive. X 461

Fig 2: Testis immunostained with anti-AQP-8 antibody. Seminiferous tubule at stage VII of the cycle. A reaction is evident in the seminiferous epithelium (SE) bordering the lumen (arrowheads) with occasional streaks of reaction (arrows) extending down to the base of the epithelium. Note that tails (T) of the sperm in the lumen (Lu) are unreactive. X 288

Figs 3a, b: Testis immunostained with anti-AQP-8 antibody. Seminiferous tubules at stage IX of the cycle at low (a) and high (b) magnification. The immunoperoxidase reaction is seen as a filamentous-like network extending from the base (arrows) of the seminiferous epithelium (SE) to the lumen (arrowheads). Residual bodies (open arrows) are unreactive. Their dark coloration is due to the counterstaining by Methylene Blue. No reaction is seen in the interstitial space (IS). Lu, lumen. a) X 288; b) X 461


SE
SE
IS
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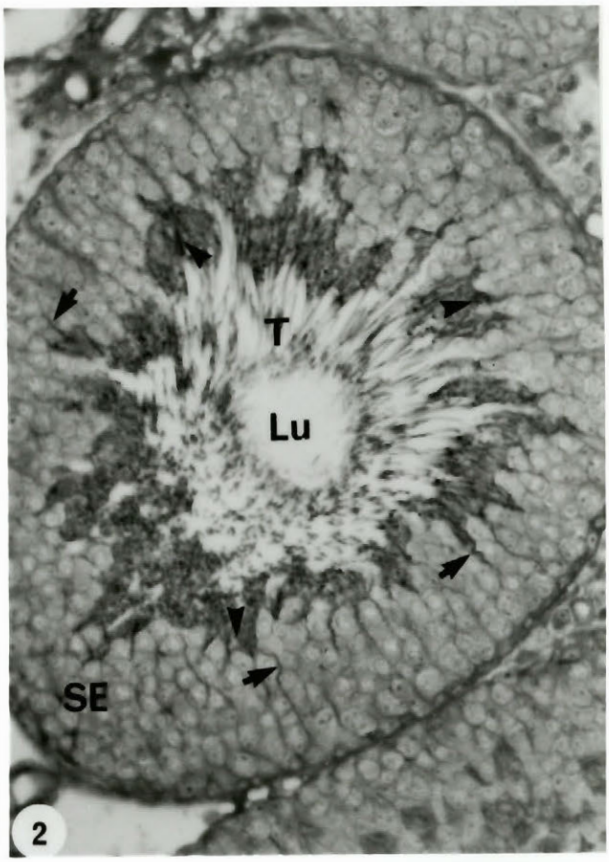
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Lu
SE
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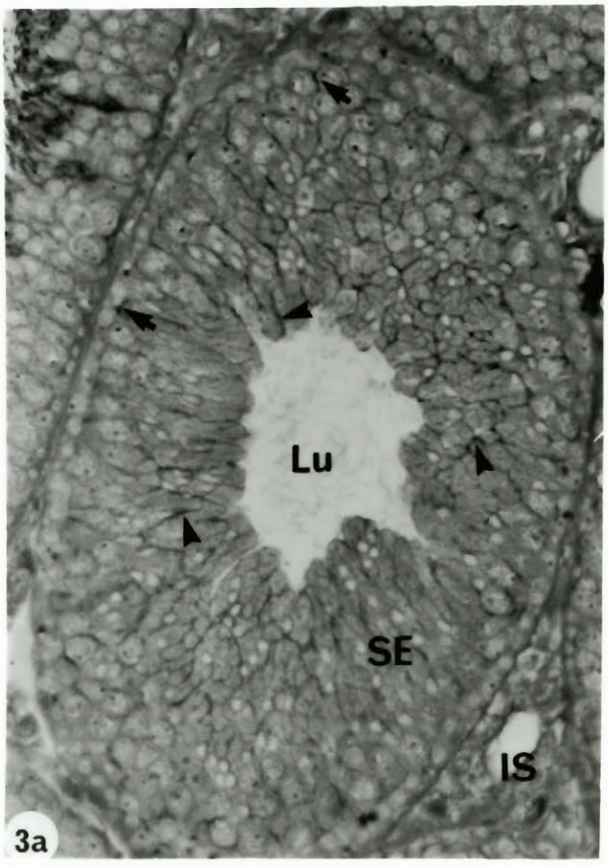

Lu
SE
IS
3a

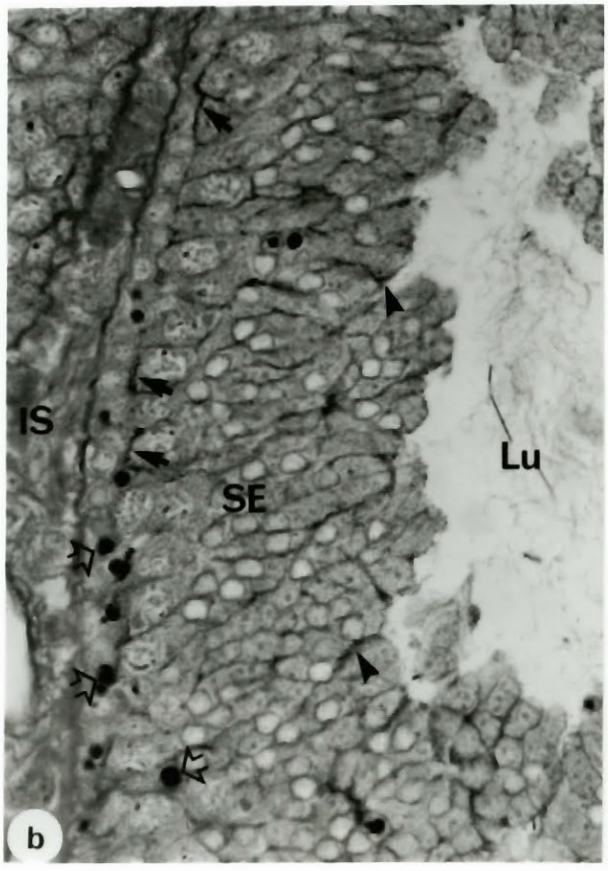

IS
Lu
SE
b

Fig 4: Efferent ducts immunostained with anti-AQP-1 antibody. Reaction is seen over the microvilli (arrowheads) of the nonciliated cells and over vesicles (circles) of their apical cytoplasm. The basolateral cell membranes (arrows) also show AQP-1 expression. Endothelial cells of vascular channels (open arrows) in the lamina propria are also reactive. Sperm (S) in the lumen are unreactive. X 461

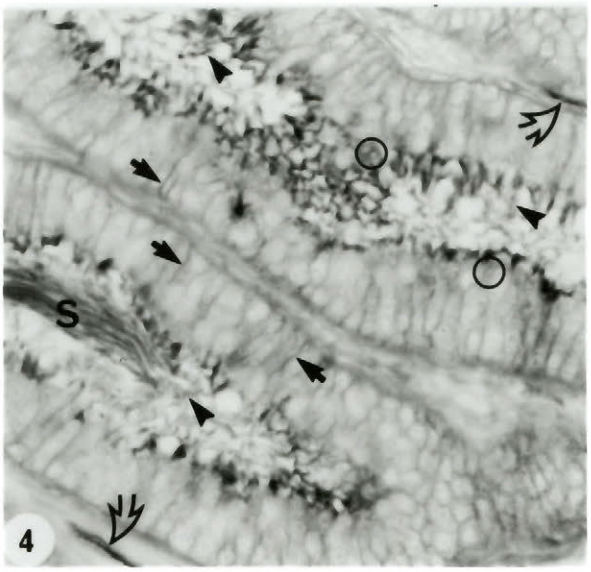
Fig 5: Efferent ducts immunostained with anti-AQP-9 antibody. Tufts of reaction (arrows) are seen over the apical rim of cytoplasm of the nonciliated cells. Microvilli (arrowheads) and basolateral plasma membranes of these cells are unreactive. E, epithelium; Lu, lumen. X 737

Fig 6: Initial segment of the epididymis immunostained with anti-AQP-9 antibody. Intense reaction is seen over the microvilli (arrowheads) of the principal cells (P) and apical cytoplasm (arrows) of some of these cells. Sperm (S) in the lumen are unreactive. IT, intertubular space. X 288

Fig 7: Corpus epididymidis immunostained with anti-AQP-9 antibody. Moderate reaction is seen on the microvilli (arrowheads) of the principal cells (P). S, sperm. X 461

Fig 8: Cauda epididymidis immunostained with anti-AQP-9 antibody. Intense reaction is seen over the microvilli (arrowheads) of principal cells (P). A cytoplasmic reaction (curved arrows) is also noted in clear cells. Note reactive granules (circles) in the apical cytoplasm of some principal cells. S, sperm. X 288

Fig 9: Cauda epididymidis immunostained with anti-AQP-1 antibody. Intense reaction is seen on the endothelial cells (open arrows) of vascular channels in the intertubular space (IT). E, epithelium; Lu, lumen. X 288


S
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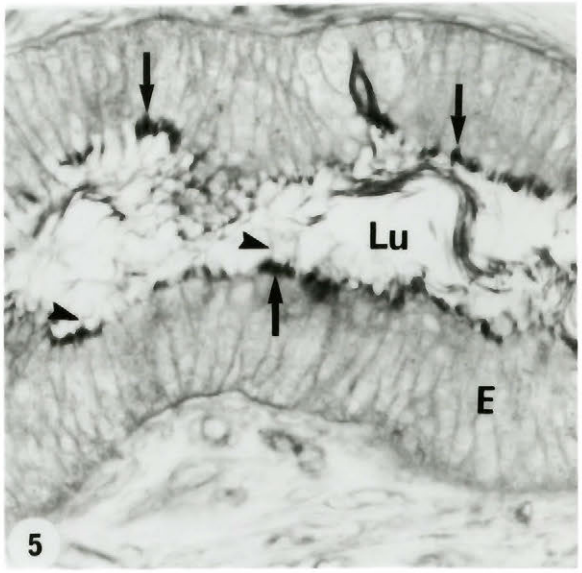
Lu
E
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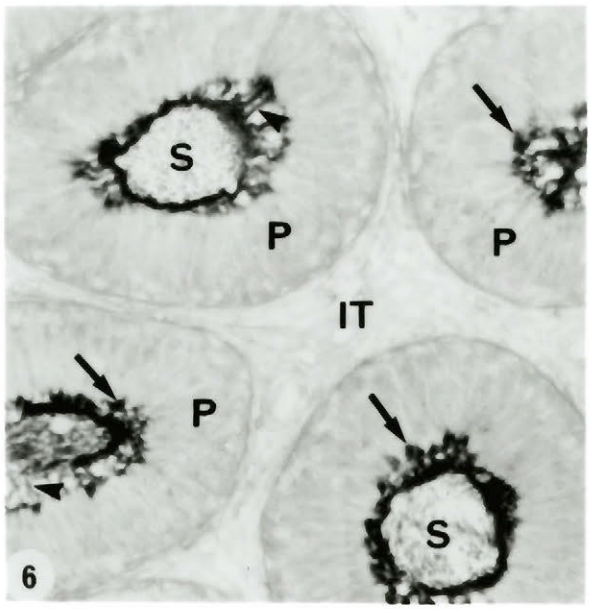
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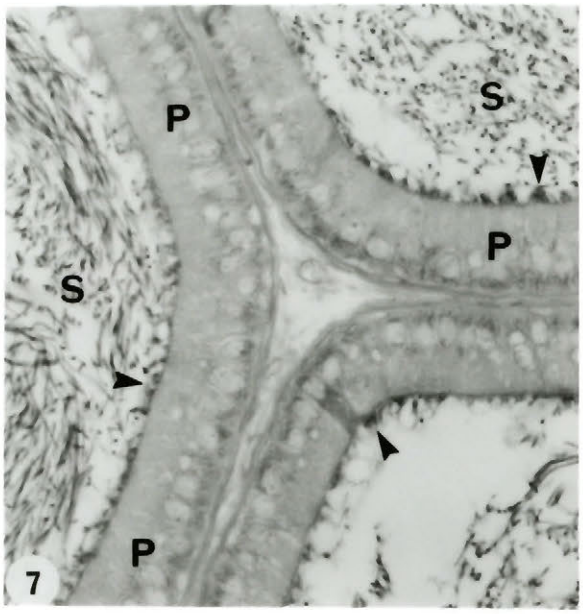
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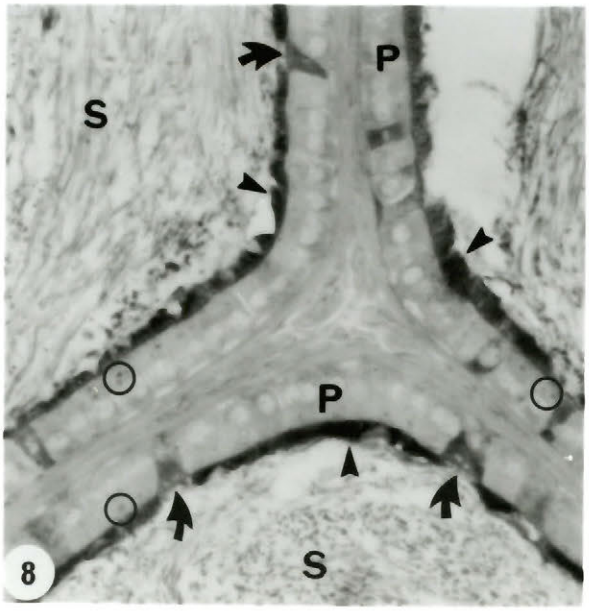

S
P
P
S
8

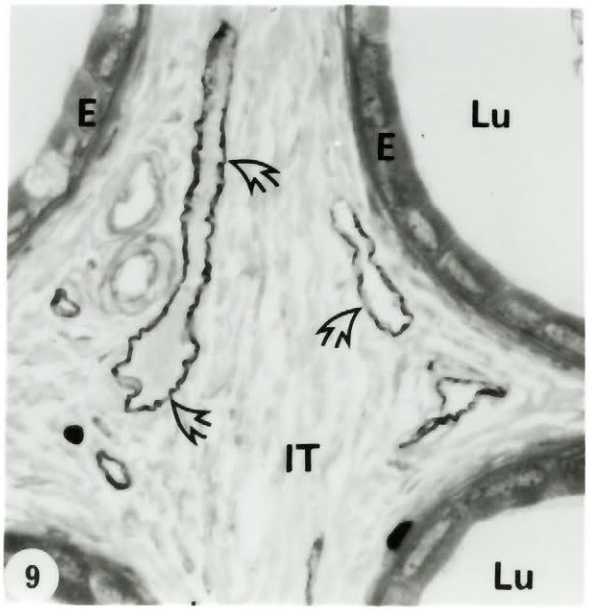
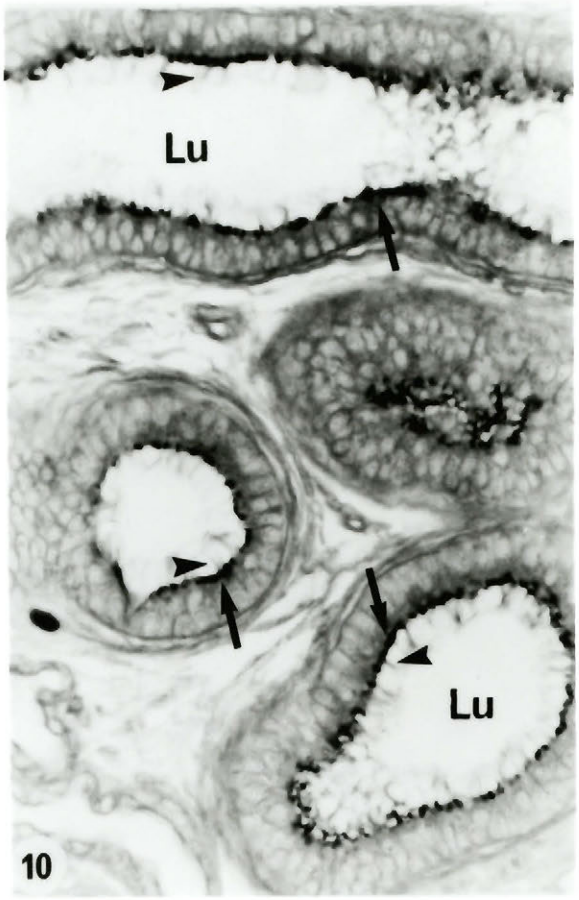

E
E
Lu
IT
Lu
9

Fig 10: Efferent ducts 14 days after orchidectomy and immunostained with anti-AQP-9 antibody. The apical rim of cytoplasm (arrows) of the nonciliated cells still displays intense reaction product. Microvilli (arrowheads) of these cells are unreactive. Lu, lumen. X 422

Fig 11: Initial segment 3 days after orchidectomy and immunostained with anti-AQP-9 antibody. The microvilli (arrowheads) of the principal cells (P) do not show reactivity. Lu, lumen. X 422

Fig 12: Initial segment 14 days after orchidectomy and immediate replacement of testosterone and immunostained with anti-AQP-9 antibody. The microvilli (arrowheads) of some principal cells (P) display reactivity. Lu, lumen. X 422

Fig 13: Cauda epididymidis 14 days after efferent duct ligation and immunostained with anti-AQP-9 antibody. Strong reactivity is present over the microvilli (arrowheads) of principal cells (P). The cytoplasm of clear cells (curved arrows) displays weak reactivity. Lu, lumen. X 264



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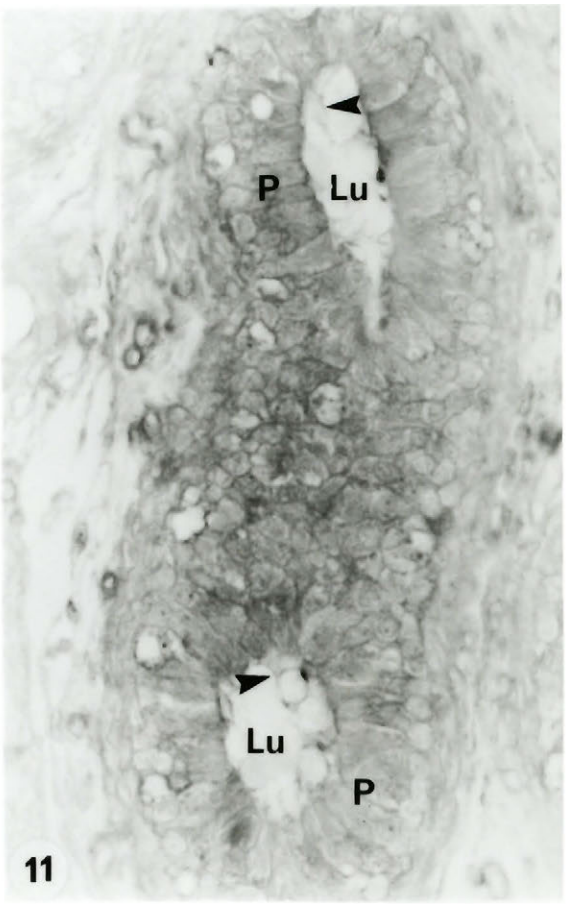

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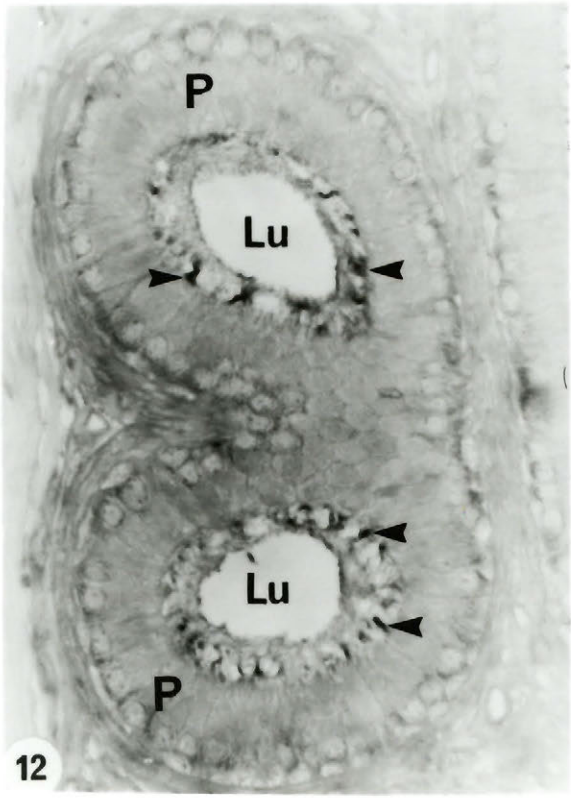

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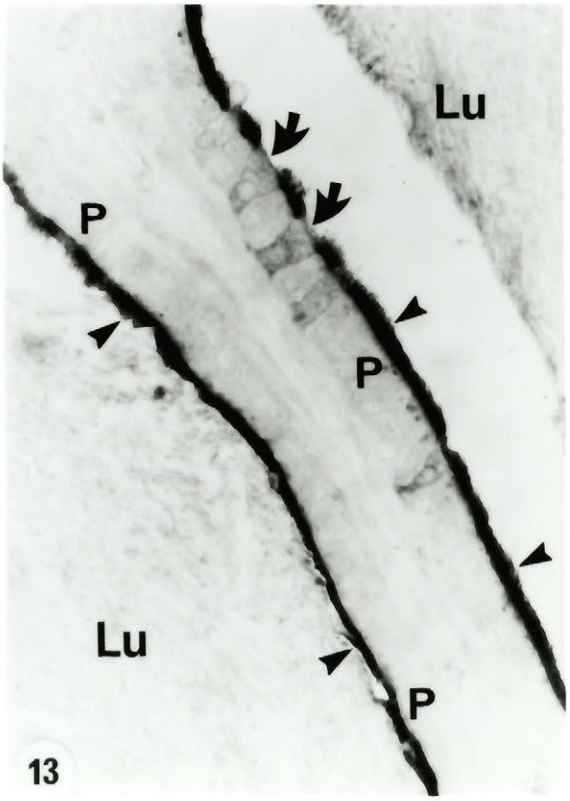
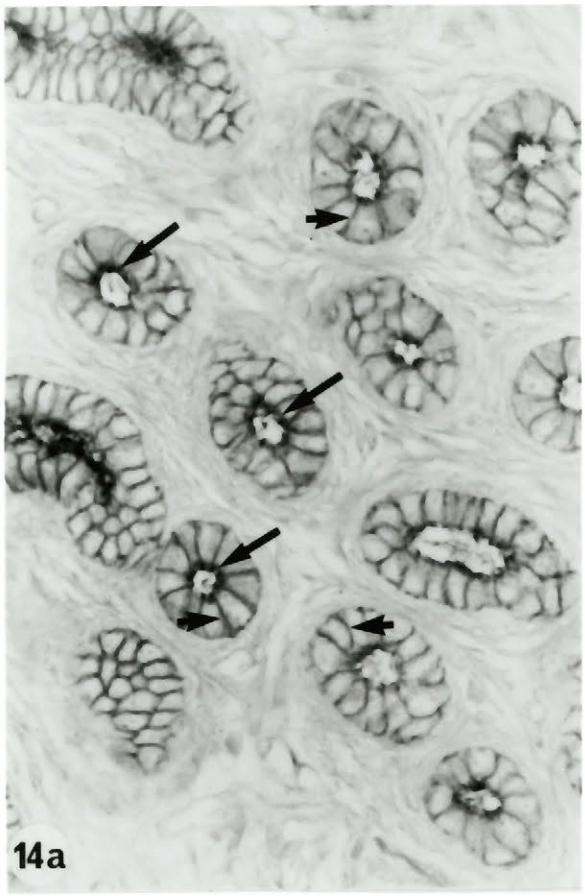


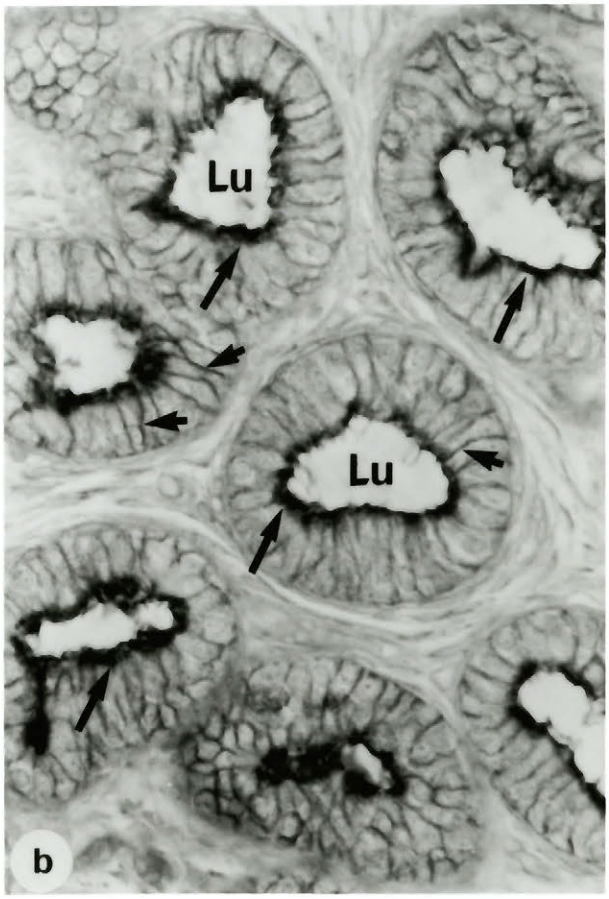
13

Fig 14a, b: Efferent ducts at postnatal day 7 (a) and day 21 (b), immunostained with anti-AQP-1 antibody. In (a), intense reactivity is seen in the apical rim of cytoplasm (long arrows) of the epithelial cells. The basolateral membranes (short arrows) of these cells also display intense reaction product. In (b), the apical rim of cytoplasm (long arrows) of the nonciliated cells displays intense reaction product. The basolateral membranes (short arrows) also show reactivity. Lu, lumen. X 422

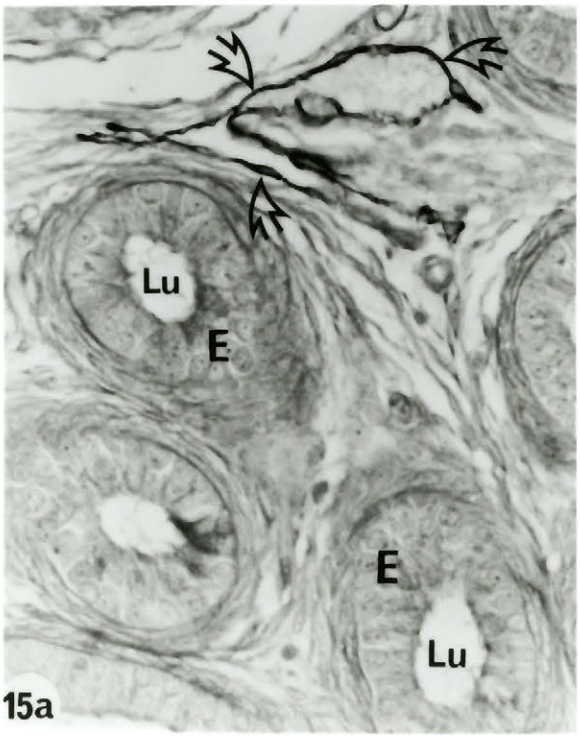
Fig 15a, b: Epididymis immunostained with anti-AQP-1 antibody. Initial segment at postnatal day 21 (a) and cauda epididymidis at postnatal day 7 (b) display reaction product in the endothelial cells (open arrows) of vascular channels. The epithelium (E) appears unreactive. Lu, lumen X 422



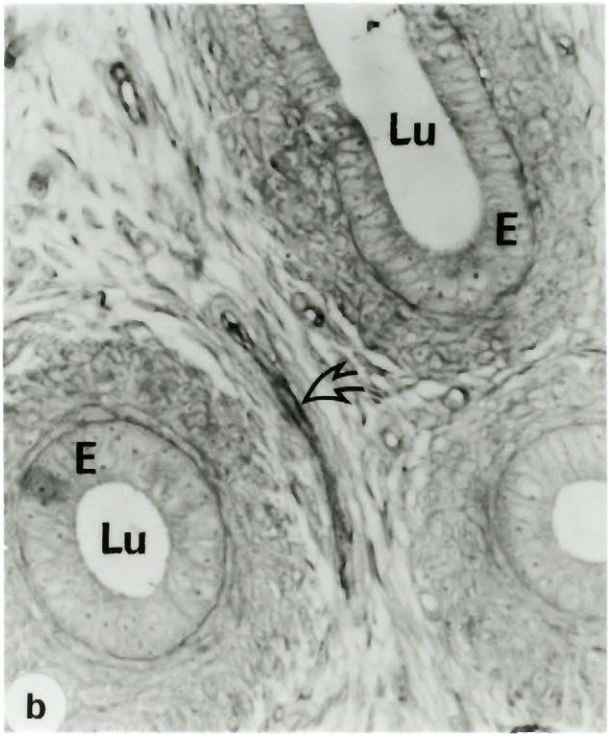
14a



b



15a

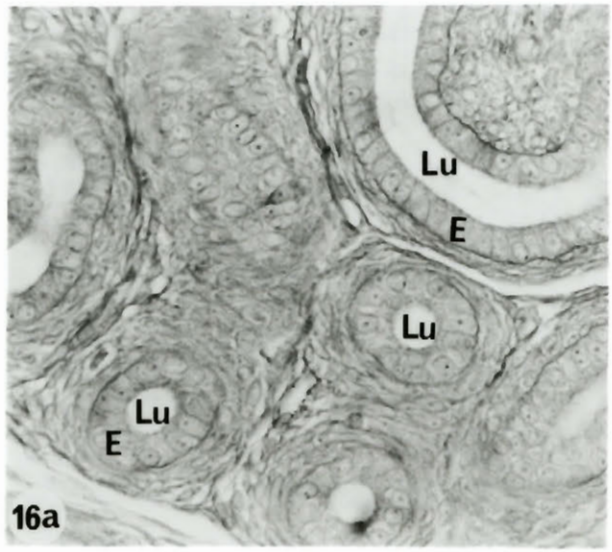


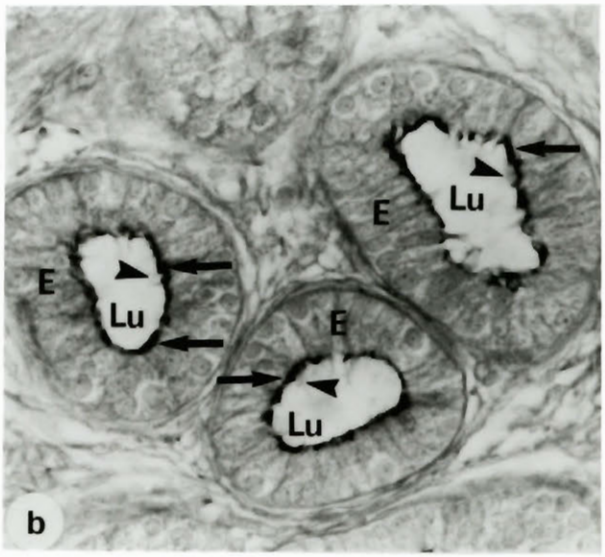
b

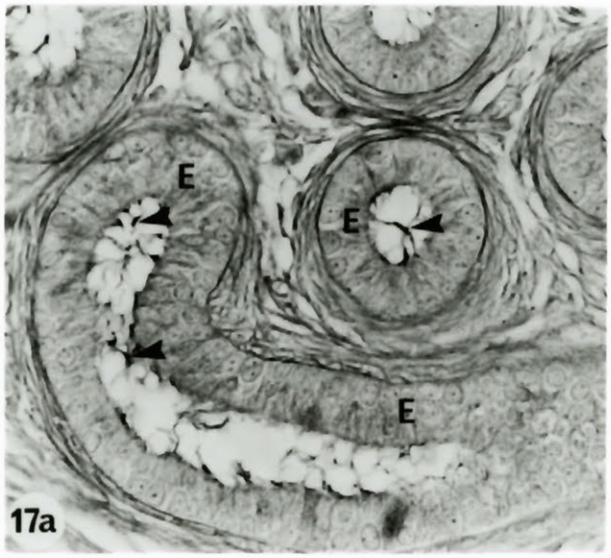
Fig 16a, b: Efferent ducts at postnatal day 7 a) and postnatal day 21 b), immunostained with anti-AQP-9 antibody. The undifferentiated nonciliated epithelial cells (E) of the tubules show no reaction at postnatal day 7 (a). However, the apical rim of cytoplasm (arrows) of the nonciliated cells shows reactivity at postnatal day 21 (b). Microvilli (arrowheads) of these cells are unreactive. Lu, lumen. X 422

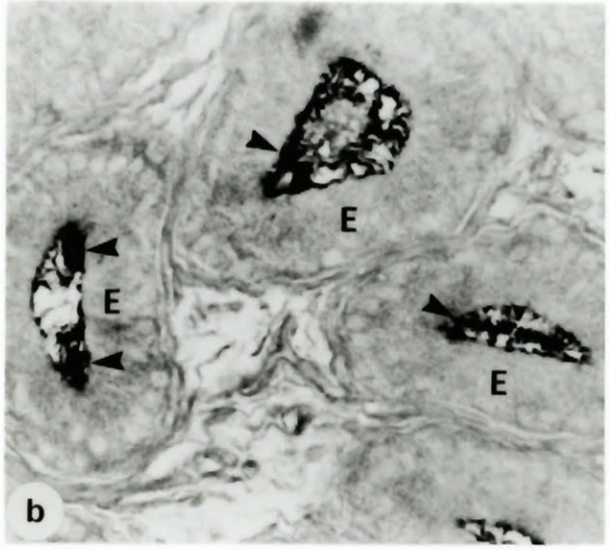
Fig 17a, b: Initial segment at postnatal day 21 a) and postnatal day 29 b), immunostained with anti-AQP-9 antibody. The microvilli (arrowheads) of the epithelial cells (E) show some reactivity at day 21 (a) but display intense reactivity by day 29 (b). X 422

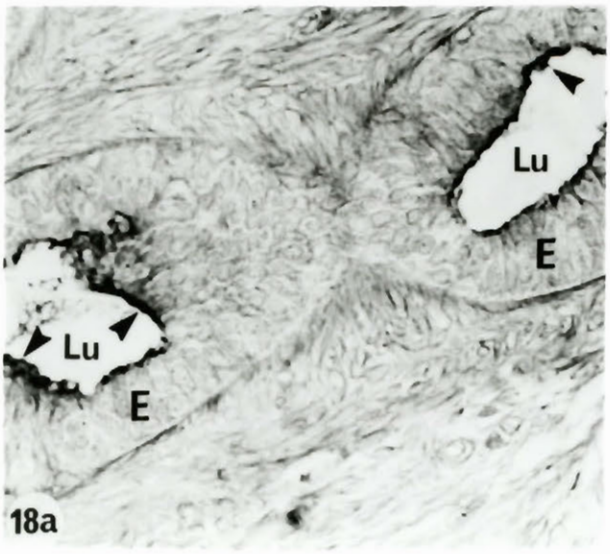
Fig 18a, b: Cauda epididymidis at postnatal day 21 a) and postnatal day 29 b), immunostained with anti-AQP-9 antibody. The microvilli (arrowheads) of the undifferentiated epithelial cells (E) display intense reaction product at day 21 (a). At day 29 (b), reactivity is seen on the microvilli (arrowheads) of principal cells (P) as well as a cytoplasmic reaction (curved arrows) in clear cells. Lu, lumen. X 422


Lu
E
Lu
Lu
E
16a


E
Lu
E
Lu
E
Lu
b


E
E
E
17a


E
E
E
b


Lu
E
Lu
E
18a

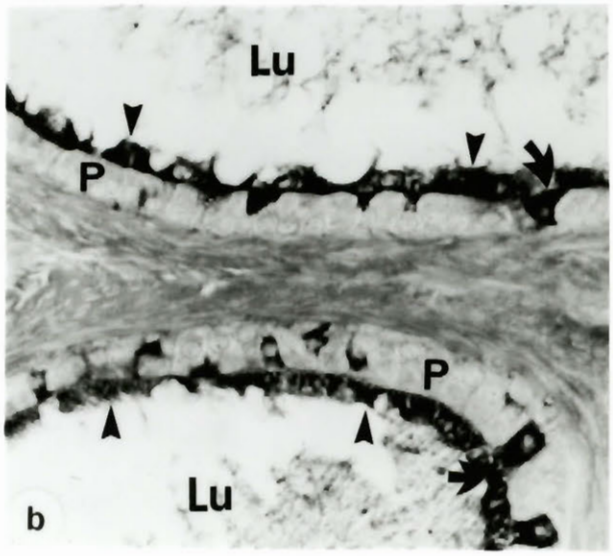
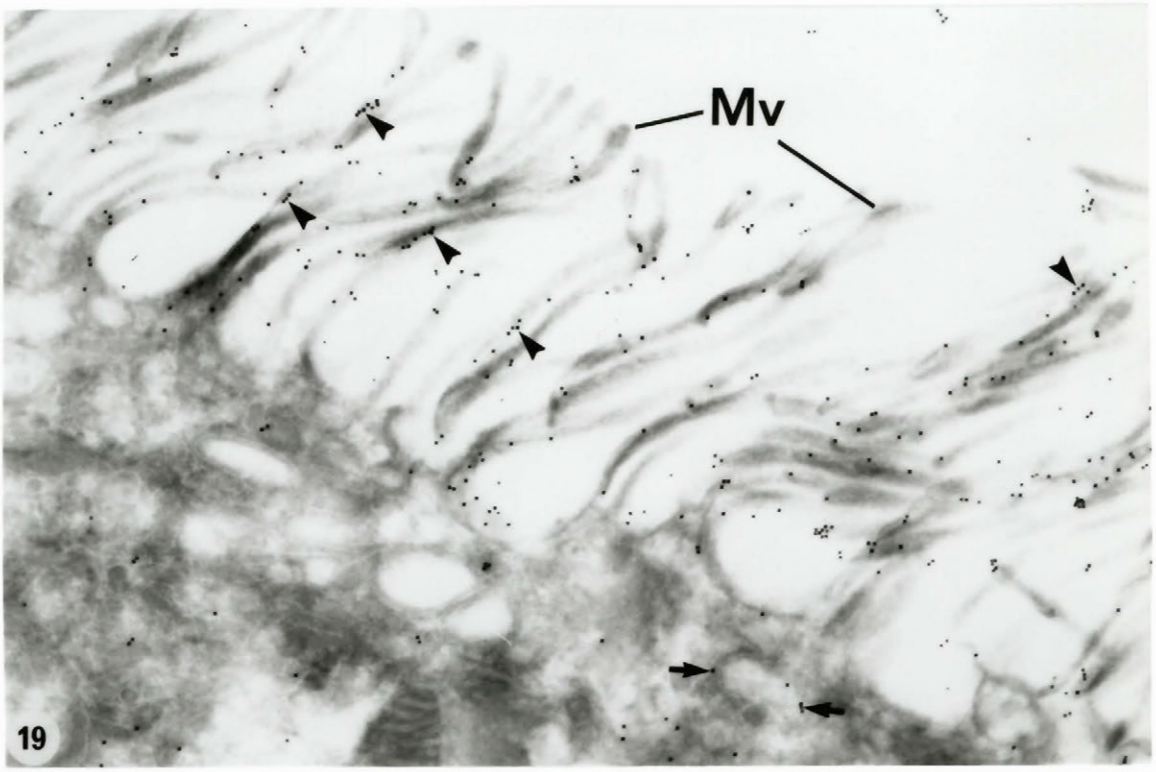

Lu
P
P
Lu
b

Fig 19: Electron micrograph of efferent ducts immunostained with anti-AQP-1 antibody. Numerous gold particles are seen on the microvilli (arrowheads) and surrounding apical subsurface vesicles (arrows) of this nonciliated cell.

Fig 20: Electron micrograph of efferent ducts immunostained with anti-AQP-9 antibody. A few gold particles are seen on the microvilli (arrowheads) of this nonciliated cells. However, numerous gold particles are seen decorating apical subsurface vesicles (arrows).


Mv
19

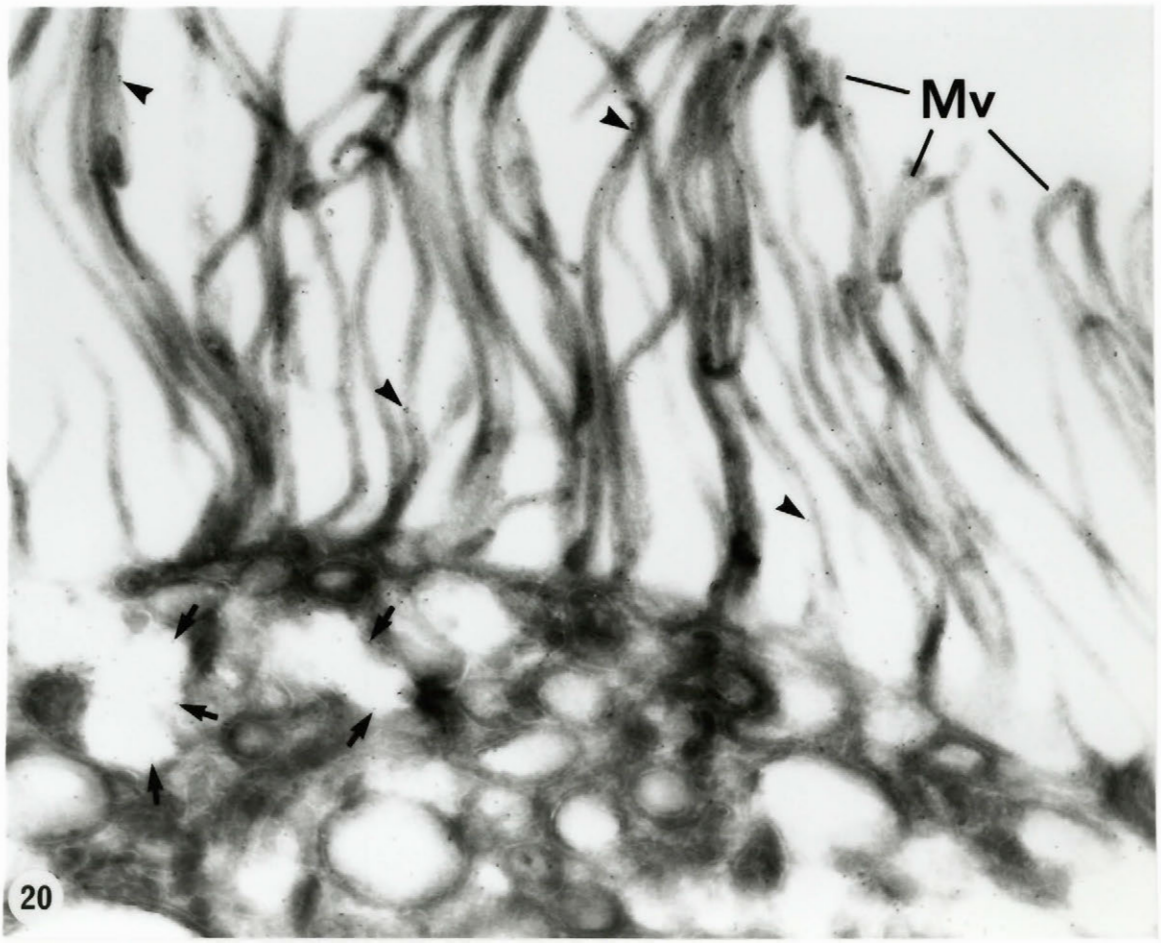

Mv
20

Table I. Expression of AQPs in testis and efferent ducts of normal animals

CELL TYPES	AQP-1	AQP-8	AQP-9
Sertoli	-[1]	++[2]	-
Leydig	-	-	++
Nonciliated	+++[3]	-	+++[4]
Ciliated	-	-	-

1) The number of plus signs is directly proportional to the intensity of the reaction with (+) being weak, (++) being moderate and (+++) being intense; (-) signifies absence of reaction.
2) Stage specific.
3) Intense on microvilli and basolateral plasma membrane.
4) Intense on apical rim of cytoplasm.

Table II. Expression of AQPs in epididymis of normal animals.

	AQP-1			AQP-8			AQP-9		
Cell types	P	C	VC	P	C	VC	P	C	VC
IS	-	NP	+++	-	-	-	+++	NP	-
Cap/ Cor	-	-	+++	-	-	-	++	-	-
Cau	-	-	+++	-	-	-	+++	+++	-

P: Principal cell; C: Clear cell; VC: Endothelial cell of vascular channels
IS: Initial segment; Cap: Caput; Cor: Corpus; Cau: Cauda
NP: Not present

Table III. Regulation of AQP-9 in principal cells of the epididymis.

	Normal	Orchidectomy				Orchidectomy + T			Ligation			
Day	N/A	3	7	14	21	3	7	14	3	7	14	21
IS	+++	-	-	-	-	-	-	-	-	-	-	-
Cap/ Cor	++	++	++	++	++	++	++	++	++	++	++	++
Cau	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

N/A: Not applicable

Table IV. Regulatin of AQP-9 in clear cells of the epididymis.

	Normal	**Orchidectomy**				**Orchidectomy + T**			**Ligation**			
Day	N/A	3	7	14	21	3	7	14	3	7	14	21
IS	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP
Cap/ Cor	-	-	-	-	-	-	-	-	-	-	-	-
Cau	+++	+++	+++	-	-	+++	+++	-	+++	+++	-	-

NP: Not present

Table V. Developmental expression of AQP-9 in epididymis.

Cell Type	**Principal**						**Clear**					
Day	7	21	29	39	49	Adult	7	21	29	39	49	Adult
IS	-	+	+++	+++	+++	+++	NP	NP	NP	NP	NP	NP
Cap/ Cor	-	+++	+++	+++	+++	+++	-	-	-	-	-	-
Cau	-	+++	+++	+++	+++	+++	-	-	+++	+++	+++	+++

NP: Not present

Table VI. Developmental expression of AQP-1 and AQP-9 in efferent ducts.

AQP	**AQP-1**						**AQP-9**					
Cell Type	**Nonciliated**						**Nonciliated**					
Day	7	21	29	39	49	Adult	7	21	29	39	49	Adult
Reaction	-	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

REFERENCES

Adamali HI, Hermo L. (1996) Apical and narrow cells are distinct cell types differing in their structure, distribution, and functions in the adult rat epididymis. *J. Androl.* 17: 208-222.

Andonian S, Hermo L. (1999) Principal cells of the vas deferens are involved in water transport and steroid synthesis in the adult rat. *J. Androl.* 20: 158-176.

Agre P, Preston GM, Smith BL, Jung JS, Raina S, Moon C, Guggino WB, Nielsen S. (1993) Aquaporin CHIP: the archetypal molecular water channel. *Am. J. Physiol.* 34: F463-F476.

Barham SS, Berlin JD, Brackeen RD. (1976) The fine structural localization of testicular phosphatases in man: the control testis. *Cell Tissue Res.* 166: 497-510.

Brown D, Katsura T, Gustafson CE. (1998) Cellular mechanisms of aquaporin trafficking. *Am. J. Physiol. Renal Physiol.* 44: F328-F331.

Brown D, Verbavatz JM, Valenti G, Lui B, Sabolic I. (1993) Localization of the CHIP28 water channel in reabsorptive segments of the rat male reproductive tract. *Eur. J. Cell Biol.* 61: 264-273.

Byers S, Graham R. (1990) Distribution of sodium-potassium ATPase in the rat testis and epididymis. *Am. J. Anat.* 188: 31-43.

Claus R, Dimmick M, Gimenez T, Hudson L. (1992) Estrogens and prostaglandin F2α in the semen and blood plasma of stallions. *Theriogenology.* 38: 687-693.

Claus R, Hoang-Vu C, Ellendorff F, Meyer H, Schopper D, Weiler U. (1987) Seminal oestrogens in the boar: origin and functions in the sow. *J. Steroid Biochem.* 27: 331-335.

Cooper TG. (1995) Role of the epidiymis in mediating changes in the male gamete during maturation. *In*: Tissue Renin-Angiotensin Systems, edited by AK Mukhopadhyay and MK Raizada, pp. 87-101. Plenum Press, New York.

Cornwall GA, Hahn SR. (1995) Specialized gene expression in the epididymis. *J. Androl.* 16: 379-383.

Crabo B (1965) Studies on the composition of epididymal content in bulls and boars. *Acta. Vet. Scand.* 6: 8-94.

Diamond JM and Tormey McD. (1966) Studies on the structural basis of water transport across epithelial membranes. *Fed. Proc.* 25: 1458-1463.

Fawcett DW, Leak LV, and Heidger PM. (1970) Electron microscopial observations on the structural components of the blood-testis barrier. *J. Reprod. Fert.* 10: 105-122.

Fisher JS, Turner KJ, Fraser HM, Saunders PTK, Brown D, Sharpe RM. (1998) Immunoexpression of aquaporin-1 in the efferent ducts of the rat and marmoset monkey during development, its modulation by estrogens, and its possible role in fluid resorption. *Endocrinol.* 139: 3935-3945.

Goyal HO, Bartol FF, Wiley AA, Neff CW. (1997) Immunolocalization of receptors for androgen and estrogen in male caprine reproductive tissues: unique distribution of estrogen receptors in efferent ductile epithelium. *Biol. Reprod.* 56: 90-101.

Gravis CJ, Yates RD, Chen IL. (1976) Light and electron microscopis localization of ATPase in normal and degenerating testes of Syrian hamsters. *Am. J. Anat.* 147: 419-432.

Griswold MD. (1993) Protein secretion by Sertoli cells: General considerations. *In*: The Sertoli cell, edited by LD Russell & MD Griswold, pp. 195-200. Cache River Press, Clearwater, FL.

Hermo L, Barin K, Robaire B. (1992) Structural differentiation of the epithelial cells of the testicular excurrent duct system of rats during postnatal development. *Anat. Rec.* 233: 205-228.

Hermo L, Lustig M, Lefrancois S, Argraves WS, Morales CR. (1999) Expression and regulation of LRP-2/Megalin in epithelial cells lining the efferent ducts and epididymis during postnatal development. *Mol. Reprod. Dev.* 53: 282-293.

Hermo L, Morales C. (1984) Endocytosis in nonciliated epithelial cells of the ductuli efferentes in the rat. *Am. J. Anat.* 171: 59-74.

Hermo L, Oko R, Morales C. (1994) Secretion and endocytosis in the male reproductive tract: A role in sperm maturation. *Int. Rev. Cyto.* 154: 105-189.

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

Hermo L, Papp S. (1996) Effects of ligation, orchidectomy, and hypophysectomy on expression of the Yf subunit of GST-P in principal and basal cells of the adult rat epididymis and on basal cell shape and overall arrangement. *Anat. Rec.* 244: 59-69.

Hermo L, Spier N, Nadler NJ. (1988) Role of apical tubules in endocytosis in nonciliated cells of the ductuli efferentes of the rat: a kinetic analysis. *Am. J. Anat.* 182: 107-119.

Hess RA, Gist DH, Bunick D, Lubahn DB, Farrell A, Bahr J, Cooke PS, Greene GL. (1997) Estrogen receptor (α and β) expression in the excurrent ducts of the adult male rat reproductive tract. *J. Androl.* 18: 602-611.

Iguchi T, Uesugi Y, Sato T, Ohta Y, Takasugi N. (1991) Developmental pattern of estrogen receptor expression in male mouse genital organs. *Mol. Androl.* 6: 109-119.

Ilio KY, Hess RA. (1992) Localization and activity of NA^+, K^+-ATPase in the ductuli efferentes of the rat. *Anat. Rec.* 234: 190-200.

Ishibashi K, Kuwahara M, Gu Y, Kageyama Y, Tohsaka F, Sugaki F, Marumo F, Sasaki S. (1997) Cloning and functional expression of a new water channel abundantly expressed in the testis permeable to water, glycerol, and urea. *J. Biol. Chem.* 272: 20782-20786.

Ishibashi K, Kuwahara M, Gu Y, Tanaka Y, Marumo F, Sasaki S. (1998) Cloning and functional expression of a new aquaporin (AQP9) abundantly expressed in the peripheral leukocytes permeable to water and urea, but not to glycerol. *Biochem. Biophys. Res. Comm.* 244: 268-274.

Ishibashi K, Kuwahara M, Kageyama Y, Tohsaka A, Marumo F, Sasaki S. (1997) Cloning and functional expression of a second new aquaporin abundantly expressed in testis. *Biochem. Biophys. Res. Comm.* 237: 714-718.

Ishibashi K, Yamauchi K, Kageyama Y, Saito-Ohara F, Ikeuchi T, Marumo F, Sasaki S. (1998) Molecular characterization of human aquaporin-7 gene and its chromosomal mapping. *Biochim. Biophys. Acta.* 1399: 62-66.

King LS, Agre P. (1996) Pathophysiology of the aquaporin water channels. *Ann. Rev. Physiol.* 58: 619-648.

Kirchoff C. (1999) Gene expression in the epididymis. *Int. Rev. Cytol.* 188:133-202.

Kuriyama H, Kawamoto S, Ishida N, Ohno I, Mita S, Matsuzawa Y, Matsubara K, Okubo K. (1997) Molecular cloning and expression of a novel human aquaporin from adipose tissue with glycerol permeability. *Biochem. Biophys. Res. Comm.* 241: 53-58.

Nielsen S, Smith BL, Christensen EI, Agre P. (1993) Distribution of the aquaporin CHIP in secretory and resorptive epithelia and capillary endothelia. *Proc. Natl. Acad. Sci. USA*. 90: 7275-7279.

Orgebin-Crist M-C. (1996) Androgens and epididymal function. *In*: Pharmacology, Biology, and Clinical Applications of Androgens, edited by S Bhasin et al., Wiley- Liss.

Orgebin-Crist M-C, Olson GE. (1984) Epididymal sperm maturation. *In*: The male in farm animal reproduction, edited by M Courot, pp. 80-102. Martines Nijhoff, Amsterdam.

Park JH, Saier MH Jr. (1996) Phylogenetic characterization of the MIP family of transmembrane channel proteins. *J. Membrane Biol.* 153: 171-180.

Parvinen M. (1993) Cyclic function of Sertoli cells. IN: The Sertoli cell, edited by LD Russell & MD Griswold, pp. 331-347. Cache River Press, Clearwater, FL.

Preston GM, Agre P. (1991) Molecular cloning of the red cell integral protein of Mr 28,000: A member of an ancient channel family. *Proc. Natl. Acad. Sci. USA.* 88: 11110-11114.

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

Russell LD. (1979) Spermatid-Sertoli tubulobulbar complexes as devices for elimination of cytoplasm from the head region of late spermatids of the rat. *Anat. Rec.* 194: 233-246.

Setchell BP, Brooks DE. (1988) Anatomy, vasculature, innervation, and fluids of the male reproductive tract. *In*: The physiology of reproduction, edited by E Knobil & JD Neill, pp. 753-836. Raven Press, Ltd., New York.

Tindall DJ, Vitale R, Means AR. (1975) Androgen binding protein as a biochemical marker for formation of the blood-testis barrier. *Endocrinol.* 97: 636-648.

Verkman AS, Van Hoek AN, Ma T, Frigeri A, Skach WR, Mitra A, Tamarappoo BK, Farinas J. (1996) Water transport across mammalian cell membranes. *Am. J. Physiol.* 270: C12-C30.

Voglmayer JK, Larsen LH, White IG. (1970) Metabolism of spermatozoa and composition of fluid collected from the rete testis of living bulls. *J. Reprod. Fertil.* 21: 449-460.

Waites GMH, Einer-Jensen N. (1974) Collection and analysis of rete testis fluid from macaque monkeys. *J. Reprod. Fertil.* 41: 505-508.

West NB, Brenner RM. (1990) Estrogen receptor in the ductuli efferentes, epididymis, and testis of rhesus and cynomolgus macaques. *Biol. Reprod.* 42: 533-538.

Wing TY, Christensen AK. (1982) Morphometric studies on rat seminiferous tubules. *Am. J. Anat.* 165: 13-25.

Wintour EM. (1997) Water channels and urea transporters. *Clin. Exp. Pharmaco. Physiol.* 24: 1-9.

CHAPTER III

Summary

SUMMARY

In the efferent ducts immunoreaction was localized to the apical cytoplasm of the nonciliated cells and to subsurface endocytic vesicles as well as microvilli. In the efferent ducts and epididymis, vascular channels of the intertubular space were also reactive.

Aquaporin-8 was exclusively localized to the testis. At all stages of the cycle of the seminiferous epithelium, the reaction was in the epithelium and not in the interstitial cells. The reaction hovered over germ cells located in the basal compartment at all stages except VII and VIII. In addition the reaction weaved its way between spermatocytes and spermatids and extended up to the lumen. At stages VII and VIII of the cycle, the reaction was seen apically and luminally where it encapsulated the late spermatids. Along with the staining pattern of the epithelium an absence of reaction over residual bodies and tails of late spermatids, it is suggested that the reaction is localized to Sertoli cells.

In the testis, immunoreaction with the anti-AQP-9 antibody was present exclusively in the interstitial space where it outlined the periphery of Leydig cells. In the efferent ducts, immunoreaction was localized to the apical rim of cytoplasm and to subsurface endocytic vesicles. In the initial segment, an intense reaction was noted on the microvilli of the principal cells as well as the apical rim of cytoplasm of some of these cells. In the caput and corpus regions of the epididymis, a weak reaction was observed on the microvilli of the principal cells. In the cauda epididymidis, a reaction was localized to the microvilli of the principal cells and few subcellular vesicles. Some clear cells were intensely reactive throughout their cytoplasm.

In conclusion, the data indicate not only cell and region specificity for the distribution of the different aquaporins in the testis, efferent ducts and epididymis, but

also subcellular distribution as well as tissue differences. The data also suggest that aquaporins are involved not only in water transport but of other molecules as well. Water transport may occur across the plasma membrane of cells but also the membranes of specifice intracytoplasmic organelles. The regulation experiments demonstrated that the aquaporins in the different tissues of the male reproductive tract may be regulated not only by androgens but also by the presence of luminal factors entering the testicular excurrent duct from the testis.