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Structural Abnormalities in the Testis and Epididymis of Cathepsin A Deficient Mice

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Structural Abnormalities in the Testis and Epididymis of Cathepsin A Deficient Mice.

To Dr. Hermo,
my mentor, teacher,
colleague and friend.

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"...and in the end, it's not the years in your life that count, it's the life in your years..."
A.L.

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Nadine Korah

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ABSTRACT

The lysosomal protective protein/cathepsin A (PPCA) forms a multi-enzyme complex with β-galactosidase and α-neuraminidase. Histological examination of the testis and epididymis of PPCA-/- mice from 2-10 months of age revealed cell- and region-specific abnormalities compared to their wild type counterparts. The testis showed a significant decrease in the diameter of seminiferous tubules, a significant increase in the size and number of interstitial space macrophages that became filled with pale lysosomes, and Leydig cells that showed an accumulation of pale lysosomes. The epithelial cells of the caput and corpus regions of the epididymis were highly vacuolated, containing numerous pale lysosomes, with some cells appearing to undergo degeneration. Halo cells of the entire epididymal epithelium were also highly engorged with pale lysosomes, and in the intertubular space large vacuolated macrophages were prominent. Taken together, the adverse effects on the testis and epididymis suggest that sperm in the lumen may be reduced in numbers and not acquire their full maturational state, accounting for reduced pregnancies noted in PPCA-/- mice.

RÉSUMÉ

La protéine lysosomale protectrice cathepsine A (PPCA) forme un complexe multi-enzymatique avec la β-galactosidase et la α-neuraminidase. L'examen histologique des testicules et des épididymes de souris PPCA -/-, âgées de 2 à 10 mois, a mis en évidence des anomalies spécifiques à la fois aux cellules et à leurs régions, en comparaison avec les souris issues de souche sauvage. Dans les testicules, une baisse significative du diamètre des tubules séminifères a été observée. Une augmentation significative dans la taille et le nombre de macrophages dans l'espace interstitiel qui sont alors remplis de lysosomes pâles a également été notée. De plus, les cellules de Leydig montrent aussi une accumulation de lysosomes pâles. Les cellules epithéliales des régions de la tête et du corps de l'épididyme comprenaient de très nombreuses vacuoles, contenant beaucoup de lysosomes pâles, et il y avait également des cellules qui semblaient subir une dégénérescence. Les cellules halo de l'ensemble de l'epithelium epididymal étaient aussi remplies de nombreux lysosomes pâles et, dans l'espace intertubulaire, les larges macrophages à vacuoles étaient très présents. L'ensemble de ces effets néfastes sur les testicules et les épididymes semblent suggérer que les spermatozoïdes dans la lumière pourraient être réduits en nombre et ne pas acquérir un état de maturité total, si nous tenons compte du nombre réduits de grossesse remarqué chez les souris PPCA -/-.

CHAPTER I

The male's reproductive role is to manufacture gametes called sperm, and to deliver them to the female reproductive tract, where fertilization occurs and offspring are produced. Sperm are produced in the testis, while the epididymis plays essential roles in the maturation, transport, protection and storage of sperm. Along the length of the male reproductive tract, a continually changing luminal environment is achieved by the secretion and endocytic activities of the cells, and is essential for sperm development. One such lysosomal enzyme is cathepsin A, also know as protective protein cathepsin A (PPCA). Thus, as PPCA appears to be a significant enzyme within the male reproductive tract, it merits further investigation.

MALE REPRODUCTIVE SYSTEM

The male reproductive organs include two testes, two epididymides each with a vas deferens, and the accessory glands. The testis is specialized in the production of testosterone and germ cells, which undergo mitotic and meiotic divisions and eventual metamorphosis into a specialized haploid cell, the sperm, which, after transit through the epididymis, becomes capable of fertilizing a viable oocyte in the female reproductive tract.

The sperm produced by the testes are immature and require exposure to the microenvironment created within the lumen of the efferent ducts and epididymis before acquiring motility and the ability to fertilize. The vas deferens is responsible for the ultimate transport of sperm to the urethra and final delivery out of the body (Robaire and Hermo, 1988). The rete testis, efferent ducts, epididymis and vas deferens collectively are known as the excurrent duct system.

The accessory sex glands are specialized in the secretion of various substances that contribute to the formation of semen, and include the prostate, bulborethral glands and seminal vesicles (Price and Williams-Ashman, 1961; Mann and Lutwak-Mann, 1981).

HISTOLOGY OF THE TRACT

A vast amount of information is known about the histology of the male reproductive tract as a result of meticulous morphological observations recorded from light and electron microscopic studies of plastic embedded tissues.

TESTIS

The testes are paired ovoid organs that, in many species, are located in an out-pocketing of skin known as the scrotum. This localization maintains the testes at a temperature lower than the rest of the body, which is required for spermatogenesis to proceed normally (Setchell, 1982). A dense connective tissue capsule known as the tunica albuginea encapsulates each testis (Davis et al., 1970; Lesson and Cookson, 1974). In some species, such as the human, extensions of a thickened area in the tunica albuginea form septa that subdivide the testis into lobules. Adjacent to the tunica albuginea is a highly vascularized loose connective tissue layer known as the tunica vasculosa.

The majority of the testis consists of numerous highly convoluted seminiferous tubules and intervening islands of interstitial tissue. The seminiferous tubules form double-open-ended loops that end into an anastamozing network of tubes known as the rete testis (Clermont and Huckins, 1961). The former are the site of spermatogenesis,

where germ cells are found at varying stages of development, along with the supporting non-dividing Sertoli cells. The periphery of these tubules is bound by a limiting membrane, which consists of connective tissue elements and contractile myoid cells (Clermont, 1958; Dym and Fawcett, 1970).

The testicular interstitial space fills up the spaces between the seminiferous tubules. It contains all the blood and lymph vessels and nerves of the testicular parenchyma (Fawcett et al., 1973). Also located in the interstitium are hormone-secreting Leydig cells and macrophages (Christensen, 1975; Mori and Christensen, 1980).

CELL TYPES AND FUCTION

Sertoli Cell

Structure

The Sertoli cell is oriented radially within the round profile of a cross-sectioned seminiferous tubule. It is the supporting or sustentacular cell of the seminiferous epithelium and is terminally differentiated (Clermont and Perey, 1957; Kluin et al., 1984). It is a tall columnar cell that extends from the basal lamina toward the central point within the lumen. The nucleus is basally located, often with frequent infoldings of the nuclear envelope, and in many species the organelles within the cytoplasm are compartmentalized (Fawcett, 1975). The Golgi apparatus is a large supranuclear organelle formed by stacks of membrane saccules. Also contained in the cytoplasm are clusters of membrane-bound lysosomes, free ribosomes, cytoskeletal elements such as

microtubules, glycogen granules, and numerous spherical and elongated mitochondria (Fawcett, 1975; Griswold et al., 1988).

The Sertoli cell possesses numerous cytoplasmic processes that interdigitate and envelope the developing germ cells, as well as deep apical invaginations into which the more mature germ cells are embedded (Wong and Russell, 1983). A unique type of actin-related intercellular adhesion junctions, known as ectoplasmic junctions, also exists in the testis (Russell et al., 1980). These junctions are related to the turnover of adhesion between Sertoli cells, to the attachment of spermatids to the seminiferous epithelium, and to sperm release. They are also part of the mechanism by which spermatids are moved through the epithelium. Sertoli cells continuously alter their structure as they mobilize germ cells from the base to the lumen and accommodate changes to the germ cells' shape along the way. Specifically, Sertoli cells extend processes beneath spermatogonia cells as they mature into primary spermatocytes, and lift them upwards as new inter-Sertoli cell junctions are formed beneath (Morales and Clermont, 1993).

Blood-Testis Barrier:

Tight junctions are formed between the Sertoli cells to create the blood-testis barrier (Dym and Fawcett, 1970; Russell and Peterson, 1985; Cyr et al., 1999). This barrier establishes two distinct compartments within the seminiferous epithelium, the basal and adluminal compartments. As a result, the seminiferous tubules are capable of excluding from the tubular lumen substances normally present in the blood and lymph. Thus, a special environment is created within the seminiferous tubules in which spermatozoa can develop. Also, the blood-testis barrier is thought to protect the haploid

germ cells from immune attack since they express antigens that are distinct from those of the somatic cells (Morales and Clermont, 1993; Millette and Bellvè, 1977).

Function: secretion

The Sertoli cell has numerous functions essential to the viability of the seminiferous epithelium. They provide physical, physiological and biochemical support for germ cell development. For instance, the Sertoli cell continuously produces fluid in which the developing germ cells are bathed, which also serves as a medium to allow sperm to enter the efferent ducts (Setchell et al, 1969; Hinton and Setchell, 1993). Aquapoprins (AQPs) are membrane protein channels that allow the rapid passage of water through an epithelium containing tight junctions. In the testis, AQP-8 is expressed exclusively to the apicolateral plasma membrane of the Sertoli cells, allowing for the passage of water from the interstitial space into the lumen (Badran and Hermo, 2002).

The Sertoli cell is also known to secrete a variety of nutrients, growth factors and proteins such as inhibin, SGP-2 and androgen-binding protein (ABP)(Griswold, 1993; Sylvester et al., 1984; Gunsalus et al., 1980), as well as protesases and antiprotesases (Fritz et al., 1993; Griswold, 1995). This secretory activity of Sertoli cells varies significantly during the cycle of spermatogenesis, thus providing the optimal luminal environment for germ cells. Such secretions also provide evidence that support the proposed "nurse cell" function of the Sertoli cell, as protein products of the Sertoli cells interact directly with germ cells.

Function: endocytosis

The Sertoli cell is also a phagocytic cell that is involved in the uptake and degradation of residual bodies (Kerr and de Krestser, 1974), degenerating germ cells

(Russell and Clermont, 1977), and other cytoplasmic elements. Sertoli cells utilize their own lysosomes and harmoniously integrate two distinct endocytic processes at their apex, fluid-phase endocytosis and phagocytosis, while receptor-mediated endocytosis takes place at the base of Sertoli cells (Morales et al., 1985; Clermont et al, 1987). The lysosomes of the seminiferous epithelium show cyclical variations, with an increase toward the time of spermiation and a decrease after the residual bodies have been digested. Furthermore, it has been demonstrated that cultured Sertoli cells are active phagocytes, and that such phagocytic activity is under negative control by follicle stimulating hormone (FSH) (Filippini A et al., 1989).

Regulation:

Spermatogenesis is a compartmentalized and continuous process that takes place sequestered within the blood-testis barrier, implicating a need for regulation by locally produced factors as well as extrinsic endocrine hormones. Gonadotropin releasing hormone (GnRH) released by the hypothalamus stimulates gonadotrophs of the anterior pituitary to release lutenizing hormone (LH) and follicle stimulating hormone (FSH). The former activates the interstitial Leydig cells to produce testosterone, whereas FSH induces Sertoli cells to synthesize androgen-binding protein (ABP) via a cAMP intermediary. Testosterone then binds to ABP and the complex is either released into the lumen of the seminiferous tubules, where the elevated testosterone concentration enhances spermatogenesis (Griswold, 1995), or it acts on its target cells, the Sertoli and myoid cells (Bardin et al., 1988; Zirkin, 1995). These target cells subsequently perform their respective functions in promoting spermatogenesis.

In addition, growth factors and cytokines produced within the seminiferous epithelium are known to influence post-meiotic stages of spermatogenesis (Eddy, 1998). Cytokines are also important for the integration of the neuro-endocrine-immune network that controls testicular function (Mayerhofer, 1996; Rivier and Rivest, 1991; Turnbull and Rivier, 1995).

Germ cells

There are three major germ cell populations that exist in the testis: spermatogonia, spematocytes, and spermatids. Spermatogonia divide by mitosis to give rise to spermatocytes that undergo two meiotic divisions to give rise to the haploid spermatids, which then undergo metamorphosis and become the fully differentiated sperm cell or spermatozoa. This entire process is referred to as spermatogenesis.

Spermatogonia are diploid cells that can be found in the basal compartment of the seminiferous epithelium (Leblond and Clermont, 1952). They divide by mitosis to renew themselves and constitute the population of cells destined for meiosis (Clermont and Leblond, 1953). Daughter cells of the latter remain connected via cytoplasmic bridges due to incomplete cytokinesis; they are referred to as intercellular bridges (Weber and Russell, 1987). Once spermatogonia differentiate into early preleptonene spermatocytes, adjacent Sertoli cells project cell processes beneath these germ cells, gradually establishing new tight junctions beneath them to move them into the adluminal compartment (Russell, 1993; Morales and Clermont, 1993; Vogl et al., 1993).

Primary spermatocytes go through a first meiotic division to produce secondary spermatocytes, which proceed with the second meiotic division. The latter reduces the

number of chromosomes in half thus giving each daughter cell one of the homologous pairs. Diploid primary spermatocytes containing twice the normal DNA content (4N) after DNA synthesis, divide into two secondary spermatocytes with a haploid chromosome number and a full amount of DNA (2N). The small, short-lived secondary spermatocytes then divide in a mitotic fashion to produce two daughter cells which each receive one chromatid of half the number of chromosomes. The result is two spermatids with a haploid chromosome number and haploid DNA content (1N) (Gartner and Hiatt, 1997).

Spermatids are haploid germ cells that no longer divide, but will differentiate, by a process known as spermiogenesis, to assume the characteristic appearance of structurally mature spermatozoa. They undergo a series of morphological changes, including the development of an acrosome, elongation and condensation of the nucleus, polarization of its cytoplasm and its organelles, and formation of the tail (Clermont et al., 1993). Prior to release into the seminiferous tubule as a nonmotile spermatozoon incapable of fertilizing an oocyte, the majority of the cytoplasm is shed, forming the residual body that is later phagocytosed by the Sertoli cell (de Krester and Kerr, 1988; Russell et al., 1993; Vogl et al., 1993).

Spermatogenesis

The process in which spermatogonia are converted into structurally mature spermatozoa is known as spermatogenesis. It has been divided into three phases – proliferative phase, meiotic phase and differentiation or spermiogenic phase – to describe the changes taking place (Russell et al., 1990).

The proliferative phase involves the numerous division taking place by the spermatogonia germ cells of which there are three types: stem cell spermatogonia, proliferative spermatogonia, and differentiating spermatogonia. The proliferative and differentiating spermatogonia are committed cells that have a high mitotic rate and divide rapidly to generate the millions of germ cells produced by the testis each day. The renewing stem cell spermatogonia divide to give rise to more stem cells, and to cells that will undergo proliferation and differentiation (Clermont and Leblond, 1953). These spermatogonial divisions increase their population one thousand fold, while the processes of meiosis and spermiogenesis build up the population by only four fold (Russell et al., 1990). In the end, the most mature spermatogonia divide to form the young primary spermatocytes; specifically preleptotene spermatocytes.

Preleptotene spermatocytes are the last cells of the spermatogenic sequence to go through the S-phase of the cell cycle, that is the replication of DNA phase. Germ cells are now ready to undergo the second phase of spermatogenesis, the meiotic phase. In this phase, genetic material is halved in each cell, while the number of germ cells is quadrupled after the completion of the two meiotic divisions (Russell et al., 1990). Genetic recombination also occurs during this phase and results in genetic variability of the resulting germ cells. The end result is the production of haploid spermatids.

The differentiation or spermiogenic phase involves cell transformations without cell divisions. The coordination of several morphogenic processes can be described from staging maps that have been organized for various species; that is, the classification of spermatogenesis and spermiogenesis is based on stages defined by grouping germ cell types at particular phases of development (Leblond and Clermont, 1952a,b). Among the

several modifications that take place, the developing spermatid acquires a flagellum, develops an acrosome, condenses its nucleus and sheds a small amount of cytoplasm (Russell et al., 1990).

Migration of a centriole pair to the cell surface forms an axoneme, a structure containing microtubules in the classic 9+2 arrangement, which causes the spermatid plasma membrane to protrude from the cell, ending in the formation of a flagellum or tail. Accessory components are added to the flagellum, and mitochondria are recruited from the cytoplasm to form a helical pattern around the flagellum (Clermont et al., 1990). Flagellar development is a continuous process, lasting from the start of the spermiogenic phase until the sperm are released. Although the flagellum imparts motility to the cell, sperm released in the testis are essentially immotile; the capability for motility is developed in the epididymis (Robaire and Hermo, 1988).

Shortly after spermatids are formed, the Golgi apparatus is involved in producing small condensing vacuoles or proacrosomal vesicles that contain a dense material or proacrosomal granules. Eventually, through coalescence of several proacrosomal granules, a single granule known as the acrosomal vesicle is formed. This vesicle is rounded until it makes contact with the nucleus, after which it flattens to conform to the shape of the nucleus. It condenses and increases in density as the spermatid matures (Gardner, 1966; Clermont et al., 1994). As time passes, the nucleus progressively elongates, as does the acrosome, while the Golgi apparatus moves towards the flagellum or tail. The acrosome is considered to be a sac of secreted enzymes necessary for penetration of the oocyte (Clermont et al., 1993; Abou-Haila and Tulsiani, 2000).

The changes seen in the shape of the spermatid head and nucleus provide a secondary means to classify spermatids as either round spermatids or elongated spermatids. The sperm head attains a characteristic shape just before release into the lumen, as does the nucleus of the spermatozoa. Furthermore, the nucleus condenses, caused by changes in histones and other specific proteins that associate with DNA (Fawcett et al., 1971), including the perinuclear theca proteins. The perinuclear theca is defined as a cytoskeletal element encapsulating the nucleus of spermatozoa. Its proteins are an assortment of cytosolic, basic and traditionally nuclear proteins that condense during development for diverse functions during spermiogenesis and fertilization (Oko et al., 2001).

The size of the spermatid also reduces in volume to approximately 25% of its original size (Sprando and Russell, 1987), through loss of water and cytoplasm. The separation of a cytoplasmic package or residual body at sperm release enables the spermatid to shed organelles and inclusions that were used earlier by the cell but are no longer necessary for survival. These cytoplasmic fragments are phagocytosed by the Sertoli cell and transported to the base of the tubule where they are digested by the Sertoli cell (Kerr and de Krester, 1974). Thus, through changes in size and shape, spermatids become hydro-dynamically streamlined to move in the fluid environment of the female reproductive tract (Russell et al., 1990).

When spermiogenesis is complete, the structurally mature spermatozoa line up along the luminal border of the seminiferous tubules in preparation for release by a process known as spermiation. This process involves breaking of specialized junctions between Sertoli and germ cells, known as ectoplasmic specializations. They are thought

to prevent premature sloughing of germ cells into the seminiferous tubule lumen. These ectoplasmic specializations consist of a layer of smooth ER and actin filaments from the Sertoli cell cytoplasm and the adjacent germ cells (Ross, 1976; Franke et al., 1978; Vogl et al., 1993). Just prior to release, the ectoplasmic specializations dissipate, and the spermatid and Sertoli cell jointly form structures called tubulobulbar complexes, which project from the ventral concavity of the head to indent the Sertoli cell (Russell and Clermont, 1976). They are thought to be a means by which cytoplasmic fluids can be eliminated at sperm release. They are also the last junctional complexes formed just before the residual body is pinched off and the spermatid is deposited into the lumen (Russell, 1993).

Staging of Spermatogenesis

As the germ cells mature, they transcend towards the lumen. Their vacated positions are simultaneously filled by germ cells of an earlier stage of development (Desjardins and Ewing, 1993). A stage or cell association is a defined grouping of germ cell types at particular phases of development in cross-sectioned tubules. Each cell types of the cell association is morphologically integrated with the others in its developmental processes (Russell et al., 1990). Leblond and Clermont (1952a,b) arbitrarily divided the cell associations into the fourteen stages of spermatogenesis in the rat, based on acrosome development and nuclear morphology. In the mouse, there are twelve stages, designated by the roman numerals I-XII, and the entire spermatogenic process takes approximately 35 days (Oakberg, 1956a, b).

The criteria set forth in staging is only a rough estimate of starting and stopping points in the continuous differentiation of germinal cells. Spermatogonia are found at all

stages of the cycle, but the type of spermatocytes and spermatids present may vary from stage to stage. Therefore, spermatids are often used to distinguish one stage from another, based on their morphological appearance (Russell et al., 1990). The development of the spermatids via the process of spermiogenesis has been divided into 16 steps. The first 12 steps can be used to identify the stage of spermatogenesis, as each of these steps is unique to the 12 stages of the cycle. Step 1-7 spermatids are recognizable by the increasing spread of the acrosomal system over the surface of the nucleus, while the nuclear shape and extent of chromatin condensation can distinguish steps 8-12.

The synchronous development of the germ cells is possible due to open cytoplasmic continuity between the cells known as intercellular bridges (Dym and Fawcett, 1971; Weber and Russell, 1987). All germ cells of a specific type will undergo the same developmental changes at approximately the same time, and these changes are also coordinated with the changes of the other germ cells found within that cell association. Intercellular bridges form when the germ cells divide and undergo incomplete cytokenesis, such that the daughter cells do not pinch off from each other by remained connected by these cytoplasmic constrictions (Dym and Fawcett, 1971). Cytoskeletal components such as actin and intermediate filaments are thought to maintain the integrity of the intercellular bridges. This would allow for the transport of substances such as mRNA through them, such that all members of a clonal population can express the same proteins (Braun et al., 1989).

Leydig Cells

Leydig cells constitute the major cell type found in the testicular interstitium. They often exist in clusters or groups that lie near blood vessels (Christensen, 1975; Mori and Christensen, 1980). Leydig cells are the main androgen-producing cell of the testis and function in the biosynthesis of testosterone in response to direct stimulation by luteinizing hormone (LH) (Zirkin et al., 1980; Wing et al., 1985). Testosterone then binds to androgen receptors on the Sertoli cell surface, which produce signals for the proper completion of spermatogenesis (Sanborn et al., 1977; Tindall et al., 1977).

Structure:

Leydig cells are often described as polygonal or fusiform cells (Clark, 1976), and their shape appears to be easily influenced by physical pressures, especially pressures caused by other cells around them (Russell and Burguet, 1977). Leydig cell nuclei often are eccentrically placed within the cell and usually display a round or irregularly oval shape. Numerous lipid inclusions in the cytoplasm serve as storage sites for cholesterol, the substrate for the synthesis of testosterone, while swirls of smooth endoplasmic reticulum (sER) serve as the site of steroidogenesis. Numerous enzymes necessary for this conversion are bound to the surface of the sER (Christensen, 1975; de Krester and Kerr, 1988).

Function: endocytosis

In addition to their endocrine secretory function, Leydig cells are actively involved in both fluid-phase and adsorptive endocytosis. Many studies have utilized different tracers to determine these various endocytic pathways in Leydig cells (Hermo et al., 1985a; Hermo and Lalli, 1988; Bozon et al., 1998). Various types of vesicular

profiles distinct from Golgi elements and sER are present in the cytoplasm of Leydig cells. Finally, autophagy, a cellular process by which small portions of cytoplasm are segregated and destroyed by lysosomal enzymes (de Duve and Wattiaux, 1966) has also been described in Leydig cells (Tang et al., 1988).

Macrophages

Structure:

Macrophages form a substantial portion of the interstitial cells (~20%) of the testis, but with none found within the seminiferous tubule. Resident macrophages have specialized functions in addition to the classical macrophage activities. Resident testicular macrophages are involved in immunological surveillance, immunoregulation, tissue remodeling and phagocytosis, and they express major histocompatibility complex class II (MHC II) molecules characteristic of normal antigen-presenting cells (Miller et al., 1983; Hutson, 1994; Hedger, 1997). However, testicular macrophages also have unique and highly specialized properties, presumably important for their interaction with Leydig cells. Though testicular macrophages have been shown to secrete cytokines, it has been suggested that they have blunted secretory response compared to peritoneal macrophages. In addition, testicular macrophages demonstrate a unique pattern of protein secretion and express follicle stimulating hormone (FSH) receptors (Orth and Christensen, 1977; Hutson and Stocco, 1989). However, the most unique feature of all is their intimate association with Leydig cells.

Leydig cell – macrophage interactions:

Intriguing and unique contact sites, commonly called digitations, exist between macrophages and Leydig cells (Christensen and Gillim, 1969; Hutson, 1992). The narrow, finger-like processes of the Leydig cell enter minute recesses of the macrophage and terminate at the concavity of a coated pit. These intercellular relationships form just prior to the pubertal surge in testosterone secretion, therefore suggesting a role in regulating reproductive functions (Hutson, 1992). Studies conducted in vitro and in vivo indicate that macrophages play an important role in regulating steroidogenesis in Leydig cells by releasing both stimulatory and inhibitory paracrine factors (Hutson, 1998; Hales, 2002). The inhibitory factors appear to be well known immune proteins, IL-1 and TNFα, while the stimulatory factor has recently been identified as 25-hydroxycholesterol, a lipophilic factor that acts through a StAR-independent pathway (Nes et al., 2000; Lukyanenko et al., 2001).

The cytotoxic drug ethane 1,2-dimethane sulphonate (EDS) causes the acute and selective destruction of Leydig cells (Morris et al., 1986; Kerr et al., 1987; Nandi et al., 1999). After EDS treatment, it has been noted that extensive phagocytosis of dead Leydig cells by testicular macrophages occurs, and a subsequent increase in proliferative activity as new Leydig cells repopulate the interstitium. At the same time, there is an increase in the number of resident macrophages and the first morphological signs of inflammation (Teerds et al, 1989; Gaytan et al, 1994; Hales, 2002). It is therefore probable that phagocytosis of necrotic or apoptotic Leydig cells, and/or other signals activate testicular macrophages to secrete growth factor(s) that stimulate the proliferative activity of the

interstitial cells, thus further supporting a dual morphological and functional relationship between these two interstitial cells.

Regulation:

Colony stimulating factor-1 (CSF-1) regulates the survival, proliferation and differentiation of cells of the mononuclear phagocytic lineage (Stanley et al., 1983; Cohen et al., 1996). Its action is mediated through a high-affinity cell surface receptor present on all cells belonging to the same lineage, including terminally differentiated macrophages. Male mice that lack CSF-1, and consequently have depleted numbers of macrophages, have reduced mating ability, low sperm counts, and 90% lower serum testosterone. Studies have revealed that low testosterone levels are due to reduced testicular Leydig cell steroidogenesis, associated with severe ultrastructural abnormalities and diminished amounts of steroidogenic enzyme proteins (Cohen et al., 1997). It has also been noted that the expression of the CSF-1 receptor appears to be restricted to macrophages of the testicular interstitium (Cohen et al., 1996); thus, macrophages may in fact regulate Leydig cell steroidogenesis.

On the other hand, factors have been identified to be secreted by Leydig cells to regulate macrophages, namely macrophage migration inhibitory factor (MIF). MIF was originally identified as a protein secreted by activated T lymphocytes associated with delayed-type hypersensitivity (Bloom and Bennett, 1966). Recently, MIF was localized almost exclusively to the Leydig cells in the interstitial regions of the rat testis (Meinhardt et al., 1996). In this study, however, they concluded that MIF acts as a paracrine regulator of Sertoli cell inhibin secretion and not the interstitial macrophage population.

EXCURRENT DUCT SYSTEM

The excurrent duct system is the arrangement of ducts that connects the seminiferous tubules with the urethra. It consists of the rete testis, efferent ducts, epididymis and vas deferens. The rete testis is a network of ducts that is in direct continuation with the seminiferous tubules of the testis. It joins with the efferent ducts, a series of 2-33 ducts (Ilio and Hess, 1994) that eventually fuse to form the single, highly coiled duct known as the epididymis (Orgebin-Crist, 1969; Robaire and Hermo, 1988). The epididymis can be anatomically divided into four regions: the initial segment, caput (head), corpus (body) and cauda (tail). Following the cauda, the duct uncoils to become a single straight tube known as the vas deferens, which eventually connects with the urethra which empties to the outside of the body.

The epithelium of the excurrent duct system is largely involved in absorption of fluid and ions, as well as secretion of proteins and small molecules (Robaire and Hermo, 1988). Absorption leads to an increase in sperm concentration, while a combination of absorption and secretion can lead to an alteration of sperm thought to complete their development in order to yield mature spermatozoa that are capable of fertilization (Hermo et al., 1994).

CELL TYPES AND FUNCTION

Rete Testis

The epithelium of the rete testis is low cuboidal and its cells possess short microvilli on their apical plasma membrane. Extensive interdigitating folds are also seen

along the lateral surfaces of the epithelial cells, as well as large basal processes containing an accumulation of mitochondria – all characteristics of epithelial cells known for their active transport of substances from the lumen to the base of the cell. Using tracers, the epithelial cells of the ret testis were found to practice both fluid-phase and adsorptive endocytosis (Morales et al., 1984; Morales and Hermo, 1986).

Efferent Ducts

The cell height of the efferent duct epithelium increases dramatically from the rete testis to tall columnar nonciliated and ciliated cells. Nonciliated cells lack cilia but possess a brush border made of numerous microvilli, while ciliated cells are recognized by the many cilia on their apical surface, and an elongated nucleus located near the apex. Both cells contain vesicular structures involved in endocytosis, such as tubular coated pits, endosomes, multivesicular bodies and lysosomes (Hermo and Morales, 1984; Robaire and Hermo, 1988). In addition, the ciliated cells are involved in the movement of the luminal contents through the ducts via the rhythmic beating of their cilia (Hermo et al., 1985b).

The major function of the efferent ducts is the reabsorption of luminal fluid, which increases the concentration of sperm several fold as they enter the epididymis. This highly active fluid reabsorption can remove between 50% and 95% of the luminal fluids (Hamilton, 1975; Ilio and Hess, 1994). The transcellular pathway via the many channels and receptors located both apically and basally appears to be the dominant mechanism for water reabsorption, including AQP-1 and –9 which have been localized to the apical and basolateral membranes respectively (Badran and Hermo, 2002).

The efferent ducts also have the capacity to reabsorb molecules through fluid-phase, adsorptive and receptor-mediated endocytosis (Morales and Hermo, 1983; Hermo and Morales, 1984). Both cell types are active endocytic cells involved in the uptake of various proteins, including SGP-1 and -2 (prosaposin and clusterin) (Hermo et al., 1985b; Morales et al., 1996). Furthermore, many proteins not present in the fluids of the rete testis are present in the initial segment or caput epididymis, indicating that the efferent duct epithelium synthesize and secrete proteins into the lumen. The regulation of the luminal contents in the efferent ducts is the first step in creating a specialized environment in which the sperm are able to mature (Robaire and Hermo, 1988; Kirchhoff, 1999).

Epididymis and Vas Deferens

The epididymis and vas deferens possess a pseudostratified columnar epithelium with stereocilia, which are microvilli. Though the cell types remain relatively consistent between the regions of the epididymis and vas deferens, the epithelial characteristics vary along the length of the duct.

Principal cells

The principal cell is the major cell type found in all four regions of the epididymis. In general, they are a columnar cell with a basally located nucleus and prominent nucleolus. The supranuclear region contains many dense granules, while a typical brush border lines their luminal aspect. Their structure and function vary dramatically between the different regions, namely in the appearance and organization of

their secretory apparatus (ER, Golgi and secretory granules), and their endocytic apparatus (coated pits, endosomes, multivesicular bodies and lysosomes) (Robaire and Hermo, 1988; Hermo and Robaire, 2002).

The principal cell of the initial segment is very tall, at times protruding into the lumen beyond the areas of the junctional specializations. It contains many numerous filaments, microtubules, glycogen and mitochondria, as well as an elaborate collection of vesicular elements reflective of their secretory function. The principal cells of the caput, corpus and cauda are shorter than those of the initial segment, yet they still contain numerous vesicles in their apical region and an abundance of rough ER in their basal region (Robaire and Hermo, 1988; Hermo and Robaire, 2002).

A major difference noted between principal cells of the caput, corpus and cauda is in the appearance of a number of large, supranuclear lysosomes. In the caput, these bodies are pale and contain loose membranous profiles; in the corpus, they take on irregular shapes and contain a uniform homogeneous electron-dense material; in the cauda, whorls of membranous profiles are embedded in dense-staining homogeneous material (Robaire and Hermo, 1988). Another dramatic difference found in principal cells of these three segments is that only in the corpus do lipid droplets become numerous in the supranuclear region of the principal cell.

The luminal environment of the epididymis is highly specialized, and tight junctions between epididymal epithelial cells regulate and distinguish the lumen from the blood circulation (Friend and Gilula, 1972). The formation of this blood-epididymal barrier is much like the blood-testis barrier; it enables the germ cells to exist in an immune privileged compartment (Cyr et al., 2002).

The fluid surrounding the spermatozoa experiences continuous and progressive changes in its composition throughout the male excurrent duct. Essentially all proteins secreted into the epididymal lumen are synthesized by principal cells and interact with the sperm. Secretion of different substances, such as SGP-2, Immobilin, Glycosidases and ABP is region-specific, and therefore the sperm encounter a constantly changing luminal environment as they proceed the length of the epididymis (Hermo and Robaire, 2002; Dacheux and Dacheux, 2002). Endocytosis of luminal contents in a region-specific manner also aids in creating this gradually changing environment. Expression of lysosomal enzymes such as β–hexosiminidase A, cathepsins A, D and B and SGP-1 is both cell- and region-specific within the epididymal epithelium (Igdoura et al., 1995; Hermo et al., 1997; Luedtke et al., 2000). This also suggests that the lysosomes of these different cell types act upon different substrates that are derived from the endocytosis of substances. Although not the major endocytosing cell of the epididymis, principal cells do contain an endocytic apparatus that is able to degrade ingested substances (Robaire and Hermo, 1988; Hermo and Robaire, 2002).

Narrow cells

Narrow cells appear only in the initial segment of the epididymis (Sun and Flickinger, 1980; Adamali and Hermo, 1996). They are narrower than the adjacent principal cells, with a wider apex and a thin process of cytoplasm reaching the basement membrane. They are characterized by numerous apically located cup-shaped vesicles that are involved in endocytosis and which secrete H^+ ions into the epididymal lumen (Robaire and Hermo, 1988; Hermo et al., 2000). The luminal fluid along much of the

epididymis is maintained at an acidic pH (Levine and Kelly, 1978). The low pH is suggested to be important for sperm maturation, by maintaining sperm in an immotile state during epididymal transit along with specific proteins, weak acids and ions, and prevention of premature activation of acrosomal enzymes (Cooper, 1986; Hinton and Palladino, 1995).

Like the principal cells, narrow cells also exhibit regional specificity in the expression of certain proteins such as glutathione S-transferases (GSTs), carbonic anhydrase II and lysosomal enzymes (Adamali and Hermo, 1996).

Apical cells

Often mistaken as narrow cells, apical cells are a distinct cell type found in the initial segment of the epididymis. They are identified in the light microscope as pale goblet-shaped cells that do not contact the basement membrane, showing a large pale-stained round nucleus in the upper half of their cytoplasm (Adamali and Hermo, 1996; Hermo and Robaire, 2002). Apical cells also differ dramatically from adjacent principal cells, showing no elaborate Golgi apparatus, ectoplasmic reticulum or secretory vesicles.

Like narrow cells, localization of various proteins and lysosomal enzymes suggests that apical cells may be involved in endocytosis; little else is known about the specific functions of these cells (Hermo and Robaire, 2002). The localization of carbonic anhydarse II, however, is exclusive to narrow cells, indicating that these two cell types are distinct, and perhaps perform different functions (Adamali and Hermo, 1996).

Clear cells

Clear cells are found in the caput, corpus and cauda epididymides, but are absent from the initial segment and vas deferens (Hamilton, 1975; Robaire and Hermo, 1988). They are large active endocytic cells identified by a highly vesiculated apical region and short and irregularly spaced microvilli. Their apex contains numerous coated pits, vesicles, endosomes, multivesicular bodies and lysosomes, while the basal region houses a nucleus, an accumulation of large dense bodies and a variable amount of lipid droplets (Robaire and Hermo, 1988; Hermo et al., 1988; Hermo and Robaire, 2002). Among a number of different proteins, clear cells are also known to take up the remnants of the cytoplasmic droplets released from the passing spermatozoa (Hermo et al., 1988). As with other epithelial cells of the epididymis, lysosomes within clear cell express different enzymes in a region-specific manner, again reflecting a variety of substrates to degrade (Flinkinger et al., 1988; Hermo et al., 1992; Vierula et al., 1995). For example, clear cells in the caput and corpus actively internalize debris and defunct proteins, while in the cauda clear cells specifically endocytose the cytoplasmic droplet, immobilin and aged spermatozoa, a process termed spermiophagy (Robaire and Hermo, 1988).

Basal cells

Basal cells appear along the entire length of the male excurrent duct system, with the exception of the efferent ducts. These nondividing cells, which do not reach the lumen, usually integrate themselves between adjacent principal cells at the base of the epithelium where they show a large area of contact with the basement membrane (Robaire and Hermo, 1988). Basal cells interact with their adjacent principal cells via

desmosomes and gap junctions containing connexin 43 (Hamilton, 1975; Cyr et al., 1996). They are round or elongated cells with large nuclei and a thin rim of cytoplasm, and appear to secrete as well as endocytose substances derived from principal cells or the blood. Furthermore, basal cells in the rat express GSTs and are thought to add anti-oxidant protection and shield sperm from harmful electrophiles (Veri et al., 1993, 1994).

Halo cells

Defined as small cells with a narrow rim of clear cytoplasm, halo cells act presumably as scavengers, possibly ingesting antigenic peptides produced by the spermatozoa (Robaire and Hermo, 1988). They are present throughout the epididymis and are usually located at the base of the epithelium, though no junctions are found between these cells and the neighboring epithelial cells. Throughout their cytoplasm, many pale-stained vesicles and lysosomes exist, indicating they are endocytic in nature (Robaire and Hermo, 1988). Using immunolabeling, halo cells have been found to consist of helper T lymphocytes, cytotoxic T lymphocytes, and monocytes, but not B lymphocytes (Flinkinger et al., 1997; Serre and Robaire, 1999; Hermo and Robaire, 2002).

CATHEPSIN A

General Properties:

The protective protein/cathepsin A (PPCA) is a serine carboxypeptidase present in lysosomes where it associates with and forms a fully functional and stable high-molecular weight multi-enzyme complex with β–D-galactosidase and N-acetyl-α-neuraminidase

(d'Azzo et al., 1982; Verheijen et al., 1982; Yamato and Nishimura, 1987; Hiraiwa, 1999). PPCA is synthesized as a 54kDa precursor that is enzymatically inactive and targeted to the lysosomes via the mannose-6-phosphate receptor. Once in the acidic lysosomal environment, this zymogen is cleaved into its mature 32/20 kDa, disulfide linked two-chain form (Galjart et al., 1988; Bonten et al., 1995). The chain on the 32 kDa subunit is necessary and sufficient for the acquisition of the mannose-6-phosphate recognition marker, while the 20 kDa subunit is crucial for the stability of the mature form (Morreau et al., 1992).

Functions:

PPCA has two distinct functions; it facilitates the intracellular routing, lysosomal localization and activation of α-neuraminidase (van der Spoel et al., 1998) and protects both β-galactosidase and α-neuraminidase against rapid proteolytic degradation in lysosomes (d'Azzo et al., 1982; Galjart et al., 1991; Morreau et al., 1992; Bonten et al., 1996; Zhou et al., 1996). Furthermore, the enzymatic activity of α–neuraminidase is contingent on its interaction with PPCA.

As a member of the serine protease family, cathepsin A exerts its own catalytic activity at an acidic pH. Together, PPCA, β-galactosidase and α-neuraminidase breakdown glycoproteins, sialyloligosaccharides and G_{M1} and G_{M2} gangliosides within lysosomes. At a neutral pH, extralysosomal PPCA exerts an esterase/c-terminal deamidase activity (Galjart et al., 1991) such that it inactivates selected neuropeptides, including substance P, endothelin I, and oxytocin (Jackman et al., 1990; Hiraiwa, 1999).

Gene structure:

The gene encoding PPCA in humans has been localized on chromosome 20q13.1 (Wiegant et al., 1991; d'Azzo et al., 1995). The amino acid sequence, as deduced from the cloned cDNA, starts with a conventional hydrophobic signal peptide of 28 residues immediately preceding the chemically determined N-terminus of the 32 kDa component, which spans 298 of the 480 amino acids in the PPCA precursor molecule. It is flanked at its C-terminal end by the N-terminus of the 20 kDa polypeptide of 154 residues (Galjart et al., 1988). The removal of a 2 kDa "linker" peptide from the carboxy terminus of the 34 kDa subunit converts the PPCA precursor into a mature and active enzyme (Bonten et al., 1995; d'Azzo et al., 1995).

Several processing steps are thought to occur during the life cycle of PPCA. In the endoplasmic reticulum, either co- or post-translationally, the single-chain 54 kDa precursor folds, and disulfide bridges between the two domains are formed. Two asparagines residues at positions 117 and 305 acquire high-mannose oligosaccharide moieties, and the resulting glycosylated precursor dimerizes. Only the oligosaccharide chain on the 32 kDa domain, that is Asn-117, gets the mannose-6-phosphate recognition marker that enables the correct intracellular segregation of the precursor molecule (Zhou et al., 1991; d'Azzo et al., 1995). Within the endosomal/lysosomal compartment, the precursor molecule is proteolytically processed into two chains, nicking the precursor at arginine-298 and trimming of the linker domain off the large chain at its C-terminus (Galjart et al., 1988). The mature and functional PPCA present in lysosomes now consists of two subunits of 32 and 20 kDa, still held together by disulfide bridges. Furthermore, like many other carboxypeptidases, the essential serine residue is activated by the

combined action of two other residues, histidine and aspartic acid, forming a "catalytic triad" (Breddam, 1986; d'Azzo et al., 1995). This triad of amino acids is highly conserved, as is much of the structure of PPCA, with yeast and plant carboxypeptidases (Galjart et al., 1988; d'Azzo et al., 1995; Hiraiwa, 1999).

Localization:

The expression of lysosomal enzymes, such as β–hexosiminidase A, cathepsins D and B, and sulfated glycoprotein-1 (SGP-1) within the various cell types of the testis and epididymis has been well characterized (Hermo et al., 1992; Igdoura et al., 1995; Hermo et al., 1997). Each of these enzymes shows a cell- and region-specific distribution along the male reproductive tract. The same is true for cathepsin A. In the testis, Sertoli cells, Leydig cells and macrophages expressed cathepsin A, but there is no expression in germ cells (Luedtke et al., 2000). In situ RNA hybridization also verified the presence of cathepsin A in Sertoli cells and Leydig cells of the testis (Rottier et al., 1998; Sohma et al., 1999). Non-ciliated cells in the efferent ducts expressed cathepsin A, while in the epididymis, lysosomes of the principle, narrow, clear and basal cells also showed cathepsin A expression, but often in a region specific manner. The varying distribution of lysosomal enzymes expressed in a cell and region-specific manner suggests that the lysosomes of these different cell types act upon different substrates that are derived from the endocytosis of substances from the lumen, therefore creating a suitable environment for sperm maturation in the epididymis.

Functional Significance:

The importance of lysosomal enzymes as constituents of lysosomes is emphasized in lysosomal storage disorders, where the absence of one or more enzymes leads to an

increase in the number and volume of lysosomes (Neufeld et al., 1975; Gravel et al., 1995; Trasler et al., 1998). Galactosialidosis is a lysosomal storage disorder associated with a combined deficiency of β–galactosidase and α–neuraminidase, secondary to a defect of the protective protein cathepsin A (d'Azzo et al., 1995). It is transmitted as an autosomal recessive trait, and three phenotypic subtypes are recognized. The early infantile form is associated with edema, visceromegaly, skeletal dysplasia and early death. The late infantile type is characterized by hepatosplenomegaly, growth retardation, cardiac involvement and absence of relevant neurologic signs. The third type is juvenile/adult form, where ataxia, mental retardation, neurologic deterioration and long survival are characteristic (Okada et al., 1983; Suzuki et al., 1988; Takano et al., 1991; d'Azzo et al., 1995; Zhou et al., 1996). Though this is a relatively rare disease, no therapy is presently available.

OBJECTIVES OF PRESENT STUDY

Cathepsin A deficient mice exhibit a phenotype similar to human galactosialidosis, where extensive vacuolation of certain cells in many organs is caused by the abnormal accumulation of undigested metabolites. Preliminary studies revealed marked vacuolation in interstitial cells of the testis and epithelial cells of the epididymis, and reduced fertility (Rottier et al., 1998). Details, however, of the cells affected, their identity and structural abnormalities at the electron microscope level have not been investigated. The production of mature sperm is a complex process requiring the presence or absence of certain factors along the whole male reproductive tract. The exchange of luminal components into and out of the testis, efferent ducts and epididymis is mediated

by numerous subcellular organelles, such as the endocytic apparatus that is dependent on many lysosomal enzymes, including PPCA. The present study, therefore, examines in great detail the effects of PPCA deficiency on various cell types of the male reproductive tract of mice at different ages (2-10 months).

In the testes of PPCA-/- mice, while Sertoli cells and germ cells appeared comparable in appearance and distribution, the mean profile area of seminiferous tubules showed a significant decrease at each age group, suggesting changes to the seminiferous tubules and their contents. In addition, pale vacuolated cells, identified as macrophages, became prominent in the interstitial space (IS) of PPCA-/- mice. Unlike those of wild type mice, these macrophages showed an increase in both size and number. Leydig cells also showed an accumulation of large pale lysosomes in PPCA-/- mice, which were not evident in wild type mice. Since a dynamic relationship exits between both these interstitial cell types, it is suggested that macrophages accumulate as a result of abnormalities occurring in Leydig cells. However, this does not exclude the possibility that macrophages may also be recruited to the testis in response to a changing seminiferous epithelium.

In the epididymis, major accumulations of lysosomes were noted in principal, narrow/apical, clear and basal cells in PPCA-/- mice, and this was especially evident in the caput and corpus regions. In addition, the base of the epithelium of these regions was greatly vacuolated, corresponding to cells that presented no identifiable features and which at times appeared to be undergoing degeneration. In addition, halo cells dispersed at various levels in the epithelium were noted to be abnormal, accumulating pale lysosomes, while numerous macrophages were observed in the intertubular space of the entire duct, presenting a large size and plethora of pale lysosomes. It is suggested that the compromised halo cells, due to PPCA

deficiency within their lysosomes, cannot function properly and as a result recruit macrophages into the intertubular space.

The working hypothesis that has risen out of the accumulated data is that PPCA may be essential for fertility. It is needed by the cells of the testis and epididymis for the breakdown of many substances that are released during sperm formation and maturation. In the absence of PPCA, sperm numbers may be altered, reflective of the change found in the seminiferous tubules. Furthermore, considering the major abnormalities seen in the epithelium of the epididymis of PPCA -/- mice, it could be hypothesized that the sperm entering the cauda are not fully mature.

Since mouse and human PPCA have a homology of about 86% (d'Azzo et al., 1995), it is hoped that our research into understanding the importance of PPCA will aid in the ongoing search for a treatment for galactosialidosis patients around the world. Furthermore, much of the data collected is revealing important information about the functions of cells within the male reproductive tract of wild type animals. The present study may contribute to the continuing efforts of understanding the processes by which sperm are made and matured.

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CHAPTER II

An increase in macrophages in the testis of cathepsin A deficient mice suggest an important role for these cells in the interstitial space of this tissue

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Abstract

Cathepsin A (PPCA) is a lysosomal carboxypeptidase that functions as a protective protein for α–neuraminidase and β–galactosidase in a multienzyme complex. In the present study, the testes of PPCA-/- mice from 2 to 10 months of age were compared with those of their wild type counterparts employing routine and immunocytochemical techniques at the light (LM) and electron microscopic (EM) levels. In the testes of PPCA-/- mice, while the cellular components of the seminiferous epithelium (germ and Sertoli cells) appeared comparable in appearance and distribution, the mean profile area of seminiferous tubules showed a significant decrease between wild type and PPCA-/- mice at each age group, suggesting changes to the seminiferous tubules and their contents. In addition, pale vacuolated cells, identified as macrophages by immunostaining with a cell surface monoclonal antibody F4/80, became prominent in the interstitial space (IS) of PPCA-/- mice. Unlike those of wild type mice, these macrophages were large, spherical and filled with pale lysosomes, often of very large size. The latter were identified as such by LM and EM immunostaining with an anti-cathepsin D antibody. Quantitative analyses of the frequency of macrophage nuclear profiles per unit area of IS in PPCA-/- mice revealed a significant increase compared to that of wild type mice at each age group; this was also the case for their mean profile area. Use of an anti-cdc47 antibody which detects cycling cells revealed a reaction over spermatogonia and early spermatocytes, while cells of the IS were unreactive. Taken together with the absence of morphological images of mitotic figures, degeneration or apoptosis of cells in the IS, the data suggest that the major recruitment of macrophages appears to be from the circulation. The abnormal accumulation of pale lysosomes in macrophages of PPCA-/- mice suggests their active role in the endocytosis and phagocytosis of substances from the IS, and their inability to degrade these substances as a result of PPCA

deficiency. In the IS, Leydig cells, identified immunocytochemically with an anti 3β-HSD antibody, also showed an accumulation of large pale lysosomes in PPCA-/- mice, which were not evident in wild type mice. Their frequency also increased significantly in PPCA -/- mice. In the EM, a close association of Leydig cell microvilli with the surface of macrophages was pronounced in PPCA-/- mice. In wild type mice, macrophages and Leydig cells interact by secreting various factors between each other. Considering the fact that Leydig cells show an accumulation of large pale lysosomes in PPCA-/- mice, it is suggested that macrophages accumulate as a result of abnormalities occurring in Leydig cells. The latter may also be responsible for changes occurring to the size of the seminiferous tubules in PPCA -/- mice. In addition, the increase in number of macrophages suggests important functions for these cells in the IS of both wild type and PPCA-/- mice.

Introduction

Hydrolases within lysosomes are involved in the degradation of macromolecules to their building blocks. More than 90% of all proteins are digested by lysosomal proteases with cathepsins being the predominant ones. To date, more than 20 cathepsins have been identified, with some also being found outside lysosomes (Hiraiwa, 1999). In addition to digestive functions, some cathepsins are involved in the proteolytic maturation of lysosomal proteins and enzymes, bone remodelling, regulation of T-cell cytolytic activity and fertilization. Cathepsins are classified into two groups,

endopeptidases and exopeptidases with some demonstrating both activities (Zhou et al, 1995; Hiraiwa, 1999).

The protective protein/cathepsin A (PPCA), carboxypeptidase A, is an exopeptidase lysosomal serine protease and member of the α/β hydrolase fold enzyme family. It is a multifunctional enzyme with distinct protective and protease functions (Galjart et al., 1991). PPCA's protective function resides in its ability to form a multienzyme complex with β-galactosidase and α-neuraminidase, contributing to the stability and lysosomal activity of both glycosidases (d'Azzo et al., 1982; Verheijen et al., 1982; Verheijen et al., 1985; Yamamoto and Nishimura, 1987). PPCA facilitates the intracellular routing, lysosomal localization and activation of α-neuraminidase and protects both β-galactosidase and α-neuraminidase against rapid proteolytic degradation in lysosomes (d'Azzo et al., 1982; Galjart et al., 1988; 1991; Morreau et al., 1992; Zhou et al., 1996; Bonten et al., 1996; van der Spoel et al., 1998; Hiraiwa, 1999). PPCA is targeted as a 54-kD precursor to lysosomes from the Golgi apparatus via the mannose-6-phosphate receptor where it is processed into its mature and active forms (Galjart et al., 1988; Bonten et al., 1995). PPCA is active at both acidic and neutral pH and functions as a multicatalytic enzyme with deamidase and esterase in addition to carboxypeptidase activities (Jackman et al., 1992; Hanna et al., 1994). PPCA can also participate in a variety of cellular processes not necessarily restricted to the lysosomal compartment (Galjart et al., 1988; Itoh et al., 1995; Rottier et al., 1998).

Galactosialidosis is a combined deficiency of both α-neuraminidase and β-galactosidase activities in lysosomes (Wenger et al., 1978; Suzuki et al., 1984; Galjart et al., 1988), that leads to storage of sialylated oligosaccharides and glycopeptides in tissues

and body fluids of patients and accumulation of lysosomes (van Pelt et al., 1988, 1989; Sohma et al., 1999). The primary genetic defect, however, lies in the gene encoding a third lysosomal enzyme, PPCA. The clinical phenotype of galactosialidosis is dictated by the loss of the protective function of PPCA with α-neuraminidase loosing its enzymatic activity, whereas β-galactosidase maintains it at about 10-15% of normal enzyme values (d'Azzo et al., 1995).

Cathepsin A knockout mice show a phenotype similar to human galactosialidosis, where extensive vacuolation of certain cells in many organs is caused by the abnormal accumulation of undigested metabolites that results primarily from the severe secondary deficiency of lysosomal neuraminidase (Zhou et al., 1995). While these mice are viable and fertile, they exhibit severe abnormalities soon after birth that closely resemble those found in galactosialidosis patients. They also develop the characteristic histopathology of the human disease. Thus this mouse model provides a unique opportunity to study the effects of PPCA-/- deficiency on cells of various tissues of the body (Zhou et al., 1995, 1996), including the male reproductive tract.

In various studies, the differential expression of several lysosomal enzymes has been investigated within cells of the testis. Sertoli cells express prosaposin, cathepsin D, β-hexosaminidase A and cathepsin L (Igdoura et al., 1995; Hermo et al., 1997; Zabludoff et al., 1990), while spermatids express cathepsin D and β-hexosaminidase A in their acrosomes (Srivastava et al., 1982; Igdoura et al., 1995). Leydig cells express prosaposin, β-hexosaminidase A and cathepsin D (Hermo et al., 1992, 1997; Luedtke et al., 2000). In the case of cathepsin A, Sertoli cells, Leydig cells and macrophages expressed cathepsin A, but there is no expression in germ cells (Luedtke et al., 2000). In situ RNA

hybridization verified the presence of cathepsin A in Sertoli cells and Leydig cells of the testis (Rottier et al., 1998; Sohma et al., 1999).

In the testis, aside from the well-known endocytic and phagocytic function of macrophages, Leydig cells have also been shown to be involved in fluid phase, adsorptive and receptor-mediated endocytosis. Electron dense non-specific tracers, as well as [^{125}I]-hCG, introduced into the interstitial space are taken up by coated pits and then became incorporated in a temporal and sequential dependent manner in endosomes, multivesicular bodies and lysosomes (Hermo et al., 1985, 1988). The latter play a role in the breakdown of substances derived from endocytosis and in autophagy, which has also been demonstrated in these cells (Tang et al., 1988).

Preliminary light microscope analysis on PPCA -/- mice revealed marked vacuolation in interstitial cells of the testis as compared to wild type mice (Rottier et al., 1998). However, details on the cells affected, their identity and structural abnormalities at the electron microscope level have not been investigated in any detail. The purpose, therefore, of the present study was to examine the effects of cathepsin A deficiency on various cell types of the testis of mice at different ages (2-10 months).

Materials and Methods

Routine Light and Electron Microscopic Preparation of Testicular Tissue

Mouse models of galactosialidosis (PPCA -/-) were developed through targeted disruption of the PPCA gene. A targeting vector for homologous recombination was constructed, which gave rise to a null mutation at the PPCA locus (Zhou et al, 1996). Wild type and PPCA -/- mice ranging from 2 to 10 months of age were utilized for this study and obtained from the lab of Dr. A. d'Azzo (Memphis, Tennessee). Wild type and PPCA -/- mice, 2-3 per age (2, 3, 5, 6, 8 and 10 months) were anaesthetized by intraperitoneal injection of sodium pentobarbital (Somnitol, MTC Pharmaceuticals, Hamilton, Ontario, Canada). In some cases, only their right testis was fixed by cardiac perfusion with 5% glutaraldehyde buffered in sodium cacodylate (0.1M) containing 0.05% calcium chloride (pH 7.4), while the left was removed prior to perfusion and immersed in Bouin's fixative (see below). After 10 minutes of cardiac perfusion, the testes were removed and cut into approximately 1 mm^3 pieces and placed in the above fixative for an additional 1h at 4°C. Thereafter, the tissues were thoroughly rinsed three times in 0.1M sodium cacodylate buffer containing 0.2M sucrose and left in buffer overnight at 4°C.

On the following day, the tissues were postfixed in potassium ferrocyanide-reduced osmium tetroxide for 1 h at 4°C to enhance staining of membranes (Karnovsky, 1971). Subsequently, the tissues were rinsed several times in buffer, dehydrated in a graded series of ethanol and propylene oxide and embedded in Epon. Thick sections (0.5

μm) of the testes were cut and stained with toludine blue and observed by light microscopy. Thin sections of selected regions of each block were cut with a diamond knife, placed on copper grids and counterstained with uranyl acetate (5 minutes) and lead citrate (3 minutes). Sections were examined with a Philips 400 electron microscope.

Light Microscopic Immunocytochemistry

Wild-type and PPCA -/- (2-3 per age) at 3, 5, 7, 8 and 10 months of age (same animals as used above) were anaesthetized with an intraperitoneal injection of sodium pentobarbital (Somnitol, MTC Pharmaceuticals, Hamilton, Ontario, Canada) and their left testis removed prior to cardiac perfusion of the entire animal. These testes were fixed by immersion in Bouin's fixative overnight. The tissues were subsequently washed thoroughly with 70% alcohol and eventually dehydrated in a series of graded ethanol solutions, followed by dioxane and embedding in paraffin wax. Sections (5μm thick) were cut, mounted on glass slides and processed for light-microscopic immunocytochemical analysis.

The following polyclonal affinity purified antibodies were used at the various dilutions for routine peroxidase immunostaining: anti-prosaposin antibody (1:200) provided by Dr. C. R. Morales, McGill University, Montreal, Canada (purified and characterized as described in Morales et al., 2000); anti-cathepsin D antibody (1:50) (purchased from Calbiochem, La Jolla, CA, and previously used on testicular tissues in Igdoura et al., 1995), and anti 3β-HSD antibody (1:50) provided by Dr. Van Luu-Thé, Laval University, Quebec (details on its characterization and purification are found in

Luu-Thé et al., 1989). Two monoclonal antibodies, along with enhancer kits, were also used in this study; F4/80 (1:10) was obtained from Abcam Ltd. (Cambridge, UK) and has been characterized and purified as described in Austyn and Gordon (1981), and mouse anti-cdc47 (1:300) antibody (Neo Markers, California), characterized and purified as described in Hiraiwa et al. (1997). Use of normal rabbit serum and incubation with 100 µl of Tris-buffered saline (TBS) and secondary antibody alone served as negative controls.

The paraffin sections were deparaffinized with Histoclear and hydrated in a series of graded ethanol solutions. Endogenous peroxidase activity was inactivated with 70% ethanol containing 1% hydrogen peroxide, while residual picric acid was neutralized in 70% alcohol containing 1% lithium carbonate. After hydration, the tissues were washed in distilled water containing 300mM glycine to block free-aldehyde groups. Prior to immunostaining, the sections were blocked for 15 minutes with 10% goat serum in TBS. Tissue sections were incubated at 37°C in a humidified chamber for 90 minutes with 100 µl of diluted primary antibody. Following several washes in TBS containing 0.1% Tween-20, the sections were blocked with 10% goat serum for 15 minutes in order to prevent non-specific binding of the secondary antibody. The secondary antibody incubation was performed at 37°C with anti-rabbit IgG conjugated to horseradish peroxidase at a dilution of 1:250 with TBS. All sections were washed and incubated with peroxidase substrate: 0.05% 3,3'-diaminobenzidine tetrahydrochloride and 0.03% hydrogen peroxide in TBS. The sections were counterstained with 0.1% methylene blue and dehydrated in a graded series of ethanol solution and Histoclear. Cover slips were mounted onto glass slides with Permount.

For the anti-monoclonal F4/80 antibody, a Vectastain elite ABC kit was utilized to enhance the reaction. After deparaffinization of the tissue sections with Histoclear and hydration in a series of graded ethanol solutions (as described before), the slides were incubated for 20 minutes with a blocking agent at room temperature. The sections were then incubated with the primary antibody diluted at 1:10 with 10mM phosphate buffered saline (PBS) for 30 minutes, followed by three rinses in PBS. The slides were then incubated with diluted secondary antibody for 30 minutes at room temperature, followed by more washing and then a final incubation with the Vectastain elite ABC reagent for 30 minutes. The same peroxidase substrate was used as described above until the desired stain intensity appeared (approximately 90 seconds), after which the sections were counterstained with methylene blue, dehydrated in ethanol and Histoclear, and mounted with cover slips using Permount.

Immunostaining with an anti-cdc47 antibody was performed using a Zymed SP kit (San Francisco, CA). Paraffin sections of 4μm were deparaffinized in toluene and rehydrated through ethanol. Endogenous peroxidase activity was eliminated by preincubation with 3% hydrogen peroxide in methanol for 20 min. A microwave retrieval technique using citrate buffer was applied (Tacha and Chen, 1994). After cooling the slides, non-specific binding sites were blocked using 10% goat serum for 30 min. Sections were then incubated with the antibody, diluted 1:300 for 1h at room temperature, washed in PBS buffer, incubated with biotinylated anti-mouse secondary antibody for 10 min and thereafter with streptavidin-peroxidase for another 10 min. Diaminobenzidine was used as the chromogen to visualize the biotin/streptavidin-peroxidase complex under light microscope monitoring. Counterstaining was performed

using #2 Gill's hematoxylin for 15 seconds; negative control experiments were performed on adjacent sections by substituting non-immune rabbit IgG for the primary antibody (1:300).

Electron Microscopic Immunocytochemistry

Two wild type and two PPCA-/- mice (7 months of age) were anesthetized with sodium pentobarbital and their right testis fixed by cardiac perfusion with a fixative containing 0.5% glutaraldehyde and 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). After their removal, the tissue was trimmed into small pieces ($0.5mm^3$), immersed for 2 h in the above fixative at 4°C, washed two or three times in 0.15M PBS (pH 7.4) and then treated with PBS containing 2.3M sucrose. The tissue was then frozen in liquid nitrogen until sectioned.

Ultrathin sections of the testis were mounted on 300-mesh, formvar-coated nickel grids (Canemco, Montreal, QC, Canada). Each grid was floated for 15 min onto a drop of 2% bovine serum albumin, 2% casein, 0.5% ovalbumin (BCO) and then incubated for 1 h on 15-µl drops of anti-cathepsin D antibody diluted 1:5 in BCO. Sections were washed six times for 5 min each in Dulbecco phosphate-buffered saline (DPBS), transferred for 15 min to drops of BCO and incubated for 30 min on 20-µl drops of goat anti-rabbit IgG antibodies conjugated to 10 nm colloidal-gold particles. The sections were subjected to six-5 min washes in DPBS, followed by six 5-min washes in distilled water. Sections were counterstained with uranyl acetate oxalate stain (pH 7) in water. Sections were

protected with a layer of 2% methyl cellulose (Anachemia, Montreal, QC, Canada). Photographs were taken on a Philips 400 electron microscope.

Quantitative Analysis

Quantitative analysis was performed using a Zeiss MOP-3 image analyser. Animals of closely related ages were grouped together due to insufficient numbers at identical ages. Photographs of seminiferous tubular cross sections of testes fixed in Bouin's and embedded in paraffin from wild type and PPCA-/- mice at 3-5, 7-8 and 10 months of age (n=3 mice per group) were taken at x128 with a light microscope, and their profile area outlined with the stylus pen of the MOP-3. Counts were obtained on 12 photographs at each age group of wild type and PPCA-/- mice. The mean profile area and standard deviations were determined using Microsoft™ Excel, while statistical analyzes (Factorial ANOVA, followed by a Post-hoc unequal N HSD t-test) were performed using Ver 6.0 of Statistica (Statsoft, Tulsa, OK); p values less than 0.05 were considered significant.

Wild type and PPCA-/- mouse testes at 2-3, 5-6 and 8-10 months of age (n=3 per group), fixed with glutaraldehyde and embedded in Epon, were used to analyze data on cell parameters of the interstitial space. This material was more suited for this analysis because the resolution of interstitial cells was better defined and more readily observed as compared to that of Bouin-fixed material. Photographs of the interstitial space were taken at x512 (12 per age group), but only those areas showing clusters of interstitial cells were photographed. Using the stylus of the MOP-3 analyzer, the profile area enclosing clusters

of Leydig cells, macrophages and any enclosed blood vessels were outlined and measured. The stylus pen followed the peripheral edges of all structures enclosed in a given cluster. The analyses did not include the entire interstitial space, such as areas devoid of cells or containing sparse interstitial cells. These data were then used to determine the mean profile area for the interstitial space of wild type versus PPCA-/- mice. The mean profile area for both Leydig cells and macrophages was also determined in both animal groups using the MOP-3. In addition, the mean frequency of nuclear profiles per unit area of interstitial space for both macrophages and Leydig cells were estimated in both animal groups. Nuclear profiles of Leydig cells and macrophages were scored if and only if 50 % or more of the nucleus was visible with a handheld magnifying glass, irrespective of the varying planes of section through the nucleus of these cells, which did not appear to change in size between wild type and PPCA-/- mice. Data were entered into Microsoft™ Excel and mean values and standard deviations were calculated for each of the above parameters, while Factorial ANOVA, followed by a Post-hoc unequal N HSD t-test, were performed using Statistica; p values less than 0.05 were considered significant.

Results

Light microscopic appearance of the testis of PPCA-/- mice.

The seminiferous epithelium of PPCA-/- mice (Fig. 1b) revealed a full complement of the different generations of germ cells and a topographical arrangement comparable to that of wild type mice at each age group examined (Figs. 1a and inset). In PPCA-/- mice, Sertoli cells, immunostained with an anti-prosaposin antibody and seen as stellate shaped cells in the LM, also presented an appearance and distribution comparable to that seen in wild type mice at the different stages of the cycle and at the different age groups (compare Fig. 2a with 2b). In addition, LM and EM images of abnormal cells as well as cell loss or degeneration were not evident at any age group of PPCA-/- mice. However, a quantitative analysis of the mean profile area of seminiferous tubules revealed a significant decrease (Table 1, line 1) with age for both wild type and PPCA-/- mice. In addition, a significant decrease (Table 1, line 2) was noted between wild type and PPCA -/- mice at each of the different age groups studied (Graph 1), suggesting that the epithelial cells of the seminiferous tubules were being affected in PPCA-/- mice.

In contrast, there was a dramatic change in the appearance of cells of the interstitial space. In wild type mice, only an occasional small pale stained macrophage was noted in routine LM sections amongst the more numerous deeply stained Leydig cells (Fig. 1a). In PPCA-/- mice, pale vacuolated cells became evident at 2 months of age and were prominent at all later ages examined (Figs. 1b, 3, 4). The distinguishing feature of these cells was the plethora of pale vacuoles that filled their cytoplasm (Figs. 1b, 3). Little else was evident except for the nucleus that was often jagged due to the

impingement of the pale vacuoles on its surface (Figs. 1b, 3). These cells were more or less spherical in appearance, pale stained and larger in size compared to the adjacent more deeply stained Leydig cells (Figs. 1b, 3). When sections were immunostained with an anti-monoclonal F4/80 antibody, a specific marker for macrophages, a reaction was observed over the cell surface of the pale vacuolated cells, indicating that they were macrophages (Fig. 5).

In wild type mice, the mean frequency of macrophage nuclear profiles per unit area of interstitial space did not change significantly between the different age groups; macrophages occupied about 10-20% of the total number of Leydig cells and macrophages combined (Graphs 3, 5). In comparison, the mean frequency of macrophage nuclear profiles per unit area of interstitial space in PPCA-/- mice was significantly increased (Table 1, line 3) as compared to that of wild type mice at the different age groups (Graph 3). In addition, the mean profile area for macrophages in PPCA-/- mice was significantly increased (Table 1, line 4) as compared to their wild type counterparts at each age group (Graph 4). The interstitial space also increased significantly (Table 1, line 5) in size between and across each age group for both wild type and PPCA-/- mice (Graph 2).

To assess whether or not the increase in frequency of macrophage nuclear profiles was the result of their division in the interstitial space, sections of the testis were immunostained with an anti-cdc47 antibody that reveals cells in the S phase of the cell cycle. In the testis of both wild type and PPCA-/- mice, spermatogonia and early spermatocytes of the seminiferous epithelium showed a reaction over their nucleus as expected, but not mid nor late spermatocytes or spermatids (Fig. 4). In the interstitial

space, Leydig cells and macrophages of wild type and PPCA-/- mice were consistently unreactive at all ages examined, indicating that both of these cell types were not dividing in PPCA -/- mice (Fig. 4).

In wild type mice, Leydig cells and macrophages were intensely reactive for the lysosomal enzyme cathepsin D (Fig. 6) and prosaposin (not shown). In PPCA -/- mice, Leydig cells maintained their reactivity for both of these enzymes, while the pale vacuolated macrophages showed a more wispy reaction, presumably because of the larger size and number of pale vacuoles in their cytoplasm (Fig. 7).

As compared to that of wild type mice, the more deeply stained Leydig cells showed vacuolation of their cytoplasm in PPCA-/- mice, although not to the extent of macrophages (Figs. 1b, 3, 7). Another consistent finding was the close approximation of Leydig cells with the surface of macrophages. As such, Leydig cells often appeared flattened and sandwiched between macrophages, revealing large areas of close investment of the surface of one cell with that of the other (Figs. 1b, 3, 7, 9inset).

Immunostaining of Leydig cells was performed in wild type and PPCA-/- mice, with an anti-3β-HSD antibody, a recognized marker of Leydig cells of the testicular interstitial space, and an enzyme involved in the conversion of various substrates that eventually lead to the synthesis of testosterone. In wild type mice of all ages, Leydig cells revealed a cytoplasmic reaction and continued to do so in PPCA-/- mice at all ages examined, despite the noted vacuolation of their cytoplasm (Fig. 9 inset).

Quantitative analyses of the mean frequency of Leydig cell nuclear profiles per unit area of interstitial space revealed a significant increase (Table 1, line 6) in PPCA -/- mice as compared to their wild type counterparts at the different age groups (Graph 5).

However, no staining of Leydig cells or other interstitial cells with an anti-cdc-47 antibody was noted in PPCA-/- mice (Fig. 5) indicative of cell division. The mean profile area for Leydig cells showed no consistent pattern of change in PPCA-/- mice as compared to wild type mice at the different age groups (Graph 6).

Electron microscopic appearance of the testis of PPCA-/- mice:

Leydig cells contained numerous profiles of smooth endoplasmic reticulum (sER) arranged as tightly packed tubular anastomotic networks and large whorls of concentric membranous profiles. These cells also contained variable amounts of lipid droplets, flattened elements of rough ER, numerous mitochondria and occasional small spherical dense lysosomes (Fig. 8). Macrophages were scattered amongst Leydig cells and contained coated pits and small subsurface endocytic vesicles, as well as spherical lysosomes of different sizes and densities, mitochondria, few flattened rough ER elements and a spherical nucleus. Several cytoplasmic processes emanated from the surface of macrophages (Fig. 8). Leydig cells displayed a close relationship with the cell surface of macrophages (Fig. 8). While the tips of some microvilli closely abutted onto the macrophage surface, others ran parallel to and were contiguous with it. Some microvilli invaginated into the macrophage surface for a short distance. In all, a large part of the macrophage surface was closely invested with the microvilli of several Leydig cells (Fig. 8).

In PPCA-/- mice at different ages, pale vacuolated cells identified as macrophages with an F4/80 marker employing LM immunocytochemistry were readily apparent (Fig. 5). These cells were large in size and contained a plethora of pale membrane bound

structures, containing few vesicular profiles and membranous elements (Figs. 9-13). In EM cryo sections of testicular tissue immunolabeled with an anti-cathepsin D antibody, these structures revealed numerous gold particles over their interior indicating their lysosomal nature (Fig. 10a). The pale lysosomes were of different sizes, and various images suggested that they fused with one another; often creating large irregular or scalloped shaped elements (Figs. 11, 12). At times, those near the cell surface appeared to have fused with it releasing their contents into the lumen (Figs. 11, 12). The nucleus was often indented as a result of the abundance of pale lysosomes (Fig. 11). Dense lysosomes were not noted in these cells, and aside from few mitochondria, small vesicles and the Golgi apparatus, other organelles were not apparent (Fig. 13). The surface of these macrophages showed few short cytoplasmic processes (Figs. 11, 12).

In the cytoplasm of Leydig cells, amongst the usual organelles seen in wild type mice, numerous large pale membrane-bound structures containing vesicular and membranous profiles were prominent in PPCA -/- mice (Fig. 9). These structures were also immunolabelled with anti-cathepsin D antibody, indicating their lysosomal nature (Fig. 10b).

Microvilli of Leydig cells of PPCA -/- mice closely approximated the surface of macrophages, which due to their large size were now devoid of their large cytoplasmic processes (Figs. 9, 13). The microvilli often ran parallel to and closely approximated the surface of macrophages for a short or considerable distance, with some being contiguous with it (Figs. 9, 13). In the space between the surface of Leydig cells and a given macrophage, membranous profiles of different sizes were evident amongst the microvilli of Leydig cells (Figs. 11, 13).

In wild type mice, cells of small size and with a pale staining appearance were noted in the various layers of the limiting membrane often sandwiched between the myoid cell and endothelial cell layers enveloping the seminiferous tubules. Such cells have been identified as monocytes in rats and humans and serve as resident cells of the limiting membrane (Hermo and Clermont, 1976; Hermo and Lalli, 1978; Hermo and Clermont, 1981). In PPCA -/- mice, these cells showed an accumulation of large pale lysosomes that were not evident in those of wild type mice (Fig. 14).

Discussion

In a previous study, using LM and EM immunocytochemistry, it was noted that cathepsin A was expressed in Sertoli cells of the seminiferous epithelium, while in the interstitial space both Leydig cells and macrophages expressed cathepsin A (Luedtke et al., 2000). In the present study, we have analyzed the consequences of cathepsin A deficiency on the various cell types of the interstitial space of the testis of PPCA-/- mice.

In the seminiferous epithelium, PPCA-/- mice did not reveal any major morphological changes to Sertoli cells at all ages examined in both the LM and EM, as compared to those of wild type mice, in terms of their distribution and topographical arrangement at the different stages of the cycle of the seminiferous epithelium. There were also no changes noted to the size, shape and distribution of their lysosomes. This finding is surprising as Sertoli cells have been shown to express high levels of cathepsin A (Rottier et al., 1998; Luedtke et al., 2000). Furthermore, Sertoli cells are active in fluid

phase endocytosis whereby different substances after being internalized from the lumen of the seminiferous epithelium are incorporated in a temporal and sequential manner into coated pits and vesicles, endosomes, multivesicular bodies and lysosomes (Morales et al., 1985). The latter would then be involved in the degradation of the substances internalized from the lumen. Sertoli cells are also phagocytic cells whereby their lysosomes are involved in the degradation of the excess cytoplasm or residual bodies emanating from late spermatids at the time of spermiation (Russell, 1993). In addition, Sertoli cells are also involved in the receptor-mediated endocytosis of substances, such as transferrin, from their basal aspect and this involves a distinct and separate lysosomal compartment (Morales et al., 1986). Thus the lysosomes of Sertoli cells carry out important functions in relation to Sertoli cells.

In addition to cathepsin A, Sertoli cells also express β-hexosaminidase A, cathepsins D and L and prosaposin (Hermo et al., 1992; Igdoura et al., 1995; Hall et al., 1996; Hermo et al., 1997; Zabludoff et al., 1990), suggesting multiple roles for their lysosomes in the degradation of internalized substances. In the case of β-hexosaminidase A, also expressed by Sertoli cells, it was noted that *hexa-/-* or *hexb-/-* mice, deficient in the α or β subunits, respectively, also showed no major changes in the distribution, topographical arrangement or ultrastructural appearance of Sertoli cells (Adamali et al., 1999a, b). Taken together, these data with that of PPCA-/- mice would suggest other mechanisms exist in Sertoli cells to compensate for the absence of either one of these enzymes, or they may be reflective of the metabolic state and type of substrates that are being catabolized by Sertoli cells.

The seminiferous epithelium also displayed the appropriate generations of germ cells at the different stages of the cycle, without any apparent signs of degeneration or inappropriate asynchronous cellular associations. This is consistent with the absence of cathepsin A expression in these cells. On the other hand, measurements of the mean profile area of seminiferous tubules revealed a significant decrease between wild type and PPCA -/- mice at the different age groups. This suggests that germ cells and/or Sertoli cells may be affected in PPCA-/- mice. Although testicular weights were not recorded in this study, at the time of their removal, no apparent change in size of the testis of PPCA-/- mice as compared to wild type mice was observed to warrant detailed analysis. However, in analyzing the numbers of interstitial cells in PPCA and wild type mice, it was noted that the interstitial space was increased in size in PPCA-/- mice at each age group, possibly accounting for the significant increase in number of interstitial cells in PPCA-/- mice. The resulting increase in size of the interstitial space may influence cells of the seminiferous epithelium and result in a diminution of their size or number resulting in a reduction in the profile area of the seminiferous tubules to maintain a normal testicular size. Future studies will have to be carried out to determine the cell types affected and significance of the changes to the seminiferous tubules, studies beyond the scope of the present investigation.

In the present study, Leydig cells were immunostained with an anti-prosaposin antibody and with the Leydig-cell specific marker, 3β-HSD. In each case, these cells and their nuclei appeared comparable in size and appearance between wild type and PPCA -/- mice. In a quantitative analysis, while the mean frequency of Leydig cell nuclear profiles remained similar in wild type mice at the different age groups, their number increased

significantly in PPCA-/- mice as compared to that of wild type mice at each age group; the mean profile area for Leydig cells, however, unlike that for macrophages, remained unchanged in wild type versus PPCA-/- mice at the different age groups. While images of mitotic figures or cycling cells were not observed in the interstitial space of PPCA-/- mice, it is not impossible that fibroblastic cells, precursors of Leydig cells (Ge et al., 1996), transformed into Leydig cells to account for their increase in number. Since a dynamic relationship exists between Leydig cells and macrophages with both cell types exchanging factors between each other, it is suggested that Leydig cell numbers increase as a response to signals derived from macrophages (see section below).

In the interstitial space, Leydig cells have been documented to be active in fluid phase, adsorptive and receptor-mediated endocytosis, whereby different substances after being internalized end up in lysosomes where they are degraded (Hermo et al., 1985; Hermo and Lalli, 1988). The latter are also involved in autophagy, a known function of these cells (Tang et al., 1988). In wild type animals, their lysosomes are small, dense and express prosaposin, β-hexosaminidase A and cathepsins D and A (Christensen, 1975; Hermo et al., 1992, 1997; Luedtke et al., 2000). In the present study, PPCA-/- mice revealed changes to Leydig cells in the form of an accumulation of large pale lysosomes that were not evident in wild type mice. The latter were immunolabeled with an anti-cathepsin D and anti-prosaposin antibody indicating their lysosomal nature. These lysosomes reveal little content except for a few small vesicular profiles and irregularly shaped membranous elements, and often show signs of fusing with one another. Thus in the absence of cathepsin A, the lysosomes of Leydig cells accumulate in number and change their appearance suggesting their inability to degrade substances internalized by

endocytosis. Thus, unlike Sertoli cells, which also express cathepsin A, Leydig cells present an abnormal phenotype.

Macrophages, which account for about 15-20% of the IS in wild type animals (Hume et al., 1984), contain numerous coated pits and vesicles, subsurface endocytic vesicles as well as small and moderate size lysosomes of varying density, involved in the uptake and eventual degradation of substances endocytozed and phagocytosed by these cells (Territo and Cline, 1975). In wild type animals, these cells express cathepsin A (Luedtke et al, 2000). In PPCA-/- mice, numerous large pale vacuolated cells revealing a cell surface reaction for macrophages were evident at all ages. However, unlike macrophages of wild type mice, these cells were filled with large pale lysosomes identified as such in the EM with an anti-cathepsin D and anti-prosaposin antibody. The latter are of various sizes, and various images suggest they fuse with each other, giving rise to the massive pale lysosomes often showing a scalloped appearance. Containing few small vesicular profiles, such lysosomes are so prominent that they often indent the surface of the nucleus. Due to the fact that these lysosomes overwhelm the cell cytoplasm, some near the cell surface appear to release their contents into the interstitial space. In fact, vesicular and membranous profiles are evident in the space between Leydig cells and macrophages, suggesting that some originate from macrophages. Macrophages with a similar appearance referred to as foamy macrophages have been described in PPCA-/- mice in other tissues such as the skin, spleen, kidney, liver, small intestine, muscle, ovary and uterus (Zhou et al., 1995; Rottier et al., 1998).

The presence of large pale lysosomes has also been documented in PPCA -/- mice in cells of epithelial/endothelial/endocrine origin such as the kidney, intestine, liver,

pancreas, ovaries, uterus, brain and pituitary gland (Zhou et al., 1995). The numerous pale lysosomes in these cells were identified as secondary lysosomes and appeared empty or filled with sparse fibrillar structures, consistent with the accumulation of low molecular weight compounds, e.g., oligosaccharides or glycopeptides (Zhou et al., 1995; Rottier et al., 1998).

The accumulation of pale lysosomes in macrophages of PPCA-/- mice suggests that in the absence of cathepsin A there is a progressive accumulation of undegraded products leading to the subsequent increase in number, enlargement and eventual fusion of lysosomes with one another. This is a classic situation noted for many other lysosomal storage diseases, such as for Hex A, Hex B and prosaposin deficient mice as well as for Gaucher and Niemann-Pick mouse models, where various cell types of numerous tissues are similarly affected (Tybulewicz et al., 1992; Horinouchi et al., 1995; Otterbach and Stoffel, 1995; Panuef et al., 1996; Adamali et al., 1999a, b).

In the present study, it was evident that the pale macrophages are already prominent at 2 months of age in PPCA-/- mice and remained so at later ages. A quantitative analysis revealed that the mean frequency of macrophage nuclear profiles per unit area of interstitial space was significantly increased at each age group as compared to that of wild type mice. The mean profile area for macrophages was also significantly increased as compared to wild type mice. To assess if these cells were dividing in the interstitial space, sections of the testis were immunostained with an anti-cdc47 antibody that detects cells in the S phase of the cell cycle. While spermatogonia and early spermatocytes of the seminiferous epithelium, known to be dividing cells, were reactive,

cells of the interstitial space did not display a reaction product, suggesting that the large pale macrophages did not increase in number as a result of cell division.

Cells showing an accumulation of pale lysosomes but being of smaller size than the interstitial space macrophages were occasionally seen in various layers of the limiting membrane in PPCA-/- mice. It has been well noted that monocytes are normal residents of the limiting membrane in several animal models, including humans (Hermo and Clermont, 1976; Hermo and Lalli, 1978; Hermo and Clermont, 1981). It is thus possible that these cells represent intermediates in the monocyte/macrophage differentiation process and that they may be headed for the interstitial space, where they would continue to differentiate into the larger pale macrophages seen in PPCA -/- mice to carry out their specific functions. However, considering the small numbers of such cells, absence of mitosis in the interstitial space and large numbers of macrophages in the interstitial space of PPCA-/- mice, it is likely that the majority of these cells emanate from the circulation.

The reason for the increased frequency of macrophages in PPCA-/- mice is not totally resolved. Macrophages play an active role in defence reactions against microorganisms and in the removal of dying cells and cellular debris (Territo and Cline, 1975). However, in the present study, the seminiferous epithelium did not show signs of cell degeneration or apoptosis of their germ cells, which in most cases are removed by Sertoli cells. In fact, macrophages do not accumulate in situations where destruction to germ cells of the seminiferous epithelium is noted, such as in vitamin E deficiency and HSL deficient mice (Bensoussan et al., 1998; Chung et al., 2001).

A close relationship exists between macrophages and the Leydig cell surface in PPCA -/- mice. Microvilli of Leydig cells often closely approximate or run parallel to the

macrophage surface. While not continuous, these two surfaces often appear to be contiguous with each other. This close relationship is a feature of wild type mice, where microvilli of Leydig cells have also been shown to invaginate into the macrophage cell surface (Miller et al., 1983; Hardy et al., 1989; Hutson, 1992). An association of one of four different types of macrophage/dendritic cells has also been reported in the testis of normal mice (Itoh et al., 1995). In the present study, several Leydig cells usually enveloped a given macrophage. In the adult rat testis, Leydig cells have been shown to secrete a factor, identified as macrophage migrating inhibitory factor (MIF), that regulates macrophage number directly (Meinhardt et al., 1998). On the other hand, macrophages secrete a lipophilic factor, identified as 25-hydroxycholesterol, that stimulates testosterone production by Leydig cells (Nes et al., 2000). In fact, elimination of macrophages from the testis causes altered testosterone production and reduced fertility (Gaytan et al., 1996). Thus macrophages and Leydig cells play crucial roles with one another.

In the present study, Leydig cells contained numerous pale large lysosomes in PPCA -/- mice, which may result in altered functions of these cells, such as a diminution of the sER, which would result in lower testosterone levels. However, we did not obtain testosterone values, as this was not an expected factor to be dealt with in the testis of PPCA-/- mice, and the eventual insufficient number of animals that were available to us as this study came to completion. However, the fact that the mean profile area for Leydig cells did not change in PPCA-/- mice suggests possible reduction in the amount of sER with the concomitant increase in the size of lysosomes of these cells. The corresponding diminished testosterone levels could be responsible for the changes to the mean profile

area of seminiferous tubules, as would be expected if testosterone values diminished (Jégou and Sharpe, 1993).

In addition, abnormalities in Leydig cells, such as the accumulation of lysosomes in their cytoplasm may reduce the secretion of the macrophage inhibiting factor, resulting in the increase of macrophages in the interstitial space. The increase in macrophage frequency and thus production of 25-hydroxycholesterol may serve to induce existing Leydig cells to produce more testosterone or to cause fibroblastic cells to transform into Leydig cells resulting in their increase in frequency in PPCA-/- mice. In either case the PPCA-/- deficiency is be a restricting factor for the maintenance of normal Leydig cell morphology and for possible normal testosterone levels. However, since macrophages are themselves dramatically affected in PPCA -/- mice, more of their numbers may be required to carry out their various functions in the interstitial space. Macrophages normally produce a colony stimulating factor that serves to increase their numbers (Cohen et al., 1996). This factor may be upregulated in PPCA-/- mice, resulting in the production of more macrophages at their sites of origin and hence provide more cells to be available where they would be required to perform their functions. In either case, while the underlying mechanisms for the increase in frequency of macrophages in PPCA-/- mice is still not fully resolved, this study strongly indicates an important role for these cells in both wild type and PPCA -/- mice. Clearly all of these points need to be addressed in future studies.

Indirect evidence for a role for Leydig cells in recruiting macrophages comes from studies on *hexb-/-* mice (Adamali et al., 1999b). In that study, macrophages revealed lysosomal accumulation, however, Leydig cells were normal in appearance. In the *hexb-/-*

mice, there was no increase in number of macrophages with age, suggesting that these cells are recruited into the interstitial space only if Leydig cells become abnormal, as noted in the present study.

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Figs. 1a,b: Light micrographs of seminiferous tubules of the testis of 6-month-old wild type (a) and PPCA-/- (b) mice. The seminiferous epithelium (SE) is comparable in appearance in both cases, with each showing a normal complement of the different generations of germ cells, such as round (small arrows) and elongating (open arrows) spermatids, as well as the supporting Sertoli cells (large arrows). In (a), the interstitial space contains deeply stained Leydig cells (large arrowheads) and occasional small pale stained macrophages (small arrowheads), while in (b), cells with a pale vacuolated appearance are prominent at 6 months of age. The latter, identified as macrophages (stars), are larger in size than those of wild type mice and filled with pale vacuoles that occupy the entire cytoplasm. In (b), Leydig cells (large arrowheads) show some vacuolation of their cytoplasm, not noted in those of wild type mice. L=lumen. 1a, b: x320.

Figs. 2a,b: Seminiferous tubules and interstitial space of the testis of wild type (a) and PPCA -/- mice (b) at 8 months of age immunostained with an anti-prosaposin antibody. In (a) and (b), the seminiferous epithelium shows intense staining of the stellate shaped Sertoli cells (S), with the reaction extending from the base of the epithelium to the lumen of each tubule. Sertoli and germ cells, such as round (arrows) and elongating (open arrows) spermatids appear to be similar in their distribution and topographical arrangement in seminiferous tubules of wild type as compared to PPCA-/- mice. In the interstitial space of (a) and (b), Leydig cells (arrowheads) reveal intense staining of their cytoplasm. L=lumen. 2a, b: x320.


1a


b


L
2a


b

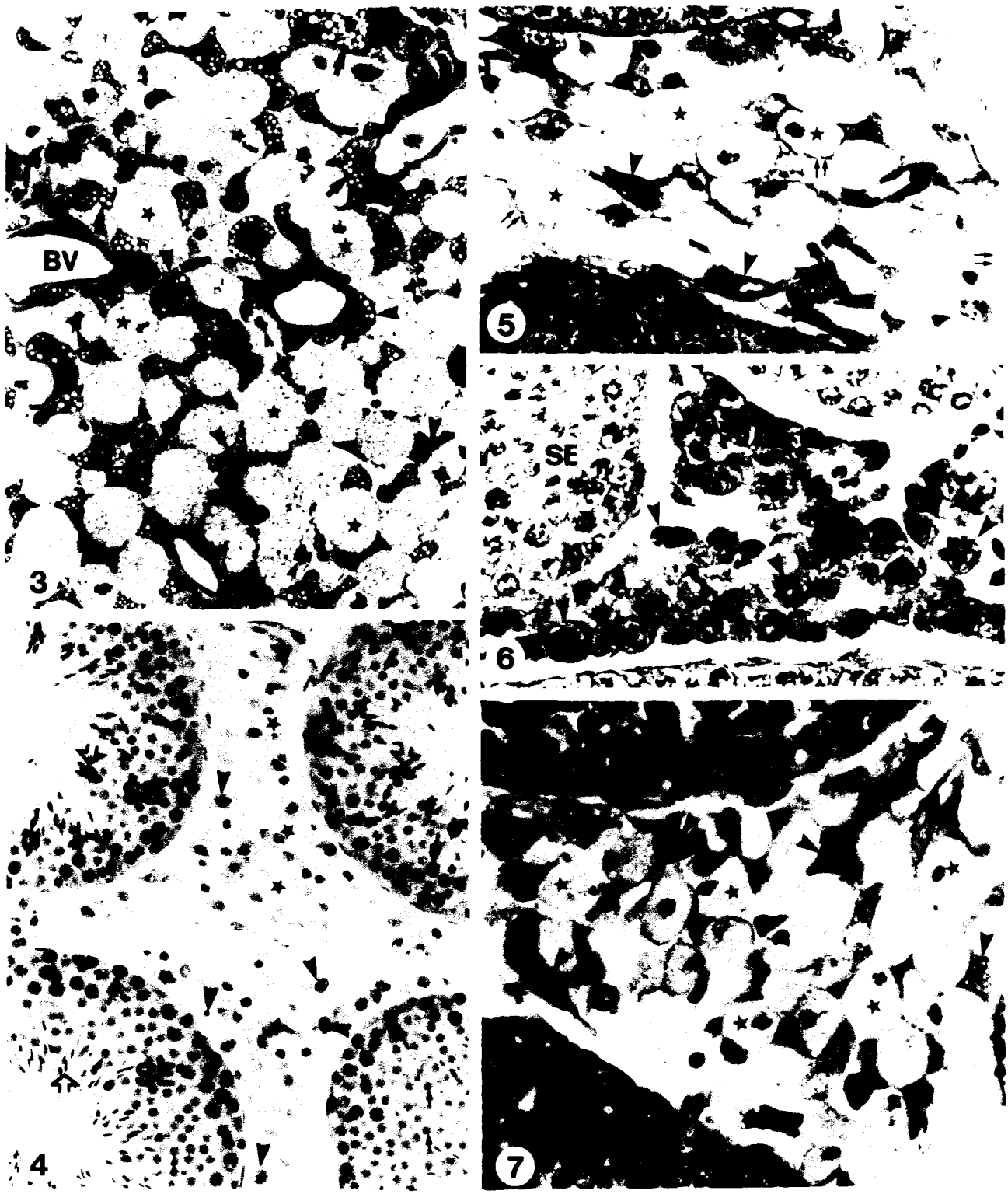
Fig. 3: High power of the interstitial space of the testis of a PPCA -/- mouse at 6 months of age revealing numerous pale vacuolated cells, identified as macrophages (stars), amongst darker staining Leydig cells (large arrowheads). Pale vacuoles fill the entire cytoplasm of macrophages and cause their nucleus (small arrowheads) to be jagged and irregular in shape. Some pale vacuoles (arrows) appear in the cytoplasm of Leydig cells. BV, blood vessel. X320.

Fig. 4: Low power light micrograph of seminiferous tubules of the testis and interstitial space of a 3-month-old PPCA-/- mouse immunostained with an anti-cdc47 antibody that detects cells in the S phase of the cell cycle. While the nuclei of spermatogonia and early spermatocytes of the seminiferous epithelium (SE) are intensely reactive (large arrows), the pale macrophages (stars) and Leydig cells (arrowheads) of the interstitial space do not display a reaction product, suggesting that both of these cell types are not dividing in the interstitial space of PPCA-/- mice. There is also no staining of round (small arrows) and elongating (open arrows) spermatids. x320.

Fig. 5: Interstitial space of the testis of a PPCA -/- mouse at 8 months of age immunostained with a monoclonal F4/80 antibody, specific for macrophages (stars). A uniform immunoperoxidase reaction product appears over the cell surface of the pale vacuolated cells, identifying them as macrophages (double arrows). No reaction is evident over Leydig cells (arrowheads) or cells of the seminiferous epithelium (SE), other than the methylene blue counterstain. X512.

Fig. 6: Interstitial space of the testis of a wild type mouse at 8 months of age, immunostained with an anti-cathepsin D antibody. An intense reaction is evident over interstitial cells (arrowheads) of the interstitial space. SE, seminiferous epithelium. X512.

Fig. 7: Interstitial space of the testis of a 5-month-old PPCA -/- mouse immunostained with an anti-prosaposin antibody. Note that the Leydig cells are intensely reactive (arrowheads), while the pale vacuolated macrophages show a wispy type of reaction throughout their cytoplasm (stars). SE, seminiferous epithelium. X512.


BV
3
4
5
SE
6
7

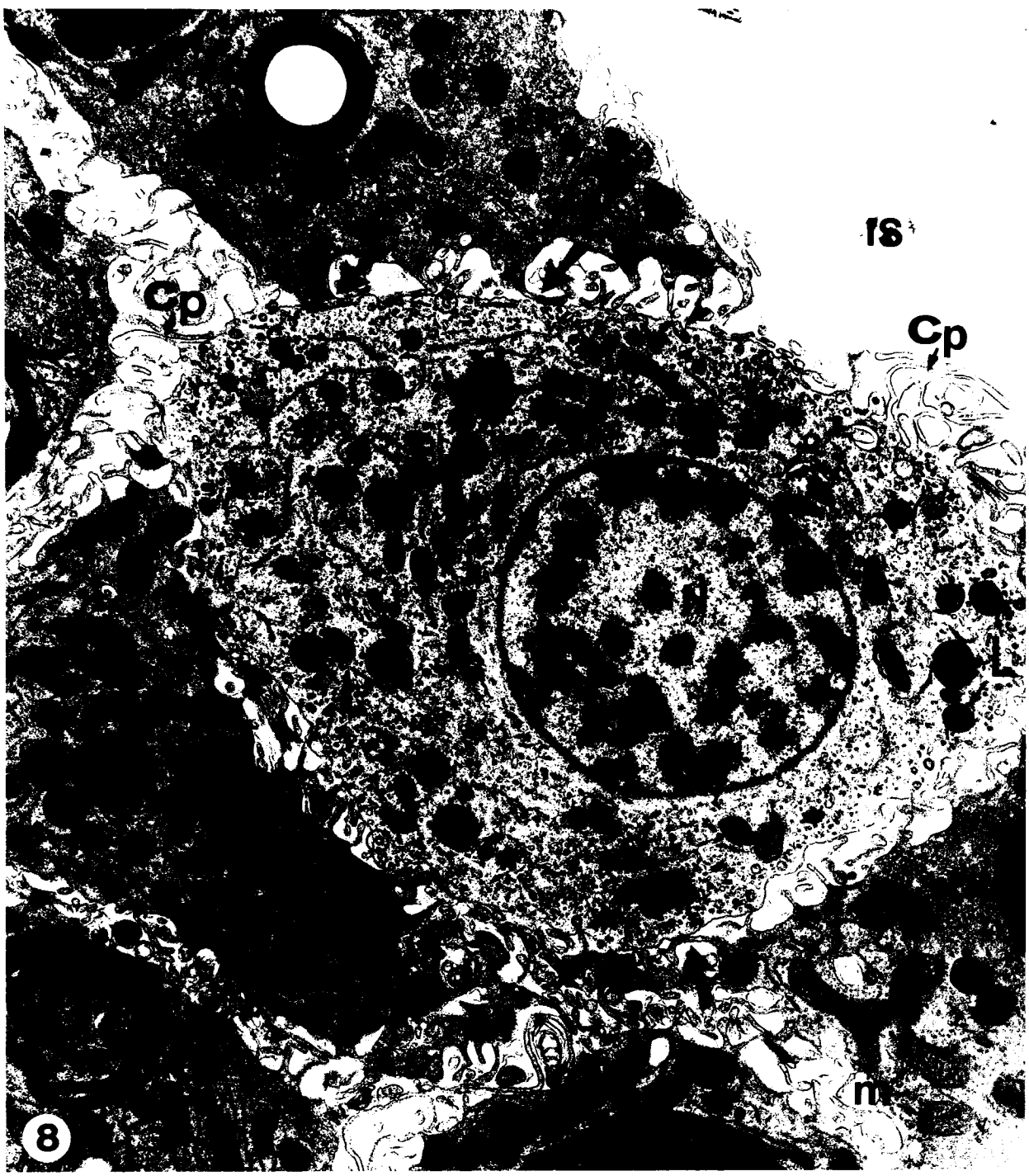

fs
Cp
Cp
m
8

Fig. 8: Electron micrograph of the interstitial space of the testis of a 6-month-old wild type mouse. Leydig cells contain an abundance of smooth endoplasmic reticulum arranged as anastomotic tubular networks throughout their cytoplasm (sER), large whorls of concentric ER elements (squares), mitochondria (m) and lipid droplets (lip). Macrophages contain coated pits (circles) and subsurface endocytic vesicles, few elongated flattened rough ER (rER) elements, as well as numerous randomly distributed dense lysosomes (L) of various sizes. The surface of the macrophage shows several cytoplasmic processes (Cp) emanating from their surface towards Leydig cells or the open interstitial space (IS). The microvilli of Leydig cells display a close relationship with the surface of macrophages. While the tips of some closely abut the macrophage surface, others run parallel to and are closely juxtaposed to it (slanted arrows); some microvilli invaginate into the macrophage surface for a short distance (arrowheads). G, Golgi apparatus; N, nucleus. x10,750.

Fig. 9: Electron micrograph of Leydig cells (Ley) and a macrophage of the interstitial space of a PPCA-/- mouse at 6 months of age. The macrophage contains a plethora of pale lysosomes (open stars), many of which appear to have fused with each other forming large irregular often scalloped-shaped elements (small arrows). The pale lysosomes show little content except for few small vesicular and membranous profiles (small arrowheads). Leydig cells, aside from numerous sER elements, also contain several pale lysosomes of different sizes (open stars), but not to the extent of that seen in macrophages. The latter also show signs of fusing with one another (arrows) and contain little more than occasional small membranous profiles. The cell surface of Leydig cells often comes into close association with that of macrophages (slanted arrows). x8,250. Inset: Interstitial space of the testis of a PPCA-/- mouse at 8 months of age immunostained with an anti-3β-HSD antibody. A reaction is noted over the cytoplasm of Leydig cells of the interstitial space. Stars, macrophages. x512.

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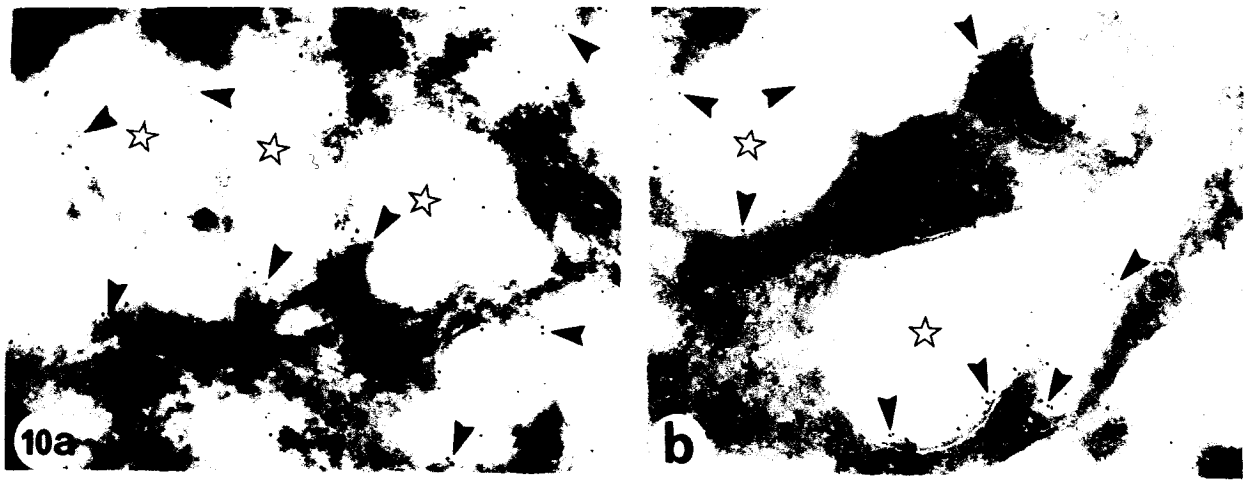
Figs. 10a-b: Electron micrographs of cryo sections of a macrophage (a) and Leydig cell (b) of a 7-month-old PPCA -/- mouse immunolabeled with an anti-cathepsin D antibody. Numerous gold particles (arrowheads) are evident over the pale lysosomes (open stars), identifying them as lysosomal elements in both of these cell types. a, b: x48,750.

Fig. 11: Electron micrograph of a macrophage of the interstitial space of the testis of a PPCA-/- mouse at 6 months of age. The macrophage is filled with pale lysosomes (open stars), with some containing small vesicular profiles and larger membranous profiles (arrowheads). The pale lysosomes are of different sizes and irregular shapes, and various images suggest that they have fused with each other (arrows). They are so abundant that those near the cell surface are closely related to it, with some appearing to have fused with the cell's plasma membrane, in this way liberating their contents into the interstitial space (curved arrows). Several irregular membranous profiles appear in the space between macrophages and Leydig cells (open arrows). The nucleus (N) is eccentric in position and deeply indented as a result of the abundance of pale lysosomes in the cytoplasm. X8,250.


10a
b

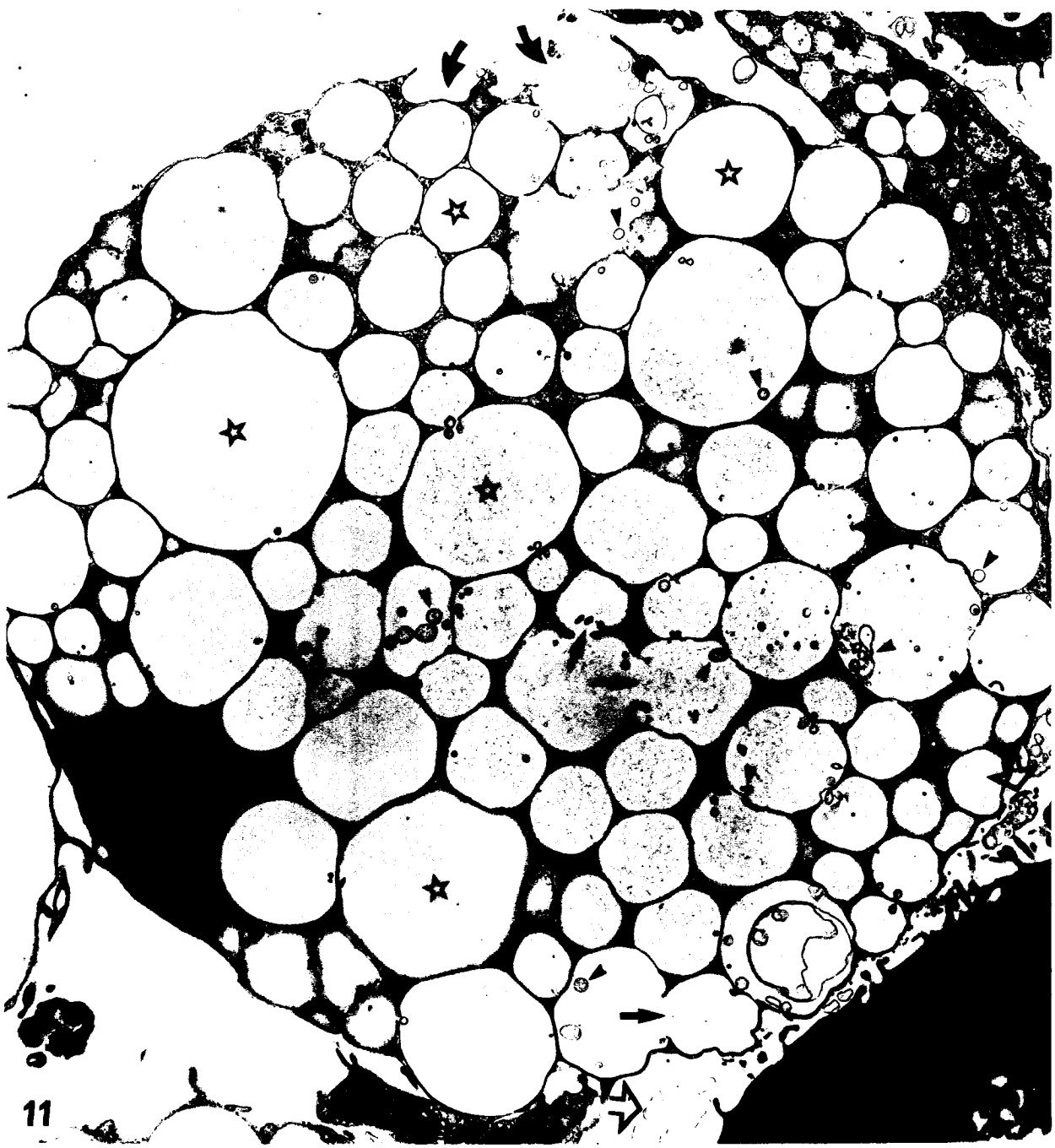

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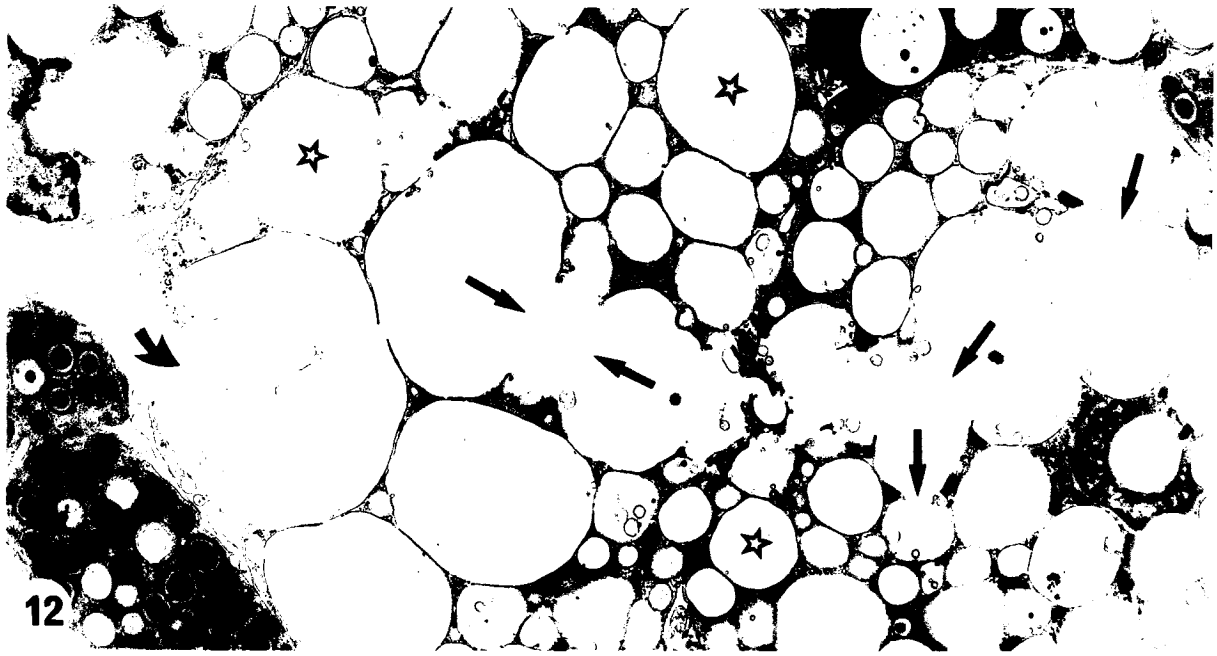
Fig. 12: Macrophage of the interstitial space of the testis of a 6-month-old PPCA-/- mouse. Note numerous pale lysosomes in the cytoplasm (stars), some with a large irregular scalloped shaped appearance that appears to have resulted from the fusion of these lysosomes with each other (arrows). A large lysosome near the cell's surface appears to be releasing its contents into the interstitial space (curved arrow). X6,250.

Fig. 13: Interface between Leydig cells and a macrophage of a PPCA-/- mouse at 6 months of age. Numerous microvilli of Leydig cells are closely associated or contiguous with the macrophage cell surface and run parallel to it for a short or considerable distance (slanted arrows). The macrophage surface does not show cytoplasmic processes; their cytoplasm is filled with pale lysosomes (stars) containing few vesicular profiles (small arrows). Note vesicular and membranous profiles in the space between the Leydig cell and macrophage surface (open arrows). G, Golgi apparatus. X17,500.



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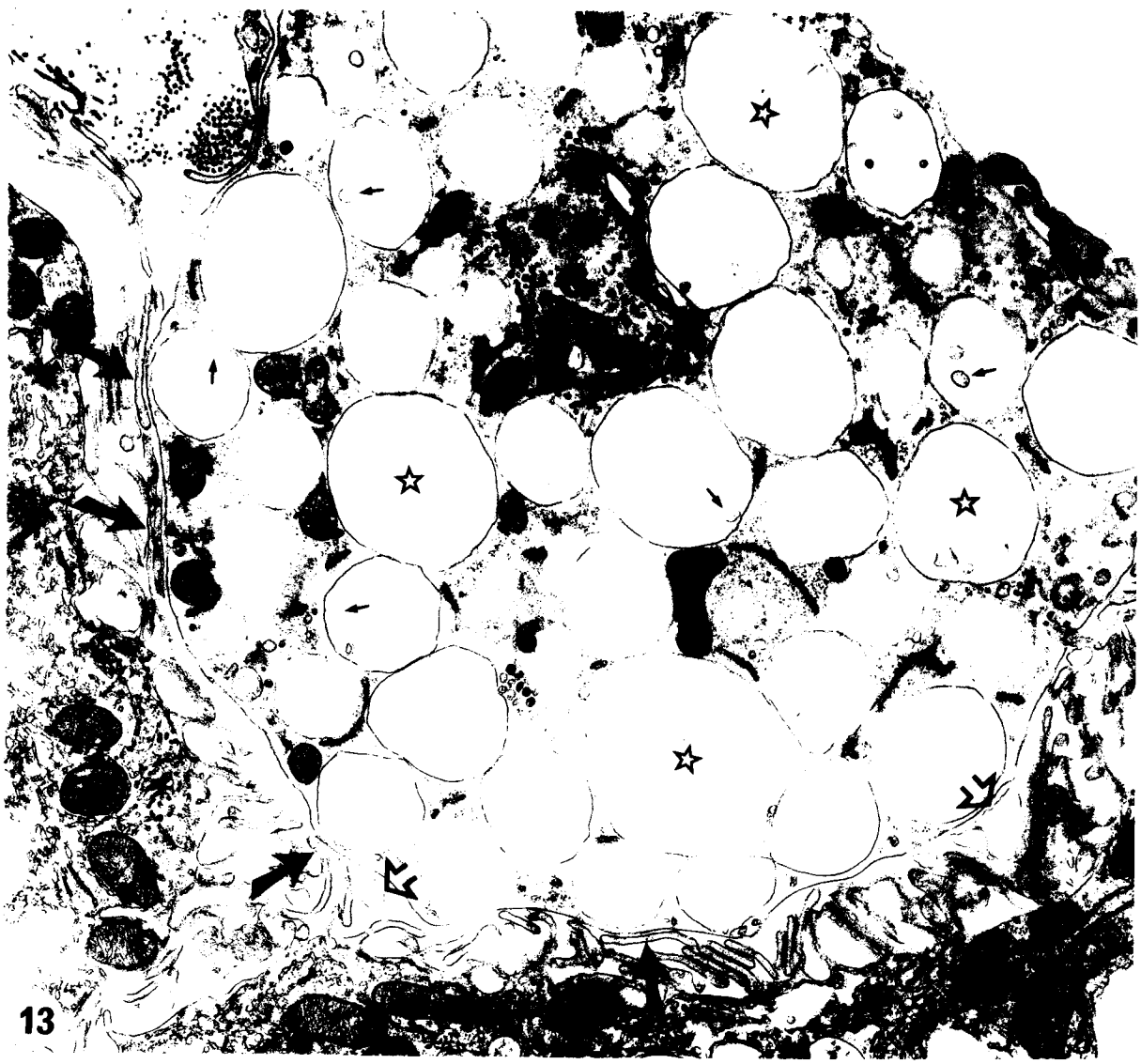

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Fig. 14: Limiting membrane of a seminiferous tubule of a PPCA-/- mouse at 8 months of age showing a monocyte lodged between a myoid cell (My) and endothelial cell (E) of the lymphatic sinusoid. The monocyte is of small size and has few mitochondria (m) and a small Golgi apparatus (G). However, this cell also contains several large pale lysosomes (stars), unlike the small dense lysosomes noted for such cells in wild type mice. Monocytes are normal components of the limiting membrane (Hermo and Clermont, 1975), and it is suggested that this cell is differentiating into a macrophage and may be headed into the interstitial space. The seminiferous epithelium appears normal and shows a germ cell (GC) and Sertoli cell cytoplasm (S) containing flattened rER elements (arrows) and extensive Sertoli-Sertoli junctional complexes (arrowheads). X13,750.

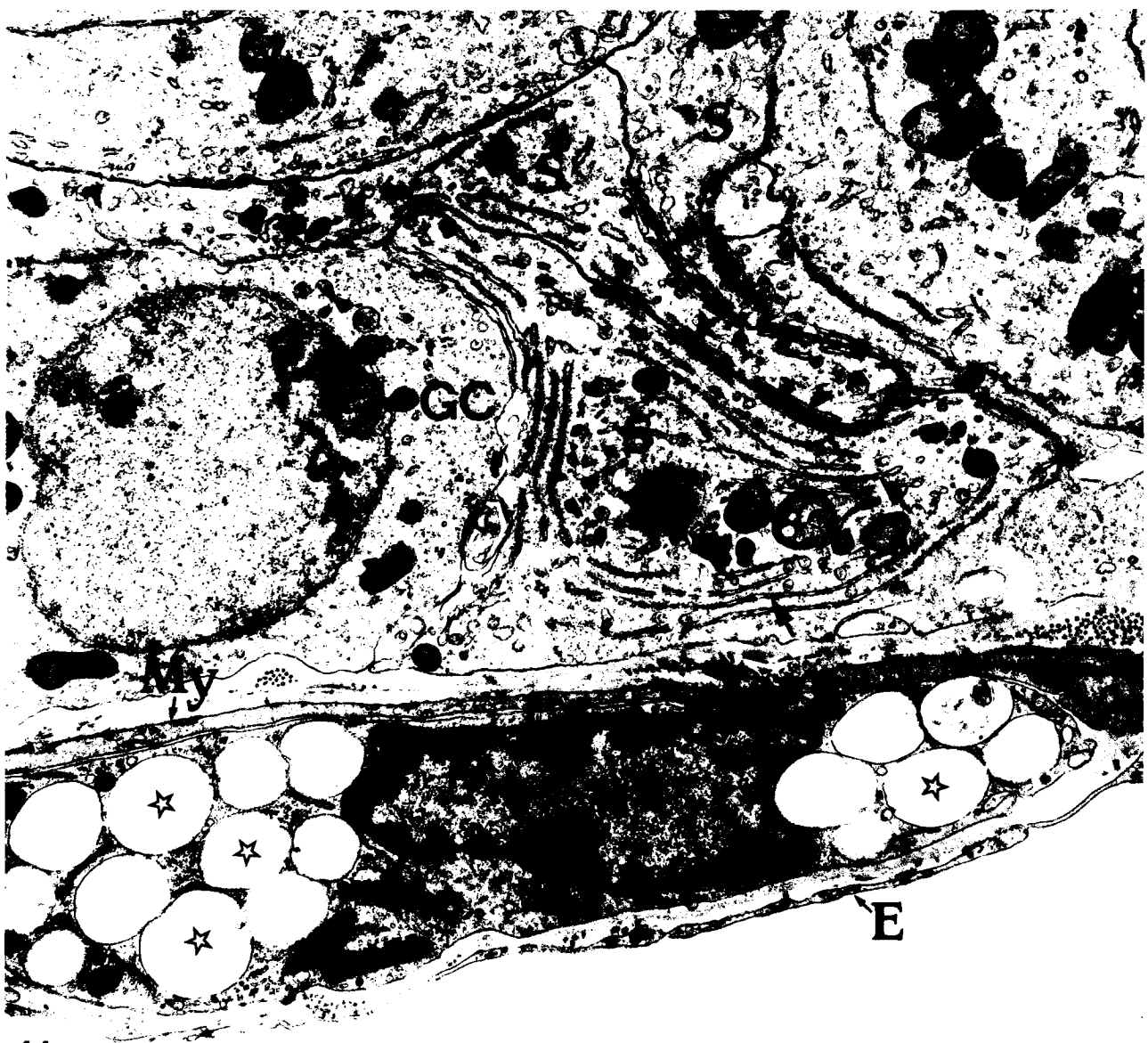

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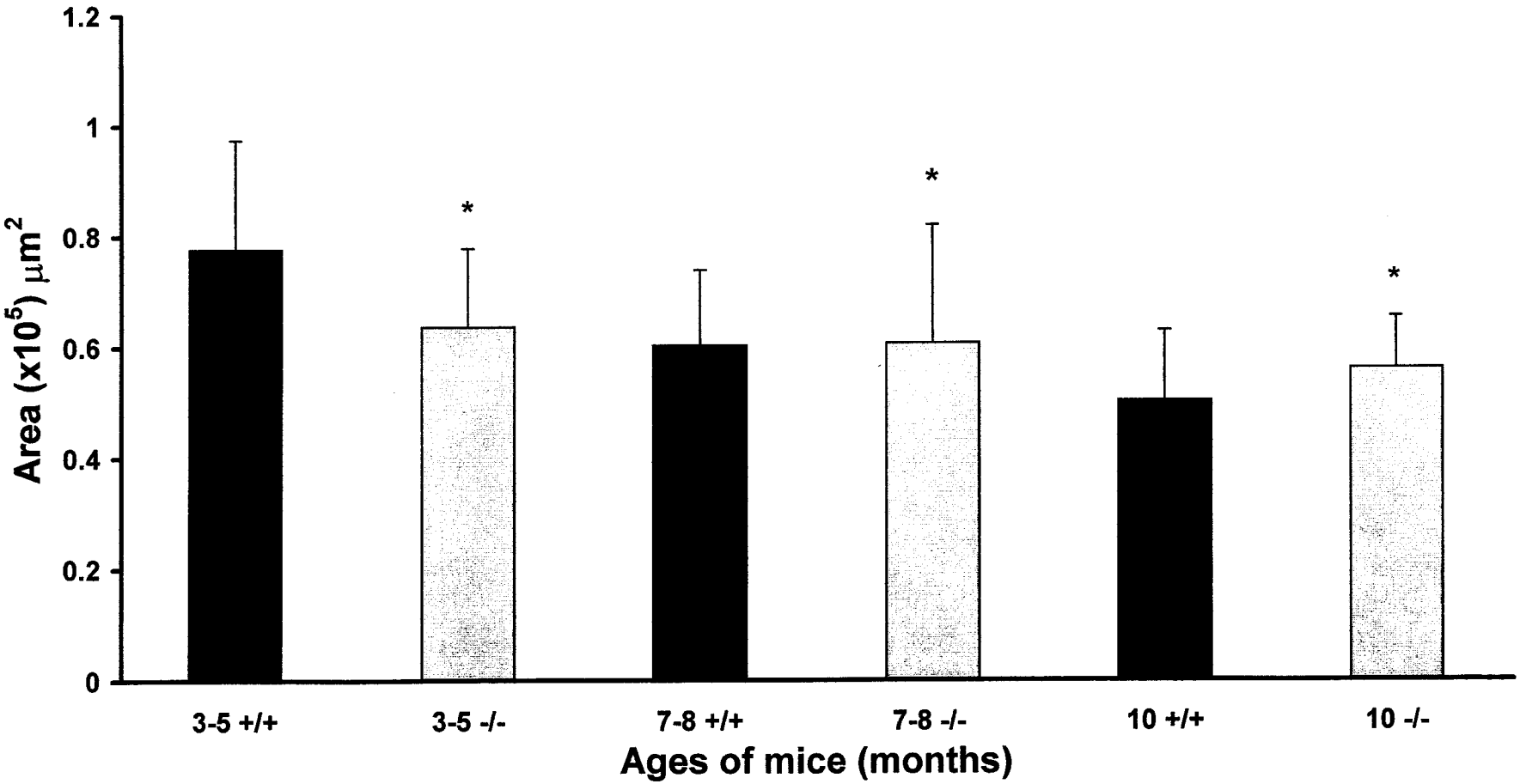
Table1: Data from ANOVA t-tests showing significance for graphs.

	Factors	Degrees of Freedom	Mean of squares	F	p value
1 (graph 1)	Age	2	2.3836	91.67	0.000
2 (graph 1)	Trmt x Age	2	0.8382	32.24	0.000
3 (graph 3)	Cell x Trmt	1	3.2201	9.755	0.002
4 (graph 4)	Cell x Trmt x Age	2	0.0617	6.040	0.002
5 (graph 2)	Trmt x Age	2	13.971	7.497	0.001
6 (graph 5)	Cell x Trmt	1	3.2201	9.755	0.002

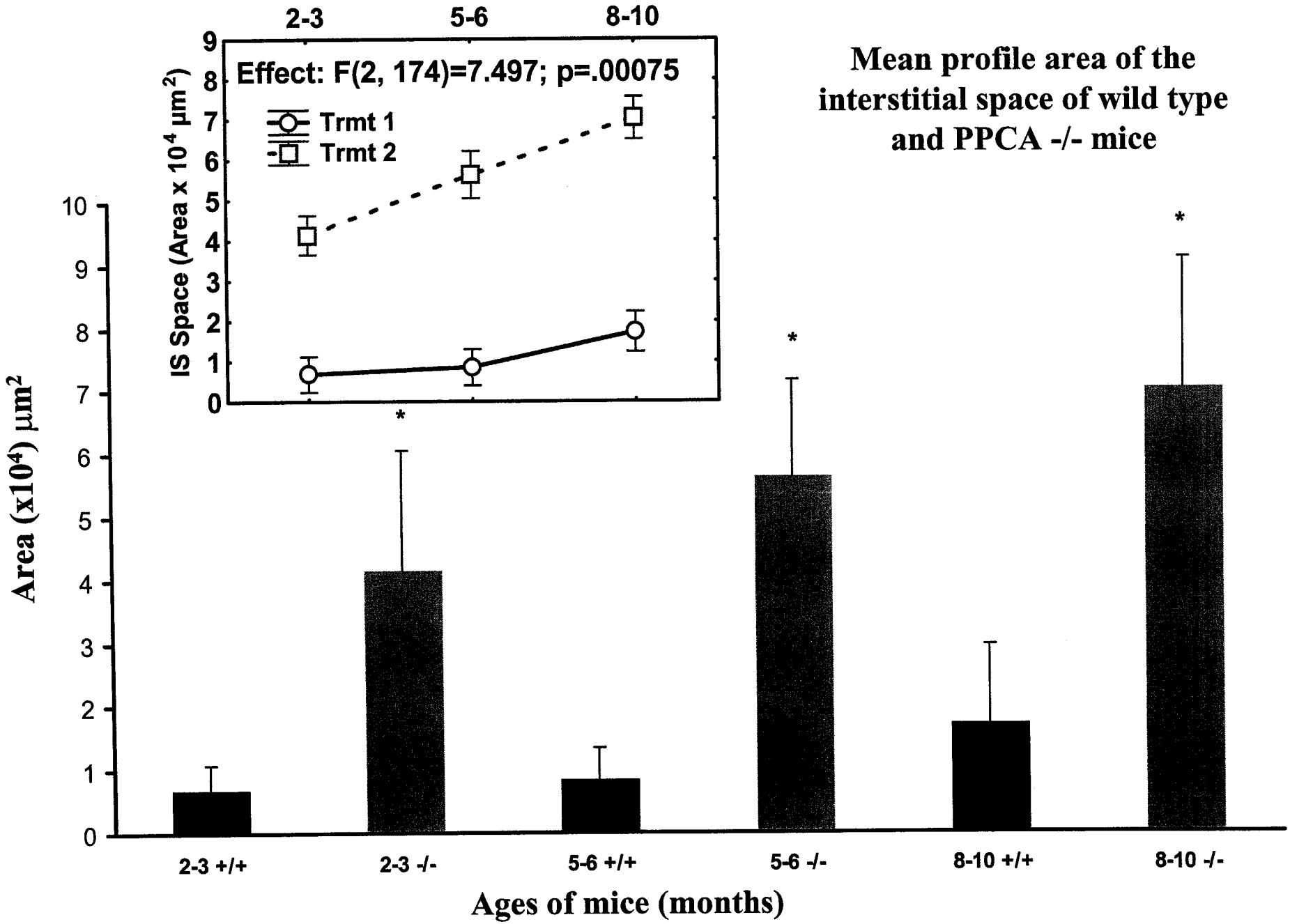
Note: Age = age of mice (months)
Trmt = treatment (wild type or knockout mice)
Cell = cell type (Leydig cell or macrophage)

Graph 1: Mean profile area of seminiferous tubules of wild type (solid bars) and PPCA -/- (shaded bars) mice at different age groups. There is a significant decrease ($p<0.000$) in the mean profile area with age in wild type mice. Furthermore, a significant decrease ($p<0.000$) is also noted between wild type and PPCA-/- mice at each age group.

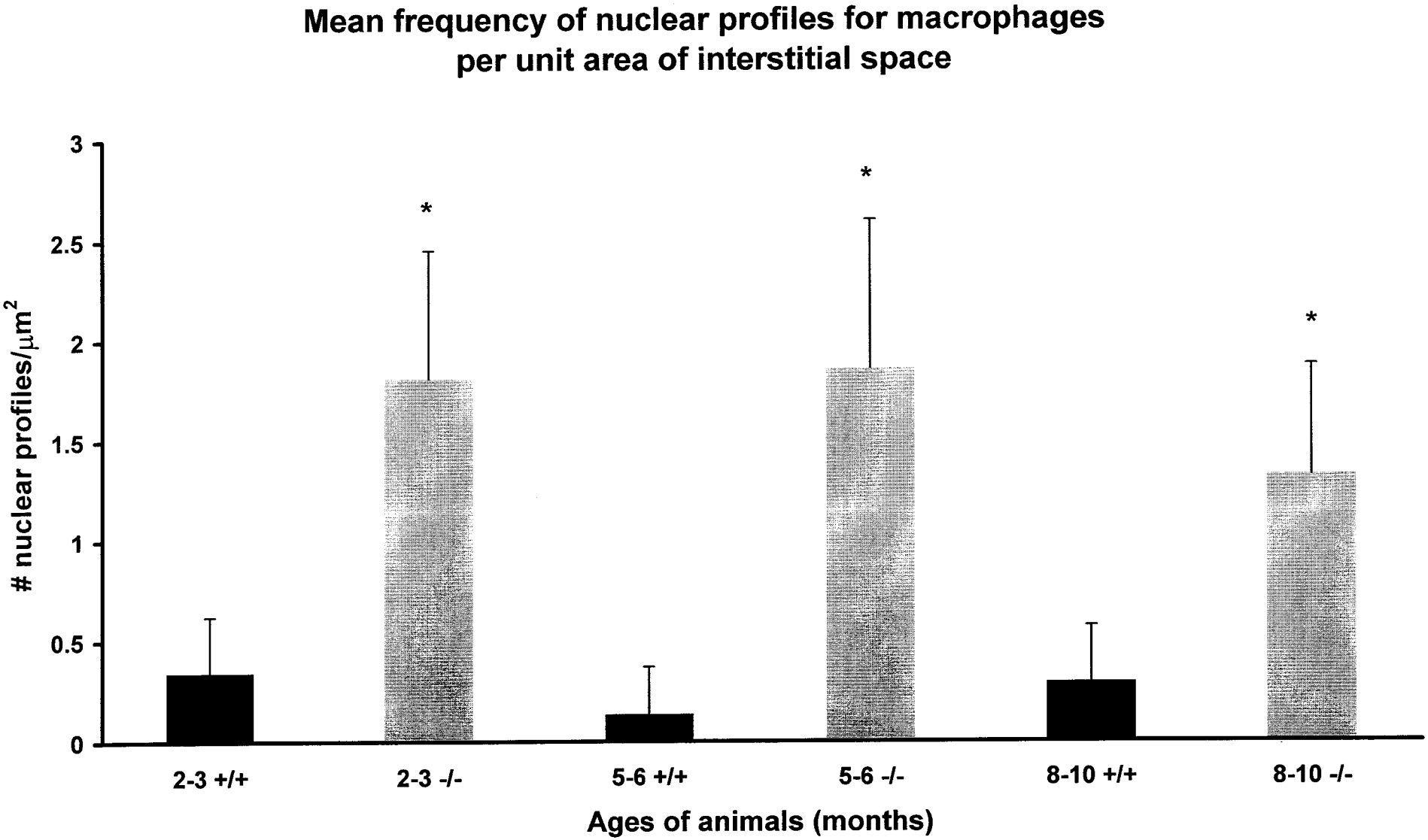
Mean profile area of seminiferous tubules of wild type and PPCA -/- mice



Graph 2: Mean profile area of the interstitial space of wild type (solid bar) and PPCA-/- (shaded bar) mice at different age groups. The interstitial space does not change with age in wild type mice, despite a trend for increase. However, there is a significant increase ($p<0.001$) in the profile area of the interstitial space of PPCA-/- mice as compared to that for wild type mice at all ages. Inset: Area data from factorial ANOVA for treatment (Trmt: wild type = 1, PPCA -/- = 2) across age (2-3, 5-6, 8-10 months). Illustrated are means ± 95% confidence intervals, F value and level of significance.

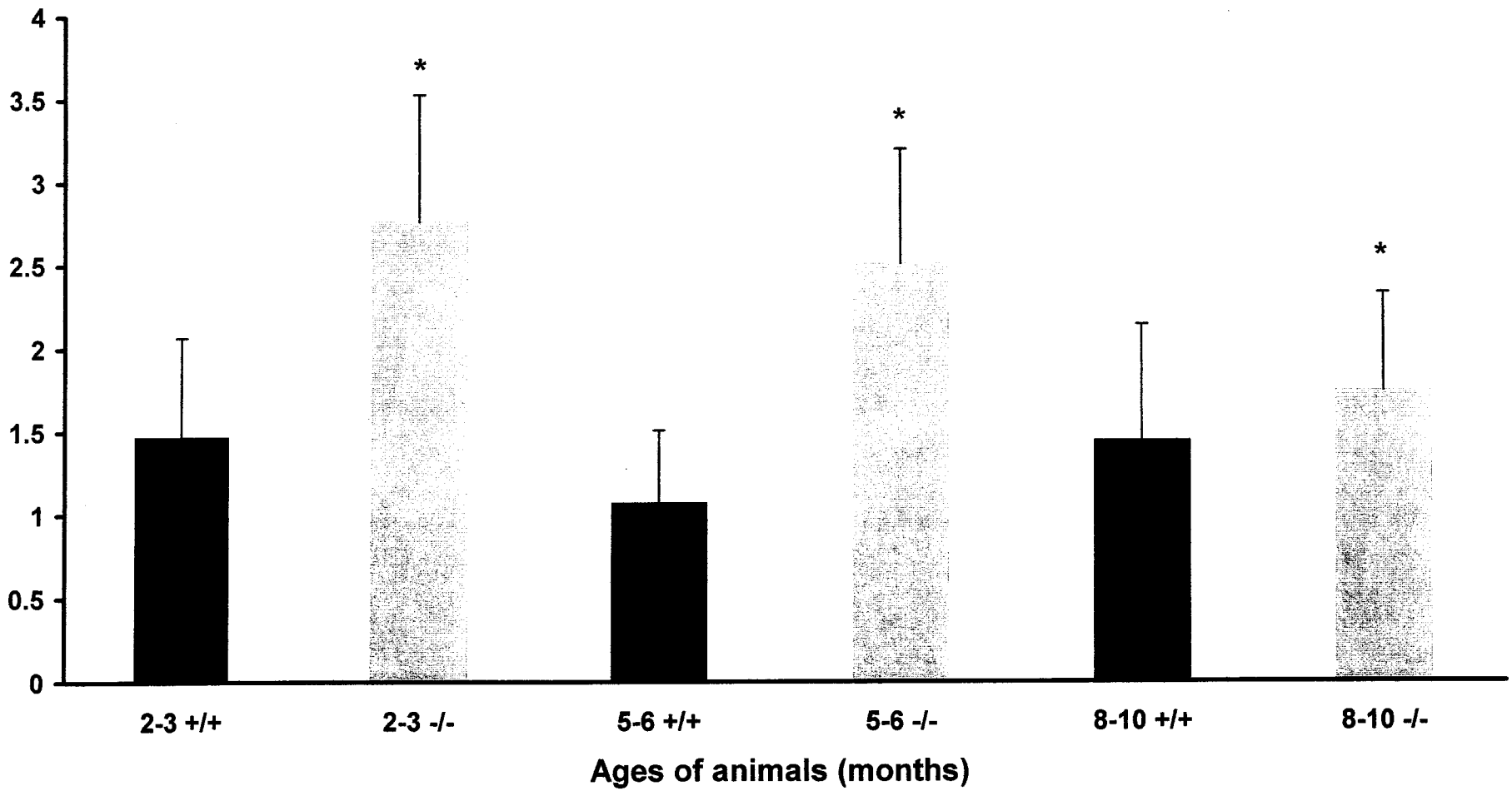

Mean profile area of the interstitial space of wild type and PPCA -/- mice
Effect: F(2, 174)=7.497; p=.00075
Trmt 1
Trmt 2
IS Space (Area x 10^4 µm^2)
2-3
5-6
8-10
Area (x10^4) µm^2
2-3 +/+
2-3 -/-
5-6 +/+
5-6 -/-
8-10 +/+
8-10 -/-
Ages of mice (months)

Graph 3: Mean frequency of nuclear profiles for macrophages per unit area of interstitial space in wild type (solid bars) and PPCA-/- (shaded bars) mice at the different age groups. The frequency of macrophages does not change with age in wild type mice. However, there is a significant increase ($p<0.002$) in the frequency of macrophages in PPCA-/- mice as compared to wild type mice at all ages.

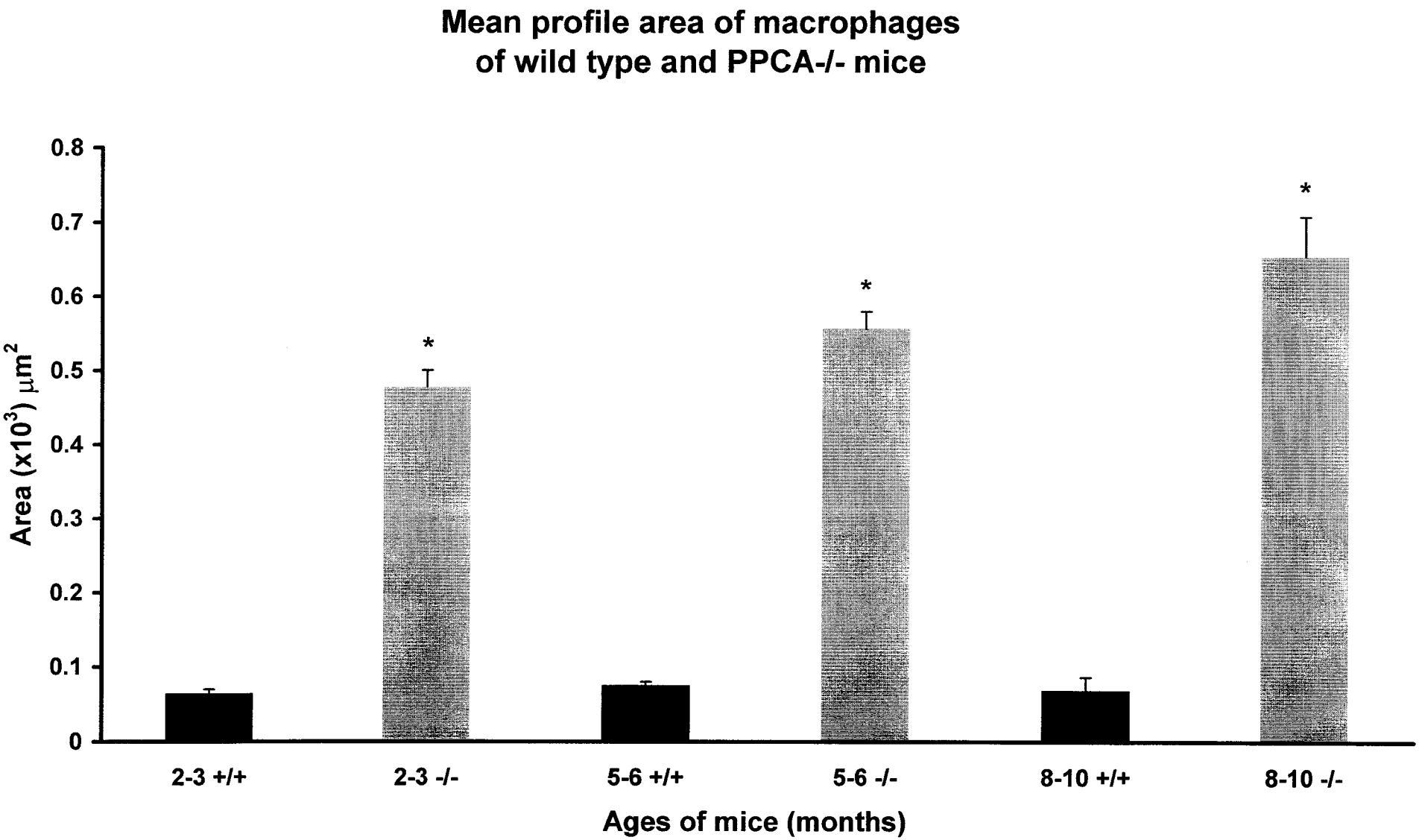

Mean frequency of nuclear profiles for macrophages per unit area of interstitial space
nuclear profiles/μm²
3
2.5
2
1.5
1
0.5
0
*
*
*
2-3 +/+
2-3 -/-
5-6 +/+
5-6 -/-
8-10 +/+
8-10 -/-
Ages of animals (months)

Graph 4: Mean frequency of nuclear profiles for Leydig cells per unit area of the interstitial space in wild type (solid bars) and PPCA-/- (shaded bars) mice at the different age groups. The frequency of Leydig cells does not appear to change with age in wild type mice. However, there is a significant increase ($p<0.002$) in the frequency of Leydig cells in PPCA-/- mice as compared to wild type mice at each age group.

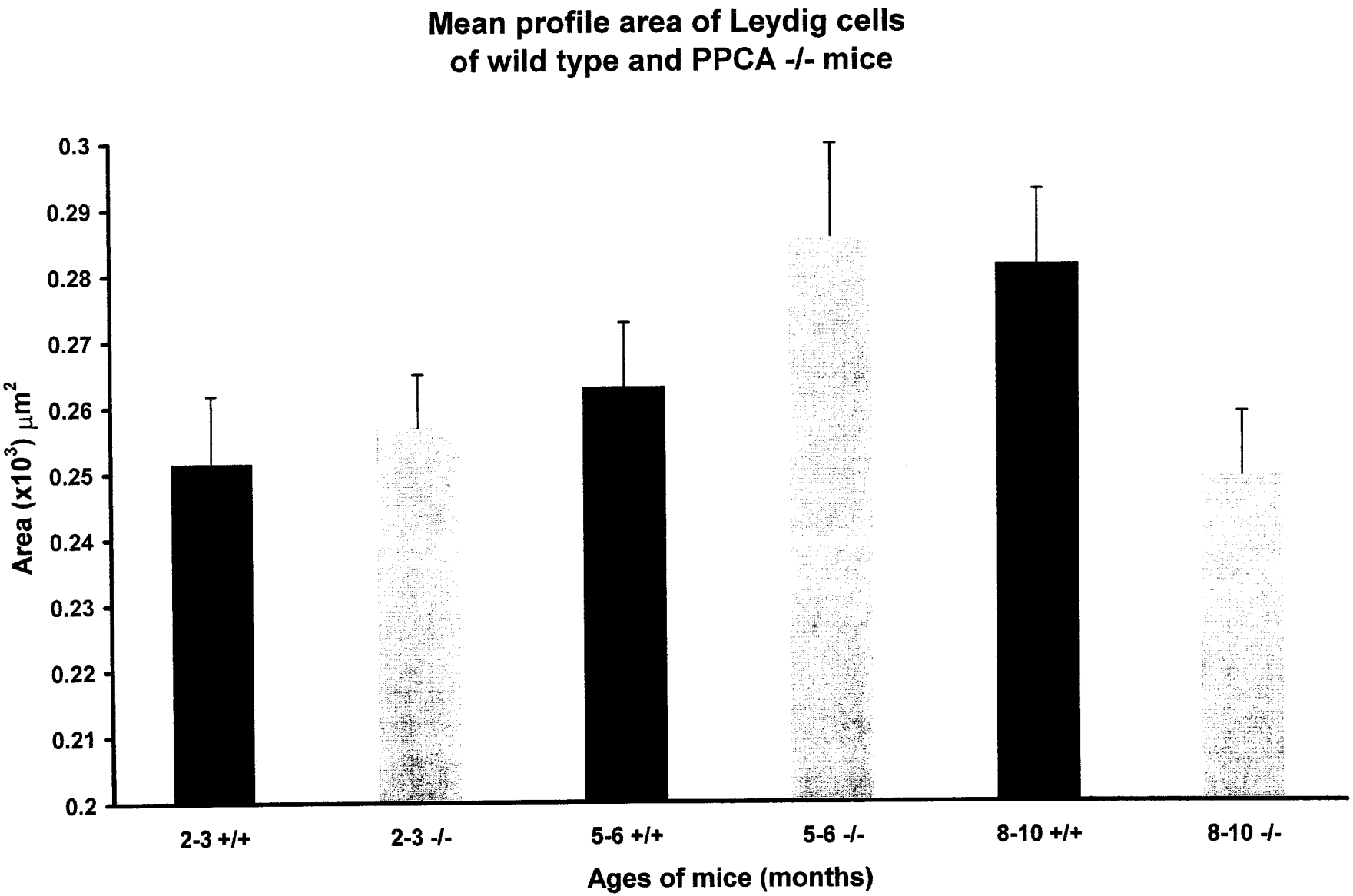
Mean frequency of nuclear profiles for Leydig cells per unit area of interstitial space



Graph 5: Mean profile area of macrophages of wild type (solid bars) and PPCA-/- (shaded bars) mice at different age groups. While the mean profile area for macrophages does not change with age in wild type mice, there is a significant increase ($p<0.002$) in PPCA-/- mice as compared to wild type mice at all ages.


Mean profile area of macrophages
of wild type and PPCA-/- mice
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Area (x10^3) μm^2
*
*
*
2-3 +/+
2-3 -/-
5-6 +/+
5-6 -/-
8-10 +/+
8-10 -/-
Ages of mice (months)

Graph 6: Mean profile area of Leydig cells of wild type (solid bars) and PPCA-/- (shaded bars) mice at different age groups. The mean profile area for Leydig cells does not change with age in wild type mice, despite a trend for increase, nor is there any significant difference between wild type and PPCA -/- mice at each age group.


Mean profile area of Leydig cells
of wild type and PPCA -/- mice
Area (x10^3) μm^2
0.3
0.29
0.28
0.27
0.26
0.25
0.24
0.23
0.22
0.21
0.2
2-3 +/+
2-3 -/-
5-6 +/+
5-6 -/-
8-10 +/+
8-10 -/-
Ages of mice (months)

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CHAPTER III

Characterization of cell- and region-specific abnormalities in the epididymis of cathepsin A deficient mice

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Abstract

Cathepsin A (PPCA), a lysosomal carboxypeptidase that functions as a protective protein for α–neuraminidase and β–galactosidase in a multienzyme complex, has been shown to be expressed in the epithelial cells of the epididymis. In the present study, the epididymis of PPCA -/- mice from 2 to 10 months of age was compared with those of their wild type counterparts. Major accumulations of pale vacuoles, corresponding to lysosomes, were noted in principal, narrow/apical, clear and basal cells in PPCA-/- mice, and this was especially evident in the caput and corpus regions, where quantitative analysis of the profile areas confirmed an apparent increase in the size of the tubules in these regions. In addition, the base of the epithelium of these regions was greatly vacuolated. The latter corresponded to cells that presented no identifiable features and which at times appeared to be degenerating. Halo cells dispersed at various levels in the epithelium and not contacting the lumen or basement membrane, also appeared to be abnormal, accumulating pale lysosomes. Since halo cells were present in regions with and without significant abnormalities, it is thus suggested that they play important roles in the epithelium of the epididymis. Furthermore, numerous macrophages, identified with a F4/80 surface marker, were observed in the intertubular space of the entire duct, presenting a large size and plethora of pale lysosomes. It is suggested that the compromised halo cells, due to PPCA deficiency within their lysosomes, cannot function properly and as a result there is a recruitment of macrophages in the intertubular space. Taken together, the data reveal major morphological abnormalities to epithelial cells of the epididymis, including the halo cells, and the recruitment of macrophages into the intertubular space.

Introduction

The epididymis is a highly coiled tube lined by several different types of epithelial cells (principal, narrow, clear and basal), showing region-specific structural features and functions. Collectively, these cells play essential roles in the maturation, concentration, protection, and storage of sperm (Hamilton, 1975; Cooper, 1986; Robaire and Hermo, 1988; Turner, 1991; Setchell et al., 1993; Hinton and Palladino, 1995).

In fine-tuning the epididymal lumen for these functions, endocytosis is an important event in the life of all the epididymal epithelial cells. Occurring along the entire length of the duct, it involves the receptor-mediated uptake of substances, such as proteins, from the lumen via coated pits. Thereafter, these proteins appear in a temporal and sequential manner in the endocytic apparatus of the cell, consisting of uncoated vesicles, endosomes, multivesicular bodies (MVBs) and lysosomes, whereupon lysosomal enzymes degrade them (Robaire and Hermo, 1988; Hermo et al., 1994; Hermo and Robaire, 2002). Along the length of the epididymal duct, the different epithelial cells at times show region-specific differences in the distribution of their lysosomal enzymes and integral membrane proteins (Suarez-Quian et al., 1992; Igdoura et al., 1994; Tomosama et al., 1994, Hermo et al., 1997). However, some lysosomal enzymes show a ubiquitous distribution in the epididymis such as sulphated glycoproteins-1 (SGP-1) (Hermo et al., 1992). This is also the case for the lysosomal protective protein/cathepsin A (PPCA), which has been shown to be highly expressed by both light and electron microscope immunocytochemistry within lysosomes of nonciliated cells of the efferent ducts, and principal, narrow/apical, clear and basal cells of the epididymis (Luedtke et al., 2000). Thus PPCA appears to be a significant player within the lysosomes of the

epididymal epithelial cells. Associating with and forming a fully functional and stable high-molecular weight multi-enzyme complex with β-galactosidase and α-neuraminidase (d'Azzo et al., 1982; Galjart et al., 1991), PPCA has two distinct functions. It facilitates the intracellular routing, lysosomal localization and activation of α-neuraminidase and protects both β-galactosidase and α-neuraminidase against rapid proteolytic degradation in lysosomes (d'Azzo et al., 1982; Galjart et al., 1991; Zhou et al., 1996; van der Spoel et al., 1998). It is targeted as a 54-kD precursor to lysosomes via the mannose-6-phosphate receptor-mediated pathway, where it is processed into its mature 32/20 kDa, disulfide linked two-chain form (Jackman et al., 1992; Hanna et al., 1994; Itoh et al., 1995; Rottier et al., 1998).

The generation of mouse models deficient in PPCA has revealed that they show a phenotype similar to human galactosialidosis, a combined deficiency of both α-neuraminidase and β-galactosidase activities (Wenger et al., 1978; Suzuki et al., 1984), that leads to the storage of sialylated oligosaccharides and glycopeptides within lysosomes, and accumulation of the latter within affected cells and tissues of the body (van Pelt et al., 1988; d'Azzo et al., 1995; Zhou et al., 1995; Sohma et al., 1999). PPCA -/- mice exhibit severe abnormalities soon after birth and develop the characteristic histopathology that resembles that found in the human disease. Thus the PPCA-/- mouse model provides a unique opportunity to study the effects of PPCA-/- deficiency on various tissues and organs of the body (Zhou et al., 1995, 1996), including the male reproductive tract, where only scant observations have been carried out (Rottier et al., 1998).

In a recent study performed on the testis of PPCA -/- mice, with PPCA having been localized to Sertoli cells of the seminiferous epithelium and macrophages and Leydig cells of the interstitial space (Luedtke et al., 2000), it was noted that there was a statistically significant decrease in the seminiferous tubular diameter from 2-10 months of age. In addition, Leydig cells showed an accumulation of pale vacuoles identified as lysosomes (Korah et al., 2003). Furthermore, macrophages, normal residents of the interstitial space of the testis (Hume et al., 1984), became grossly abnormal. They became filled with pale lysosomes and showed a statistically significant increase in their size and number from 2 to 10 months of age. It was suggested that the increase in number of macrophages in PPCA-/- mice may be a consequence of the abnormalities taking place in Leydig cells (Korah et al., 2003), as these two cell types, in normal animals, closely interact with each other by producing factors that mutually regulate their numbers and functions (Hutson et al., 1992; Hales et al., 2002).

However, in the epididymis, detailed studies on the structural abnormalities of the various epithelial cell types in the different regions have not been carried out in PPCA-/- mice. In fact, Rottier et al. (1998) only mentioned that the epithelial cells of the epididymis became vacuolated as revealed by light microscopy, and that while sperm were present, it was noted that the frequency of pregnancies was decreased compared to that for wild type mice (Rottier et al., 1998). Thus much remains to be discovered on the effects of PPCA deficiency in the epididymis and its possible consequences on sperm functions.

The purpose of the present study was to examine the structural abnormalities of PPCA deficiency on the various epithelial cells in the different epididymal regions using

routine light and electron microscopic analysis. A quantitative analysis was also carried out to assess differences in epididymal tubular profile areas (epithelium versus lumen) between PPCA-/- and wild type mice, along with statistical analysis. In addition, various markers in conjunction with LM immunocytochemistry were employed to characterize the different cell types present in the epithelium as well as the intertubular space, and to determine whether or not these cells were dividing in PPCA-/- mice. Lastly, cryo EM immunocytochemistry, with lysosomal markers, characterized the accumulated pale vacuoles in the cytoplasm of the various cell types of PPCA-/- mice as lysosomes.

Materials and Methods

Routine Light and Electron Microscopic Preparation of Testicular Tissue

Mouse models of galactosialidosis (PPCA -/-) were developed through targeted disruption of the PPCA gene. A targeting vector for homologous recombination was constructed, which gave rise to a null mutation at the PPCA locus (Zhou et al, 1996). Wild type and PPCA -/- mice of strain 129Sv, ranging from 2 to 10 months of age (2-3 per age), were utilized for this study and obtained from the lab of Dr. A. d'Azzo (Memphis, Tennessee). Prior to perfusion, the mice were anaesthetized by an intraperitoneal injection of sodium pentobarbital (Somnitol, MTC Pharmaceuticals, Hamilton, Ontario, Canada). In some cases, only the efferent ducts and epididymis of the right side were fixed by cardiac perfusion with 5% glutaraldehyde buffered in sodium cacodylate (0.1M) containing 0.05% calcium chloride (pH 7.4), while those of the left side were removed prior to perfusion and immersed in Bouin's fixative (see below). After

10 minutes of cardiac perfusion, the efferent ducts and epididymides were removed. The epididymis was divided into its major regions, i.e. the initial segment, caput, corpus and cauda (Hermo et al., 1991a), with each being cut into approximately 1 mm^3 pieces, as were the efferent ducts, and subsequently placed in the above fixative for an additional 1h at 4°C. Thereafter, the tissue was thoroughly rinsed three times in 0.1M sodium cacodylate buffer containing 0.2M sucrose and left in buffer overnight at 4°C.

On the following day, the tissues were postfixed in potassium ferrocyanide-reduced osmium tetroxide for 1 h at 4°C to enhance staining of membranes (Karnovsky, 1971) and subsequently processed for routine light and electron microscopic analyses, as described previously (Hermo et al., 1991b).

Light Microscopic Immunocytochemistry

Wild-type and PPCA -/- mice at 3, 5, 7, 8 and 10 months of age were anaesthetized with an intraperitoneal injection of sodium pentobarbital (Somnitol, MTC Pharmaceuticals, Hamilton, Ontario, Canada). While the right reproductive tract was kept intact for cardiac perfusion (as described above), the left side was removed and fixed by immersion in Bouin's fixative overnight and subsequently processed for light microscopic immunocytochemical analysis as described previously (Hermo et al., 1992b).

The following polyclonal affinity purified antibodies were used at the various dilutions for routine peroxidase immunostaining: anti-SGP-1 antibody (1:200) provided by Dr. C. R. Morales, McGill University, Montreal, Canada (purified and characterized as described in Morales et al., 2000); anti-cathepsin D antibody (1:100) purchased from

Calbiochem (La Jolla, Ca) (Igdoura et al., 1994); anti-LRP-2 antibody (1:100) obtained from Dr. S.A. Argraves, see details in Hammad et al., 2000; anti YF-GST antibody (1:100) purchased from Stressgen (details on its characterization and purification are found in Eimoto et al., 1988); and an anti-SGP-2 antibody (1:100), kindly provided by Dr. M.D. Griswold, Washington State (Sylvester et al., 1984). Two monoclonal antibodies, along with enhancer kits, were also used in this study; F4/80 (1:10) was obtained from Abcam Ltd. (Cambridge, UK) and has been characterized and purified as described in Austyn and Gordon (1981), and cdc47 (Neo Markers, California), characterized and purified as described in Hiraiwa et al. (1997). Their controls consisted of incubation only with 100µl of Tris-buffered saline (TBS), i.e., no primary antibody was added. In addition, normal rabbit serum or omitting the primary antibody was used as controls for the polyclonal antibodies at similar dilutions. In all cases, the tissue sections containing epithelial cells and interstitial space were devoid of any staining.

The paraffin sections were deparaffinized with Histoclear and subsequently processed for LM immunocytochemical analysis as described previously (Hermo et al., 1992b). The tissue sections were incubated at 37°C in a humidified chamber for 90 minutes with 100µl of diluted primary antibody. The sections were counterstained with 0.1% methylene blue and dehydrated in a graded series of ethanol solution and Histoclear. Cover slips were mounted onto glass slides with Permount.

For the anti-monoclonal F4/80 antibody, a Vectastain elite ABC kit was utilized to enhance the F4/80 reaction, while a Zymed SP kit (San Francisco, CA) was used for enhancing the cdc-47 reaction. After deparaffinization of the tissue sections with Histoclear and hydration in a series of graded ethanol solutions (Hermo et al., 1992b),

the slides were incubated for 20 minutes with a blocking agent at room temperature. The sections were then incubated with the primary antibody diluted at 1:10 with 10mM phosphate buffered saline (PBS) for 30 minutes, followed by three rinses in PBS. The slides were then incubated with diluted secondary antibody for 30 minutes at room temperature, followed by more washing and then a final incubation with the Vectastain elite ABC reagent for 30 minutes. A peroxidase substrate was used until the desired stain intensity appeared (approximately 90 seconds), after which the sections were counterstained with methylene blue, dehydrated in ethanol and Histoclear, and mounted with cover slips using Permount (Hermo et al., 1992b).

Electron Microscopic Immunocytochemistry

Two additional wild type and two PPCA-/- mice (7 months of age) were anaesthetized with sodium pentobarbital and their epididymidis were fixed by cardiac perfusion with a fixative consisting of 0.5% glutaraldehyde and 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). After their removal, the tissue was trimmed into small pieces ($0.5mm^3$), immersed for 2 h in the above fixative at 4°C, washed two or three times in 0.15M PBS (pH 7.4) and then treated with PBS containing 2.3M sucrose. The tissue was then frozen in liquid nitrogen until sectioned.

Ultrathin sections of the caput epididymidis were mounted on 300-mesh, formvar-coated nickel grids (Canemco, Montreal, QC, Canada) and processed as described previously (Luedtke et al., 2000). Photographs were taken on a Philips 400 electron microscope. Incubation without primary antibody served as controls, with

images from these sections being devoid or showing sparse gold particles representing low background levels of labelling.

Quantitative LM studies

Quantitative analyses were performed in wild type and PPCA-/- mice at 2-10 months of age using a Zeiss MOP-3 image analyser (Carl Zeiss Canada Ltd. Montreal, QC). Randomly selected fields of initial segment, corpus/caput, and cauda regions in cross sections of epididymidal tubules of glutaraldehyde-fixed and Epon-embedded tissue were photographed at x320 with a light microscope. The outer peripheral and inner luminal cross sectional profile areas of the tubules in these regions were outlined with the stylus of the MOP-3 and area measurements were recorded in Microsoft™ Excel. The profile area of the epithelium forming the walls of the tubules in each region was computed as the difference between the outer peripheral and inner luminal area measurements in each field. Statistical analyzes including power tests of sample sizes for comparing two means of independent samples, distribution test, and nonparametric tests (Mann-Whitney U test and Kolmogorov-Smirnov test) were done using Ver 6.0 of Statistica (Statsoft, Tulsa, OK). For the latter tests, p values less than 0.05 were considered statistically significant.

Results

Structural features of the efferent ducts in PPCA-/- mice

The epithelial cells of the efferent ducts of PPCA-/- mice were similar in appearance to those of wild type mice at all ages examined (Figs. 1a, b). The nonciliated

cells showed a basally located nucleus and few small dense supranuclear lysosomes, while ciliated cells presented an apically located nucleus. However, in PPCA -/- mice, spherical cells with a cytoplasm filled with pale lysosomes were often noted at the base of the epithelium (Fig. 1b). Such cells did not contact the lumen, and were never seen in wild type mice; they will be referred to as halo cells. Functionally, the epithelial cells expressed various lysosomal enzymes such as SGP-1, cathepsin D and low-density lipoprotein receptor-related protein-2 (LRP-2), a cell surface receptor, at levels comparable to that of wild type mice (Fig. 6).

Structural features of the epithelial cells of the epididymis

In the initial segment of PPCA-/- mice, principal cells appeared comparable to that of wild type mice; they extended from the base of the epithelium to the lumen, showed a basally located nucleus and little accumulation of pale lysosomes (compare Fig. 2a with 2b). However, some cells, with a more apically positioned nucleus, presented a highly vacuolated cytoplasm in PPCA-/- mice, which was not observed in wild type mice; they were referred to as narrow/apical cells (Figs. 2a, b). In addition, other smaller vacuolated cells resided exclusively near the base of the epithelium in PPCA-/- mice and corresponded either to halo or basal cells (Fig. 2b), as will be discussed below. From a structural point of view, principal cells expressed various lysosomal enzymes, such as SGP-1 and cathepsin D, as well as LRP-2 on their apical cell surfaces (Fig. 6a) and Yf-glutathione-s-transferase (Yf-GST) in their cytoplasm (Fig.6b), comparable to that seen in wild type mice. The highly vacuolated narrow/ apical cells were not reactive for LRP-2

and did not express Yf-GST as seen in wild type mice, thus allowing the distinction of these cell types from principal cells (Fig. 6b).

The caput and corpus epididymidis were the most seriously affected regions of the epididymis in PPCA-/- mice as compared to wild type mice at all ages examined (Figs. 3a, b, 4a, b, 6c-e). In both regions, principal cells were highly vacuolated, showing numerous pale lysosomes that filled their entire cytoplasm, that were not noted in wild type mice (Fig. 3a, b, 4a, b). In addition, clear cells were greatly enlarged, revealing a cytoplasm engorged with pale lysosomes of various sizes (Figs. 4a, b). These cells generally contacted the lumen and basement membrane, however, their distinct foamy appearance allowed for their identification, despite the fact that in some planes of sections they did not contact the lumen or basement membrane. Furthermore, clear cells were not reactive for SGP-2 or cathepsin D, as were the adjacent principal cells, allowing the distinction of one from another (Figs. 6c, e).

Also evident in the epithelium were extensive areas of vacuolation. Such vacuolated territories resided mainly near the base of the epithelium and did not contact the lumen and were filled with lysosomes, some of gigantic size (Figs. 3b, 4b, 6c-e). Their identity was problematic as they did not contain identifiable features or organelles and did not stain for various markers of the epithelial cells; they will be discussed below. Their presence resulted in a dramatic redistribution of the epithelial cell population in any given tubular cross section of these regions (Figs. 4a, b, 6d). This was especially evident in epididymal sections, where the various epithelial cells were immunostained as for the case with SGP-1 (Fig. 6d).

In the cauda epididymidis of PPCA-/- mice, principal cells showed numerous pale lysosomes especially in their infranuclear region. Clear cells were also vacuolated (Fig.5b), however, not to the extent of that noted for these cells in the caput and corpus epididymidis of PPCA-/- mice (Figs. 4a, b). Extensive basal vacuolation of the epithelium was not pronounced in the cauda region, as compared to the caput and corpus regions. There was no comparable vacuolation of epithelial cells in wild type mice (Fig. 5a).

Despite the serious consequences to the epithelium, the lumen always contained sperm, even though at times the lumen appeared to be greatly reduced in size. In the intertubular space, numerous pale vacuolated cells were evident in PPCA-/- mice (Figs. 3b, 4a,b, 6c,e) that were never noted in wild type mice (Figs.2a, 3a). They were identified as macrophages due to the fact that their cell surface immunostained with a monoclonal F4/80 antibody, specific for macrophages (Fig. 6f). They did not react with cdc-47, a cell cycle marker, suggesting that these cells were not dividing in the intertubular space (6g).

Control sections employing normal rabbit serum or omitting the primary antibody revealed in all cases, a complete absence of reaction over the epididymal epithelium, luminal contents or intertubualr space (not shown). Comparable images can be found in our other studies (Hermo et al., 1992a; Hermo et al., 2000).

Electron microscopic appearance of the epithelium of PPCA-/- mice

In the efferent ducts, the ultrastructural appearance of the epithelial nonciliated and ciliated cells was comparable to those of wild type mice (not shown). This was also the case for principal cells of the initial segment of the epididymis of PPCA-/- mice.

These cells were comparable in the size, appearance and distribution of their organelles to those of wild type mice and showed no accumulation of pale lysosomes in their cytoplasm (Fig. 13).

In the caput, corpus and cauda epididymidis of PPCA-/- mice however, principal cells were markedly different from those of wild type mice. Notable was the presence of numerous pale membrane bound structures in their cytoplasm, containing few vesicular and membranous profiles (Fig. 7). These structures revealed numerous gold particles representing anti SGP-1 and cathepsin D antibodies, indicative of their lysosomal nature (Fig. 11a). Control sections immunolabeled with normal rabbit serum or omitting the primary antibody revealed only an occasional gold particle over a given field of section, comparable to that considered as background levels of labelling (not shown). These lysosomes are of variable sizes, extending from the apical to the infranuclear regions of the cell, and sites of fusion between them were often noted (Fig. 7). Such lysosomes were not present in wild type mice. Despite the accumulation of pale lysosomes, the cytoplasm of principal cells displayed a conspicuous Golgi apparatus and cisternae of endoplasmic reticulum. However, the presence of electron dense lysosomes of small and moderate sizes, as compared to those described in principal cells of wild type animals (Abou-Haila et al., 1984; Adamali et al., 1999a, b), was not evident in principal cells of PPCA-/- mice. The apical region of principal cells contained coated pits and vesicles (Fig. 7), which appeared to be as prominent as that seen in wild type mice.

Clear cells of the caput and corpus epididymidis were dramatically affected in PPCA-/- mice (Fig. 8). Absent were the numerous dense lysosomes seen in wild type mice (Adamali et al., 1999a,b). Instead, pale membrane bound structures, identified as

lysosomes with lysosomal markers (Fig. 11c), occupied their entire cytoplasm. They were so numerous that they appeared to result in a dramatic increase in the overall size of the cell. The pale lysosomes, containing few small vesicular and membranous profiles, often appeared to fuse with each other. The presence of coated pits and vesicles in the apical region suggested that endocytosis was an ongoing event in clear cells of PPCA-/- mice.

In the initial segment, narrow/apical cells of PPCA-/- mice were markedly different from those of wild type mice. These cells showed an abundance of pale lysosomes throughout their cytoplasm, which were identified as such by lysosomal markers (Fig. 11 b). In some cases, their supranuclear region took on the appearance of one gigantic lysosome, containing vesicular profiles of different sizes (Fig. 9). Numerous small spherical and c-shaped vesicles, characteristic of these cells in wild type animals (Adamali et al., 1999a,b), occupied their apical region. The overall size of these cells appeared to be greatly increased in PPCA-/- mice, as compared to that of wild type mice.

Along the entire epididymis, basal cells also contained pale lysosomes, immunolabeled with anti SGP-1 antibody (not shown). They were identified as basal cells since they resided on the basement membrane and did not reveal any contact with the lumen (Fig. 10).

In the caput and corpus epididymidis, occasional extensive vacuolated territories were noted at the base of the epithelium (Fig. 12). At times such areas merely contained gigantic pale membrane-bound structures enveloped by a thin rim of cytoplasm. Similar areas, in appropriate planes of section also revealed a small pyknotic nucleus and numerous small pale lysosomes (Fig. 12). These territories appeared to represent cells in

degeneration, and due to the lack of distinguishable features, their identity could not be determined, even with epithelial cell markers.

In the efferent ducts and entire epididymis, well-defined cells were evident near the base of the epithelium (Figs. 13, 14a, b). Such cells showed no features typical of principal, clear or narrow/apical cells, and were not classified as basal cells as they did not contact the basement membrane. Due to their small size, and meandering distribution and positioning within the epithelium, they were classified as halo cells. Grossly altered in their appearance in PPCA-/- mice, such halo cells were often sandwiched between adjacent principal cells, revealing an abundance of pale lysosomes that dominated their cytoplasm and identified as such with anti SGP-1 antibody. While lysosomes are a component of halo cell cytoplasm in wild type mice, they are small and dense in appearance (Adamali et al., 1999) and never take on the pale appearance and numbers as that seen in PPCA-/- mice. Occasionally, halo cells with a more normal looking appearance were seen in PPCA -/- mice (Fig. 14b), but even these revealed several small pale lysosomes. The true identity of these cells was especially apparent in the efferent ducts and initial segment (Fig. 13), where extensive epithelial cell vacuolation was not apparent.

In the intertubular space of both the efferent ducts and epididymis, numerous large pale macrophages were evident in PPCA-/- mice. Such cells were engorged with pale lysosomes that filled their entire cytoplasm (Fig. 15). Normal looking macrophages of smaller size and containing dense lysosomes of various sizes were not evident in PPCA-/- mice. In fact, macrophages are not a resident cell type of the intertubular space of wild type mice. The number of macrophages in PPCA-/- mice appeared to be

significant, as they were present in almost all fields examined of the intertubular space of the entire efferent ducts and epididymis.

Quantitative analysis of tubular profile areas of different epididymal regions of wild type and PPCA-/- mice

Preliminary analyses indicated that the profile areas of tubules and lumens measured in all regions of the epididymis did not follow normal distributions in either wild type or PPCA-/- mice. Some improvements toward normality were obtained using simple $\log_{10}$ transformations (Sokal and Rohlf, 1981), and this transformation was applied to all area measurements prior to comparing results by conventional nonparametric tests for 1 variable (area) and 2 groups (wild type versus PPCA-/-). Based on number of observations, means, and standard deviations of area measurements, statistical tests done in the corpus/caput region had considerable power (greater than 97%) but those done in the initial segment were less conclusive (about 70% power) due to inherent high variation and limitations in the number of profile area measurements that were possible in the wild type and PPCA-/- mice.

The quantitative measurements indicated that the profile areas of tubules in PPCA-/- mice increased by about 20% above wild type mice in both the initial segment and the corpus/caput regions of the epididymidis. These changes were due to increases in profile areas of the epithelium, which were significantly larger in both regions ($p<0.05$). In the initial segment, there were no apparent differences in the luminal areas of PPCA-/- and wild type mice, whereas in the corpus/caput region luminal areas appeared to have shrunk by 50% (graphs 1, 2). This decrease was significant ($p<0.05$).

Discussion

In the present study, it was noted that PPCA deficiency resulted in major cell- and region-specific abnormalities. The efferent ducts, in which the nonciliated cells express PPCA (Luedtke et al., 2000), demonstrated no noticeable abnormalities in PPCA-/- mice. These cells are highly endocytic cells and possess numerous supranuclear lysosomes (Hermo and Morales, 1984), however, no apparent changes to the number, size and distribution of lysosomes were detected. Previous studies have revealed the entry of tracers, introduced into the lumen of these ducts, into coated pits and eventually lysosomes (Hermo and Morales, 1984). Thus, the absence of a phenotype in PPCA-/- mice suggests that either the substrates acted upon by PPCA in nonciliated cells are absent, or that other lysosomal enzymes may take over the role of PPCA in its absence.

In the initial segment, narrow/apical cells were adversely affected in PPCA-/- mice. Narrow cells are slender attenuated cells that have been shown to be active in endocytosis, possess numerous small dense supranuclear lysosomes and express PPCA (Adamali and Hermo, 1996; Hermo et al., 2000; Luedtke et al., 2000). Unlike apical cells, which reside apically, narrow cells contact the basement membrane via a thin foot like process. Because we were not able to systematically differentiate narrow from apical cells in the present study, due to varying planes of section, we will therefore simply refer to these cells as narrow/apical cells. In the present study, narrow/apical cells were greatly enlarged and filled with pale lysosomes of various sizes, some of gigantic size. They were identified by the presence of numerous small spherical and c-shaped vesicles in their apical region, which is not a feature of principal cells (Abou-Haila and Fain-Maurel, 1984; Adamali and Hermo, 1996). In addition, unlike principal cells, they did not express

either LRP-2 or Yf-GST, as noted in wild type animals, (Papp et al., 1995; Adamali and Hermo, 1996; Hermo et al., 1999). The fact that narrow/apical cells appear abnormal in PPCA-/- mice suggests that these cells are dependent upon PPCA and in its absence the substrates acted upon by PPCA accumulate in their lysosomes. Thus narrow/apical cells appear to be actively involved in modifying the luminal contents via endocytosis as sperm travel through the initial segment in their quest for maturation.

Principal cells of the epididymis displayed a differential response to the absence of cathepsin A despite the fact that these cells expressed PPCA along the entire duct (Luedtke et al., 2000). In the initial segment, no vacuolation of their cytoplasm was noted, and while principal cells of the caput and corpus regions were grossly abnormal, those of the cauda region were only moderately abnormal. The abnormalities were seen as an accumulation of pale lysosomes throughout their cytoplasm, as compared to those of wild type mice, which show only several small dense lysosomes restricted to their supranuclear region (Abou-Haila and Fain-Maurel, 1984; Adamali and Hermo, 1996). Indeed, principal cells are active endocytic cells and take up various substances by coated pits that eventually end up in lysosomes where they are degraded (Moore and Bedford, 1979; Hermo et al., 1994). The accumulation of lysosomes by these cells indicates their inability to degrade substrates endocytosed by them and as a consequence lysosomes begin to fuse with one another and gradually occupy the apical and infranuclear areas of the cytoplasm, where lysosomes are not normally found. Endocytosis appears to be ongoing as coated pits and vesicles still persist on the principal cell surface. Thus principal cells appear to be involved in modifying the luminal contents as sperm acquire their maturational features in these regions of the duct. The region specificity of a

phenotype suggests that other enzymes may compensate for the absence of PPCA in these cells or that the specific substrates acted upon by PPCA are absent or diminished in these regions.

In the caput and corpus regions, clear cells were grossly altered in PPCA -/- mice. Normally large cells as compared to the adjacent principal cells of wild type animals (Abou-Haila and Fain-Maurel, 1984; Adamali et al., 1999a,b), clear cells became gigantic in size and as a result greatly displaced the principal cells. Clear cells were identified as such by their absence of staining for LRP-2 and Yf-GST, as both of these are markers for principal cells and are not expressed in clear cells of wild type animals (Papp et al., 1995; Hermo et al., 1999). Clear cells are highly endocytic, as compared to principal cells (Moore and Bedford, 1979), and possess numerous dense lysosomes supranuclearly (Abou-Haila and Fain-Maurel, 1984; Robaire and Hermo, 1988; Adamali et al., 1999a,b). In the present study, clear cells, as seen in the EM, were packed with pale lysosomes that occupied all regions of the cytoplasm and that fused with each other. As they express PPCA in normal animals (Luedtke et al., 2000), its absence in PPCA-/- mice indicates that it plays a dramatic role in lysosomes in the degradation of substrates endocytosed from the lumen. In fact, clear cells have been shown to endocytose the contents of cytoplasmic droplets released from sperm as they travel down the duct, in addition to various specific proteins (Hermo et al., 1988; 1992b; Rankin et al., 1992; Vierula et al., 1995). Interestingly, clear cells of the cauda region were not as affected even though they also express PPCA (Luedtke et al., 2000), suggesting that substrates for PPCA in this region are not as prominent, or that other enzymes compensate for its absence. In fact, the

majority of sperm have already shed their cytoplasmic droplets by the time they enter the cauda (Hermo et al., 1988).

In the epithelium of the efferent ducts and epididymis of PPCA-/- mice, cells other than of epithelial origin, were grossly abnormal. Such cells did not contact the lumen, or present any features characteristic of neighboring principal, clear or narrow/apical cells, nor did they contact the basement membrane, as did basal cells. Due to their size, shape and positioning at different levels of the epithelium, and the fact that they did not show junctions with adjacent epithelial cells, they were classified as halo cells; cells well recognized to be components of the epithelium of the efferent ducts and epididymis of normal animals (Dym and Romrell, 1975; Abou-Haila and Fain-Maurel, 1984; Robaire and Hermo, 1988). While the majority of halo cells were of larger size than wild type and filled with lysosomes, at the expense of other organelles, some halo cells contained only a few small pale lysosomes, and under these conditions, the true halo cell-like morphology could be visualized. Furthermore, halo cells were readily apparent in the epithelium of the efferent ducts and initial segment, where abnormalities to the other cell types at times did not overshadow their presence. However, in the caput and corpus epididymidis, due to the gross abnormalities of the other epithelial cells of this region, their distinct identity was more problematic. While halo cells have been identified with specific markers and shown to represent lymphocytes and monocytes (Flickinger et al., 1988; Serre and Robaire, 2000), in our hands, we could not detect staining for either of these cell types in the epithelium of PPCA-/- mice. One possibility could be that the secretory apparatus of halo cells in PPCA-/- mice, due to their enlarged size and abnormalities, is compromised and that they express low levels of surface markers. In

any case, the identity of halo cells versus principal, clear, narrow/apical and basal cells could be established in some cases by routine EM analysis, as well as the use of specific markers for principal versus clear cells, confirming that halo cells are dramatically altered in PPCA-/- mice. However, as was more readily apparent in the efferent ducts and initial segment, the number of halo cells did not appear to increase in PPCA-/- mice.

The fact that halo cells are abnormal suggests an important role for these cells in the epithelium. This is especially evident in the case of the efferent ducts, where abnormalities to the epithelial cells were not apparent. Such observations suggest that halo cells, identified as monocytes and/or lymphocytes, well known phagocytic cells, normally perform this function in the epithelium of wild type mice (Robaire and Hermo, 1988; Flinkinger et al., 1997). This becomes especially evident in PPCA-/- mice, where they become filled with pale lysosomes suggesting their active role in the uptake of substances from the epithelium, but inability to break them down in the absence of PPCA.

In the intertubular space of PPCA-/- mice, large vacuolated cells appeared in all regions of the epididymis, including the efferent ducts. Such cells showed a cell surface reaction for the macrophage marker F4/80, indicating their macrophage lineage. However, unlike typical macrophages, which show numerous dense lysosomes, those of the intertubular space were filled with pale lysosomes. Macrophages with a similar appearance, referred to as foamy macrophages, have been described in PPCA-/- mice in other tissues (Zhou et al., 1995; Rottier et al., 1998). In this respect, they are also similar to those described in the interstitial space of PPCA-/- mice of the testis (Korah et al., 2003). There, macrophages accumulated and increased in size and number with age and

accumulated a plethora of lysosomes in their cytoplasm due to PPCA deficiency. It was suggested that they entered from the blood, as division of these cells in the interstitial space was not observed. In addition, it was suggested that the increase in macrophage number was a consequence of abnormalities seen in Leydig cells as the latter accumulated lysosomes (Korah et al., 2003), and since over the years it has been demonstrated that macrophages closely interact with Leydig cells both structurally and functionally (Miller et al., 1983; Morris et al., 1986; Hutson, 1992; Hales, 2002). However, it may also be a result of a significant decrease in size of seminiferous tubular profile areas; however, major structural abnormalities to the seminiferous epithelium were not evident (Korah et al., 2003).

In the present study, macrophages appeared in large numbers in the intertubular space of both the efferent ducts and epididymis of PPCA-/- mice, while they were never seen in wild type mice. Macrophages express PPCA (Luedtke et al., 2000), and as a result, lysosomes accumulate in its absence leading to major abnormalities in these cells, which may cause more of their numbers to be recruited into the intertubular space. In fact, there was no evidence of their division in the intertubular space, suggesting that they enter from the blood. Nevertheless, macrophages were never seen in the lumen of the efferent ducts or epididymis, nor were they observed in the epithelium.

In the efferent ducts, no abnormalities were noted to the epithelium, while in the epididymis, gross abnormalities were observed in PPCA-/- mice. In either case, halo cells presented abnormal features. It may be suggested that while halo cells in the epithelium are actively involved in the uptake of substances, they may not be able to effectively carry out their functions as they become seriously compromised due to their engorgement

with lysosomes in PPCA-/- mice. As a consequence, macrophages may be recruited into the intertubular space to help alleviate this problem.

While the precise nature of the stimulus for macrophage infiltration is unknown in PPCA deficient mice, an interesting correlation can be made from the β-hexosaminidase A deficient mice. In the case of the latter, it was noted that *HexA* -/- or *HexB* -/- mice, deficient in the α or β subunits, respectively, also showed major gross abnormalities to the epithelial cells of the epididymis, as well as the efferent ducts. However, while halo cells were also grossly abnormal in the epithelium, macrophages never appeared in the intertubular space (Adamali et al., 1999a,b). The reason for this is unclear, but suggests differences in the types of substrates generated and released by the grossly abnormal cells of the epithelium of PPCA-/- mice as compared to that of β-hexosaminiadse A deficient mice.

The presence of large pale lysosomes has also been documented in PPCA-/- mice in cells of epithelial/endothelial/endocrine origin such as the kidney, intestine, liver, pancreas, ovaries, uterus and brain (Zhou et al., 1995). The numerous pale lysosomes in these cells were identified as secondary lysosomes and appeared empty or contained sparse fibrillar material, consistent with the accumulation of low molecular weight compounds, e.g., oligosaccharides or glycopeptides (Zhou et al., 1995; Rottier et al., 1998). The accumulation of pale lysosomes in epithelial cells of the epididymis as well as halo cells and macrophages suggests that in the absence of PPCA, there is a progressive accumulation of undegraded products leading to the subsequent increase in number, enlargement and eventual fusion of lysosomes with each other. This is a classic situation noted for many other lysosomal storage diseases, where various cell types of numerous

tissues are similarly affected (Wenger et al., 1978; Tybulewicz et al., 1992; Horinouchi et al., 1995; Otterbach and Stoffel, 1995; Phaneuf et al., 1995; Morales et al., 2000).

In the caput and corpus epididymidis, extensive areas of vacuolation were observed in PPCA-/- mice. Such vacuolated territories resided mainly near the base of the epithelium and were filled with lysosomes, some of gigantic sizes. Their identity was problematic as they did not contain identifiable features or organelles in the EM and did not stain for various markers of the epithelial cells. Several images suggested that such cells were degenerating as evidenced by a pyknotic nucleus. The presence of such large vacuolated territories and the greatly enlarged size of clear cells and presence of highly vacuolated principal and halo cells of the caput and corpus epididymidis resulted in a dramatic alteration of the appearance of any given tubular cross section of these regions. This was confirmed by quantitative analyses, where it was noted that while the profile area of tubules of both of these regions was significantly increased as compared to that of wild type mice, there was a significant decrease of the tubular lumen of these regions. The increase in size of the tubular epithelium was not due neither to division of cells of the epithelium, as revealed by absence of staining for cdc-47 a cell cycle marker, nor an influx of halo cells or macrophages from the intertubular space, as discussed above. Rather, it appears to be a consequence of the accumulation of lysosomes in the resident cells of the epithelium, some of which become grossly enlarged prior to degeneration, resulting in an overall enlargement of their size and corresponding increase in epididymal tubular profile areas.

Acknowledgments

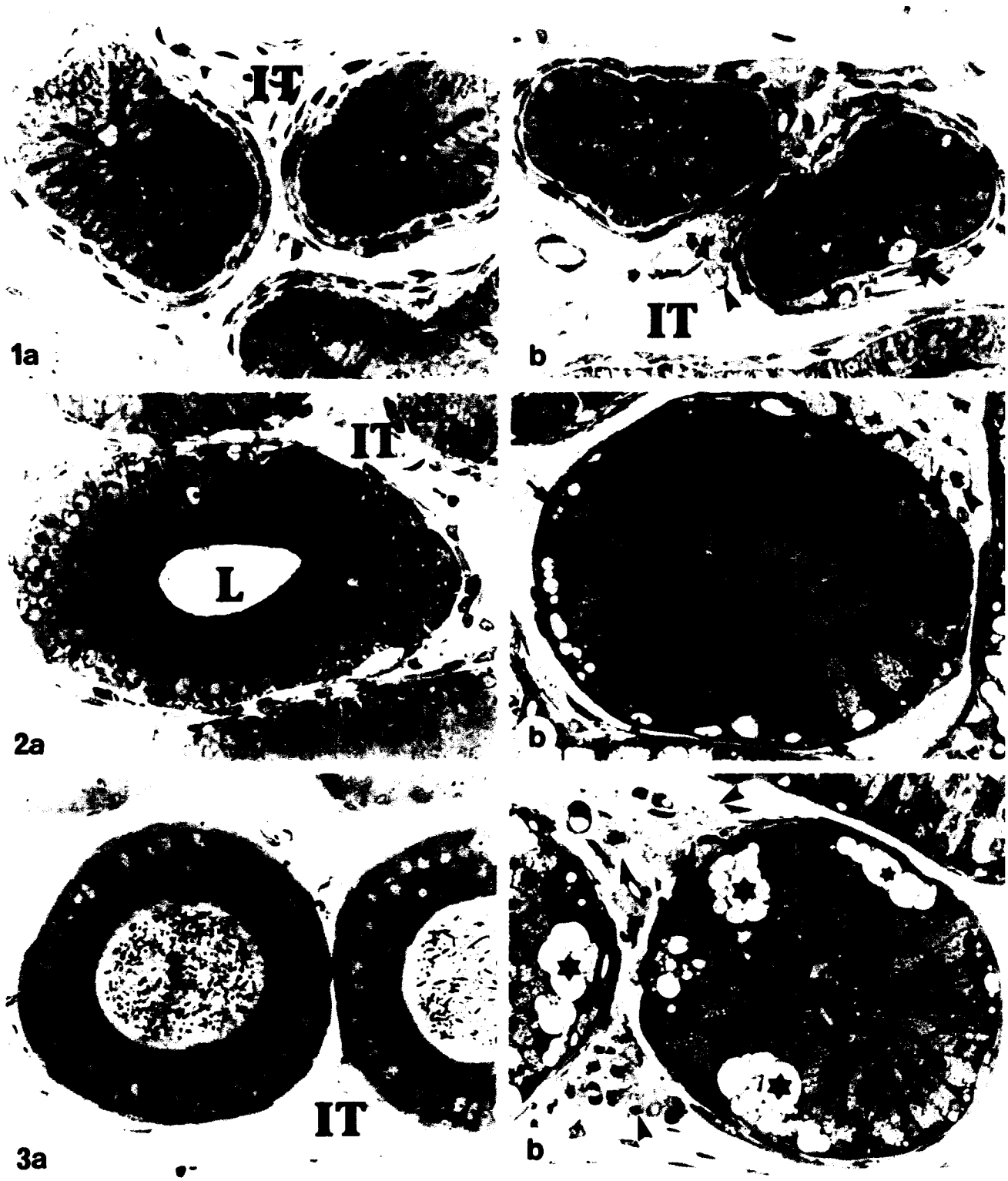
We are most appreciative for the generous contributions of all the antibodies provided to us: the prosaposin, LRP-2 and SGP-2 antibodies from Drs. C.R. Morales, S. A. Argraves and M.D. Griswold, respectively.

Figs. 1a, b: Light micrograph of the efferent ducts of 5-month-old wild type (a) and PPCA -/- (b) mice. The epithelial cells (E) are comparable in their appearance and distribution, with no discernible differences between the two groups of animals. However, in (b), highly vacuolated cells appear at the base of the epithelium (slanted arrow) and in the intertubular space (arrowheads). No such cells are present in wild type mice. IT, intertubular space. X448.

Figs. 2a, b: Initial segment of the epididymis of wild type (a) and PPCA -/- mice (b) at 10 months of age. The tubular profile areas of this region in (b) appear larger than those of wild type mice (a). The tall columnar principal cells (P) in (b), do not show major changes in their morphological appearance as compared to their wild type counterparts seen in (a). However, there is extensive vacuolation of cells (arrows) confined to the base of the epithelium in (b), which are not evident in (a). Narrow cells (N) in (b) show extensive vacuolation of their apical/supranuclear cytoplasm, and in appropriate planes of section of their basal region as well, all of which correspond to lysosomal accumulation. These cells are identified by their more apically located nucleus (n); the same cell type in (a) shows no vacuolation. In the intertubular space (IT) of PPCA-/- mice, numerous pale vacuolated macrophages (large arrowheads) are a consistent finding, not noted in wild type mice (a). Note the presence of sperm in the lumen (L) of PPCA -/- mice. Capillaries (small arrowheads). X448.

Figs. 3a, b: Caput epididymidis of 5-month old wild type (a) and PPCA -/- (b) mice. The tubular profile areas of this region in (b) appear larger than those of wild type mice (a). In (b), principal cells (P) contain numerous pale lysosomes that fill their apical, supranuclear and infranuclear cytoplasm. Such pale lysosomes and their ubiquitous

distribution within the cytoplasm are never seen in principal cells of wild type animals (a). At the base of the epithelium of PPCA-/- mice, there is extensive vacuolation of cells, some of which are grossly exaggerated (stars). No such vacuolation is present in wild type mice (a). Numerous pale vacuolated macrophages (arrowheads) are evident in the intertubular space (IT) of PPCA-/- mice, but are never present in wild type mice (a). Sperm are present in the lumen (L) of both wild type and PPCA-/- mice. X448.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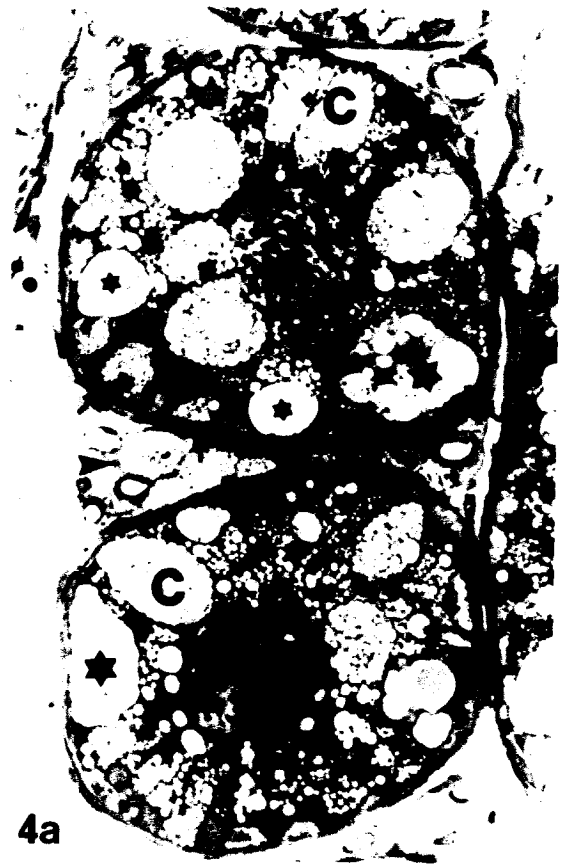
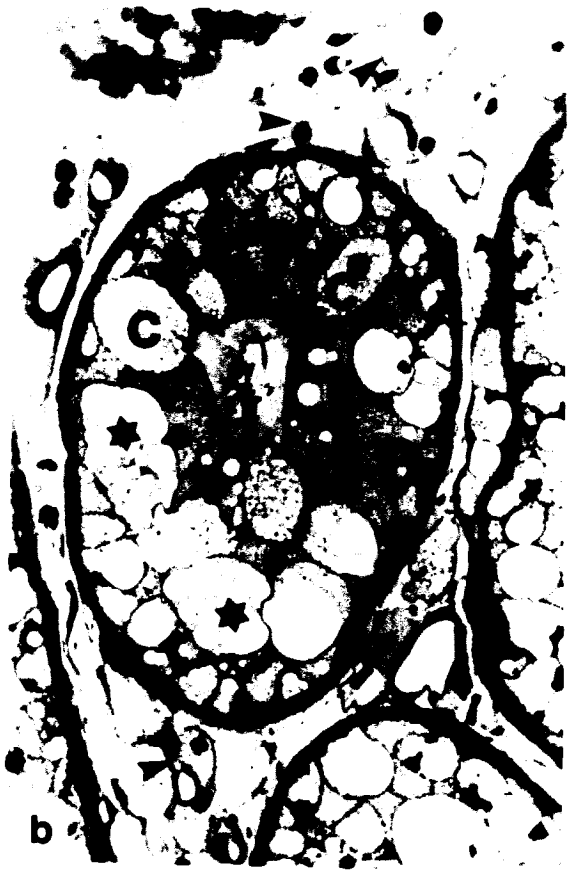
Figs. 4a, b: Corpus epididymidis of PPCA -/- mice at 6 (a) and 10 (b) months of age. The epithelium is grossly altered due to PPCA deficiency in this region, even at earlier ages. Principal cells (P) show extensive vacuolation of their cytoplasm, but this appears to be insignificant compared to that noted in clear cells (C). The latter are identified due to the fact that they extend from the basement membrane to the lumen (L), show an apically located nucleus and are greatly enlarged. Clear cells are so abnormal that they appear to affect the distribution and topographical arrangement of principal cells encompassing the tubular diameter. Extensive vacuolation of some cells also occurs at the base of the epithelium (stars). A few smaller vacuolated cells are also noted near the basement membrane (arrows), corresponding to basal cells. Despite the extensive vacuolation of the epithelium and small size of the lumen (L) in some tubular cross sections, sperm are still present. The intertubular space (IT) displays a plethora of macrophages (arrowheads), which are never noted in wild type mice (a). X448.

Figs. 5a, b: Cauda epididymidis of 6-month old wild type (a) and PPCA-/- (b) mice. While the epithelium shows vacuolation, it is never as exaggerated as that seen in the caput and corpus regions at any age group examined. In (b), principal cells (P) show vacuolation of their cytoplasm especially in their infranuclear area, which is never seen in (a). Clear cells (C) are also vacuolated as compared to their wild type counterparts in (a), but not to the degree of those seen in the caput and corpus regions. Vacuolation of cells residing at the base of the epithelium in (b, stars), although evident, is not as prominent in the cauda region as compared to that seen in the caput and corpus regions. The lumen (L) of the cauda region is large and shows numerous sperm in both types of animals. IT, intertubular space. X448.



4a



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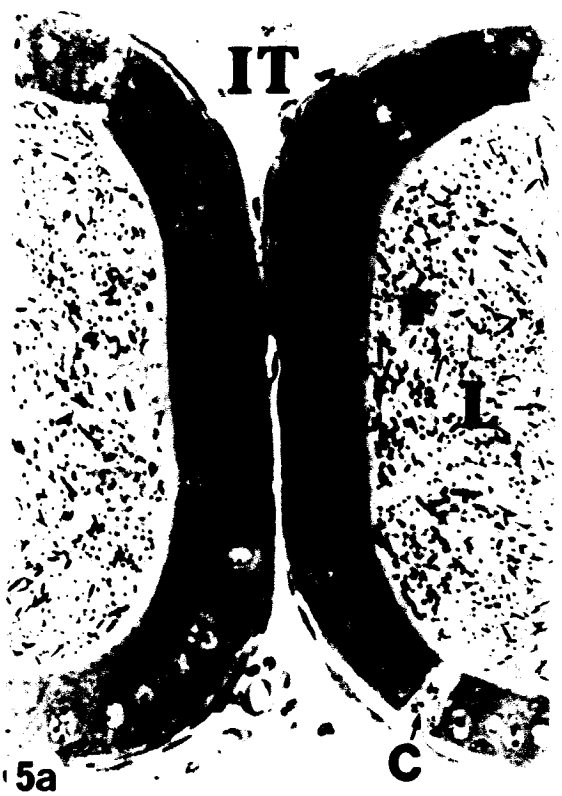
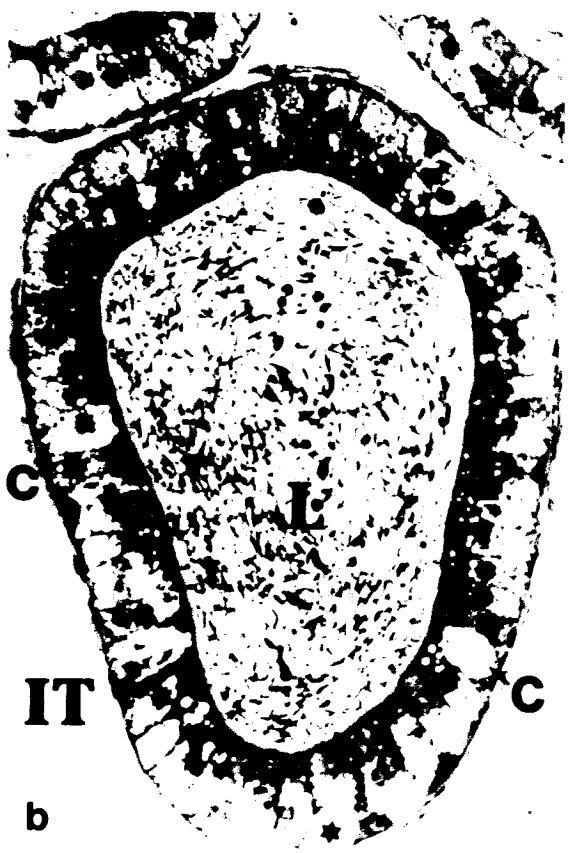


5a



b

Figs. 6a-g: (a): An efferent duct tubule of an 8-month old PPCA-/- mouse, immunostained with an anti-LRP-2 antibody shows a distinct band of reaction product (curved arrow) over the apical plasma membrane of the epithelial cells (E) and no apparent vacuolation of the epithelium. **(b):** Initial segment of the epididymis of a PPCA -/- mouse at 8-months of age, immunostained with an anti-Yf-GST antibody. Principal cells (P) show a cytoplasmic reaction in their cytoplasm. Narrow cells (N) are highly vacuolated and do not display a reaction product. One large vacuolation appears in the epithelium (star). **(c):** Caput epididymidis of a 7-month old PPCA -/- mouse, immunostained with an anti-SGP-2 antibody. Principal cells (P) show a reaction (curved arrows) apically and supranuclearly and a vacuolated unreactive cytoplasm infranuclearly. Some highly vacuolated cells, extending from the base of the epithelium to the lumen, are unreactive and as such correspond to clear cells (C); others appear to be restricted to the base of the epithelium and may represent either basal or halo cells (stars). **(d):** Corpus epididymidis of a 5-month old PPCA -/- mouse, immunostained with an anti-SGP-1 antibody. An intense reaction (curved arrows) is evident over the vacuolated principal cells (P), while a wispy reaction is evident over the cytoplasm of clear cells (C) reaching the lumen. Extensive vacuolation of some cells not contacting the lumen is also evident (stars). The latter take on an enormous size, which appears to dramatically affect the shape and distribution of neighbouring principal cells. **(e):** Corpus epididymidis of an 8-month old PPCA -/- mouse, immunostained with an anti-cathepsin D antibody. The vacuolated principal cells (P) show a reaction (curved arrows) for cathepsin D in their cytoplasm. Clear cells (C) are grossly abnormal in size and are unreactive for cathepsin D. **(f):** Intertubular space of the caput epididymidis of a 7-month old PPCA -/- mouse

immunostained with a monoclonal F4/80 antibody. Note reaction product outlining the plasma membrane of the pale vacuolated cells (double arrows), indicating that these cells correspond to macrophages. In (b-e), sperm are plentiful in the lumen (L), and in the intertubular space (IT) of (a, c, e), pale vacuolated macrophages are evident (arrowheads). **(g):** Intertubular space of the caput epididymidis of a 5 month old PPCA -/- mouse, immunostained with cdc-47, a cell cycle marker. Note the absence of staining on the nuclei (n) of the epithelial cells, as well as from the intertubular macrophages (arrowheads). a, b, d, e, f, X 512; c, g X 320.

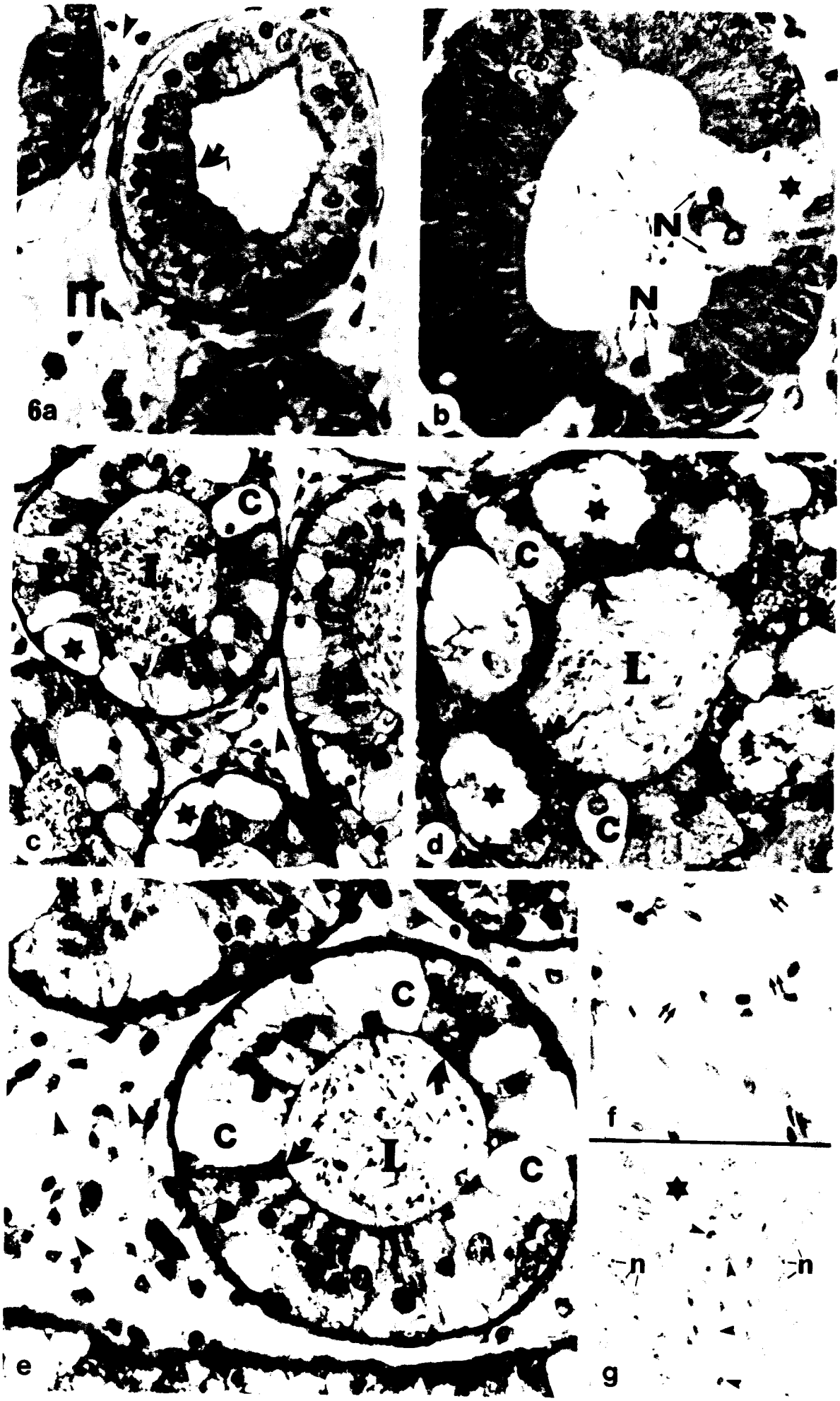

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Fig. 7: Electron micrograph of a principal cell of the corpus region of a PPCA-/- mouse at 6 months of age demonstrating numerous pale membrane-bound lysosomes (Ly) in their cytoplasm. Such lysosomes appear in the apical, supranuclear and infranuclear cytoplasm, and signs of fusion between them are evident (arrows). Lysosomes contain few small vesicular or membranous profiles (arrowheads). Microvilli (mv) flank the apical cell surface and coated pits (small arrowheads) are still apparent on the apical plasma membrane. The Golgi apparatus (G) is still elaborate in principal cells. The nucleus (N) is partially indented by lysosomes. J, junctional complexes; m, mitochondria. X 9,460.

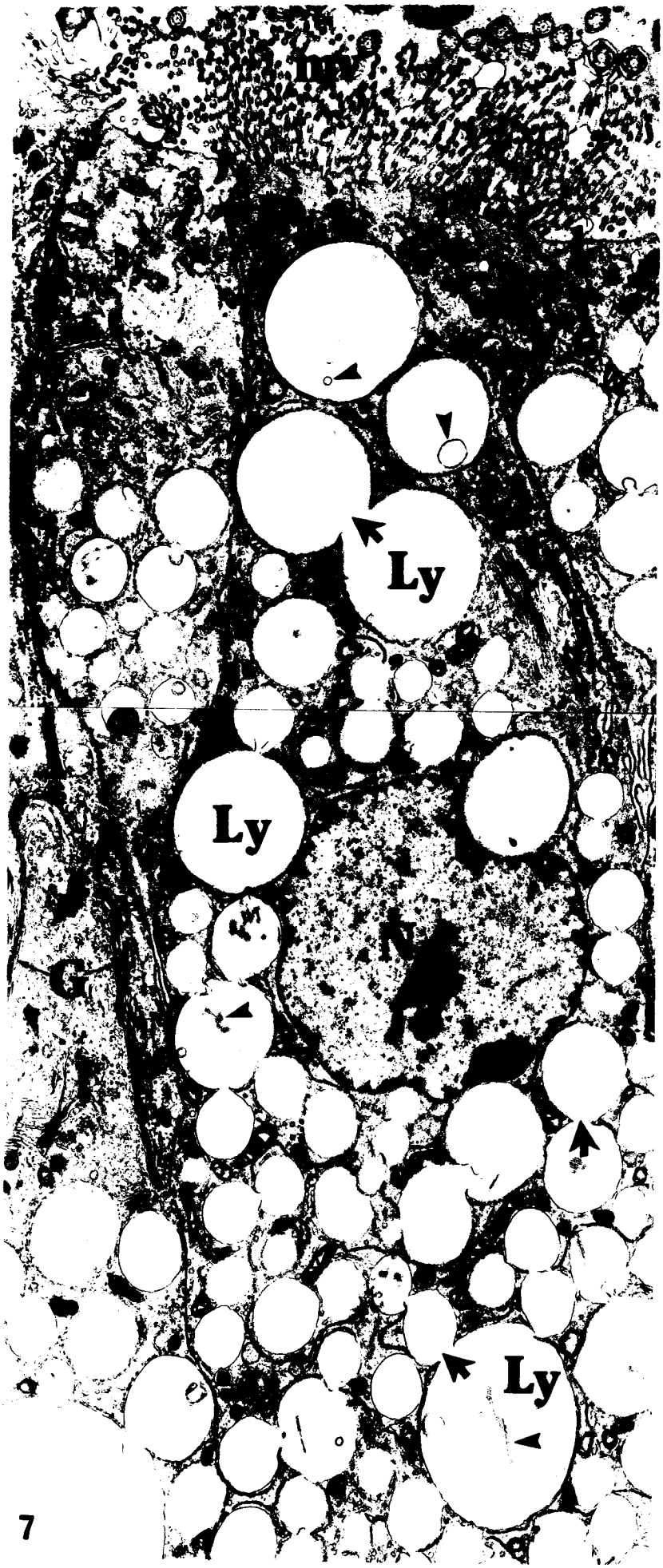

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Fig. 8: Electron micrograph of a clear cell of the corpus region of a 10-month old PPCA -/- mouse, extending from the basement membrane (arrows) to the lumen (L). The cytoplasm of the clear cell is packed with pale lysosomes (Ly), and the nucleus (N) is positioned apically. Lysosomes show signs of fusion with each other (large arrows) and are so numerous that they indent the nucleus; they contain few small vesicular and membranous profiles (arrowheads). Microvilli (mv) do not flourish on the apical surface of clear cells as they do on principal cells. X 6000

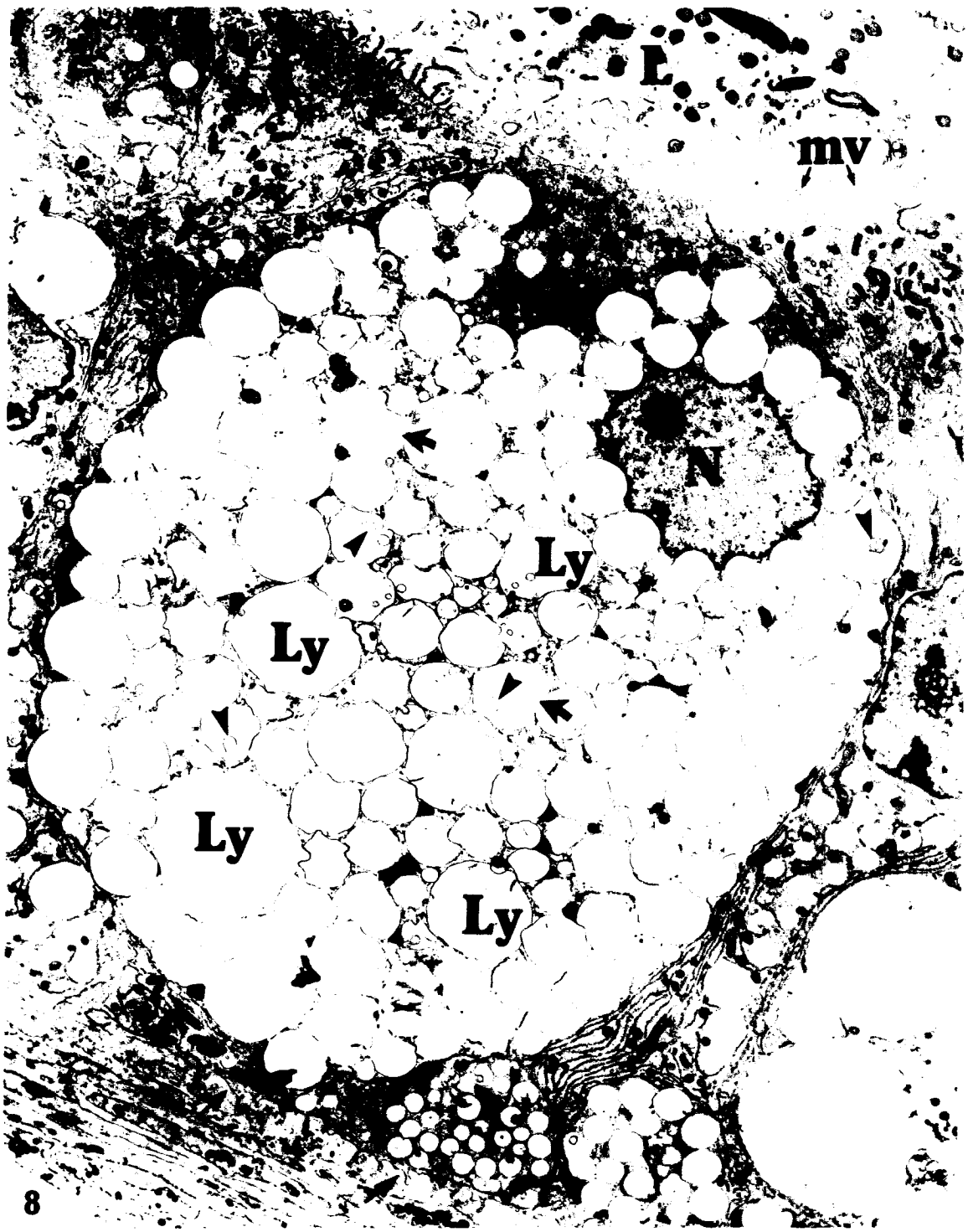

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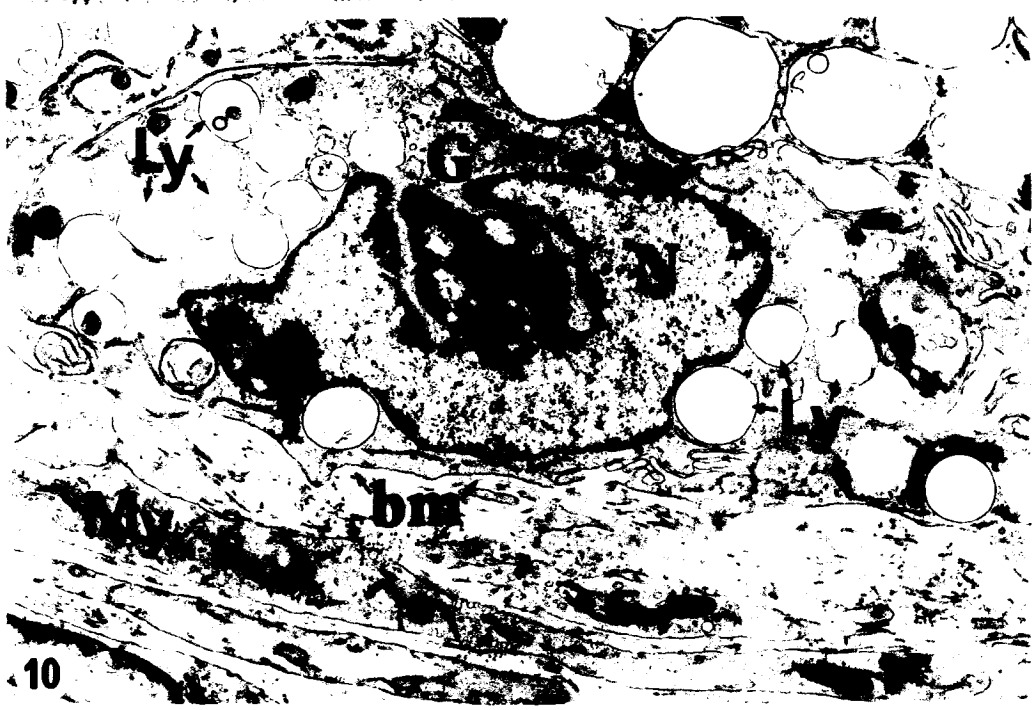
Fig. 9: Electron micrograph of a narrow cell of the initial segment of a 2-month old PPCA -/- mouse. Apically, this cell contains numerous small vesicular profiles (arrows) typical of that seen in wild type mice. The supranuclear area contains one gigantic lysosome (Ly), revealing few vesicular and membranous profiles (arrowheads), and that indents the surface of the nucleus (N). The rest of the cytoplasm shows numerous mitochondria (M) and a Golgi apparatus (G). X 12,375.

Fig. 10: Electron micrograph of a basal cell of the corpus region of a PPCA -/- mouse at 10 months of age. This cell is in contact with the basement membrane (bm) and resides exclusively at the base of the epithelium. Prominent in the cytoplasm are numerous pale lysosomes (Ly) and the Golgi apparatus (G). The nucleus (N) is highly irregular in form. My: myoid cell. X 11,825

9



10

Fig. 11a-c: Electron micrographs of cryo sections of a principal (a), narrow (b) and clear cell (c) of a 7-month-old PPCA -/- mouse. Both a and b are immunolabeled with an anti-cathepsin D antibody, while c is immunolabeled with an anti-SGP-1 antibody. Numerous 10nm gold particles (arrowheads) are evident over the pale vacuoles, identifying them as lysosomal elements (Ly) in all of these cell types. Control experiments revealed only 1-3 gold particles in comparable fields as shown in 11a-c. X53,200.

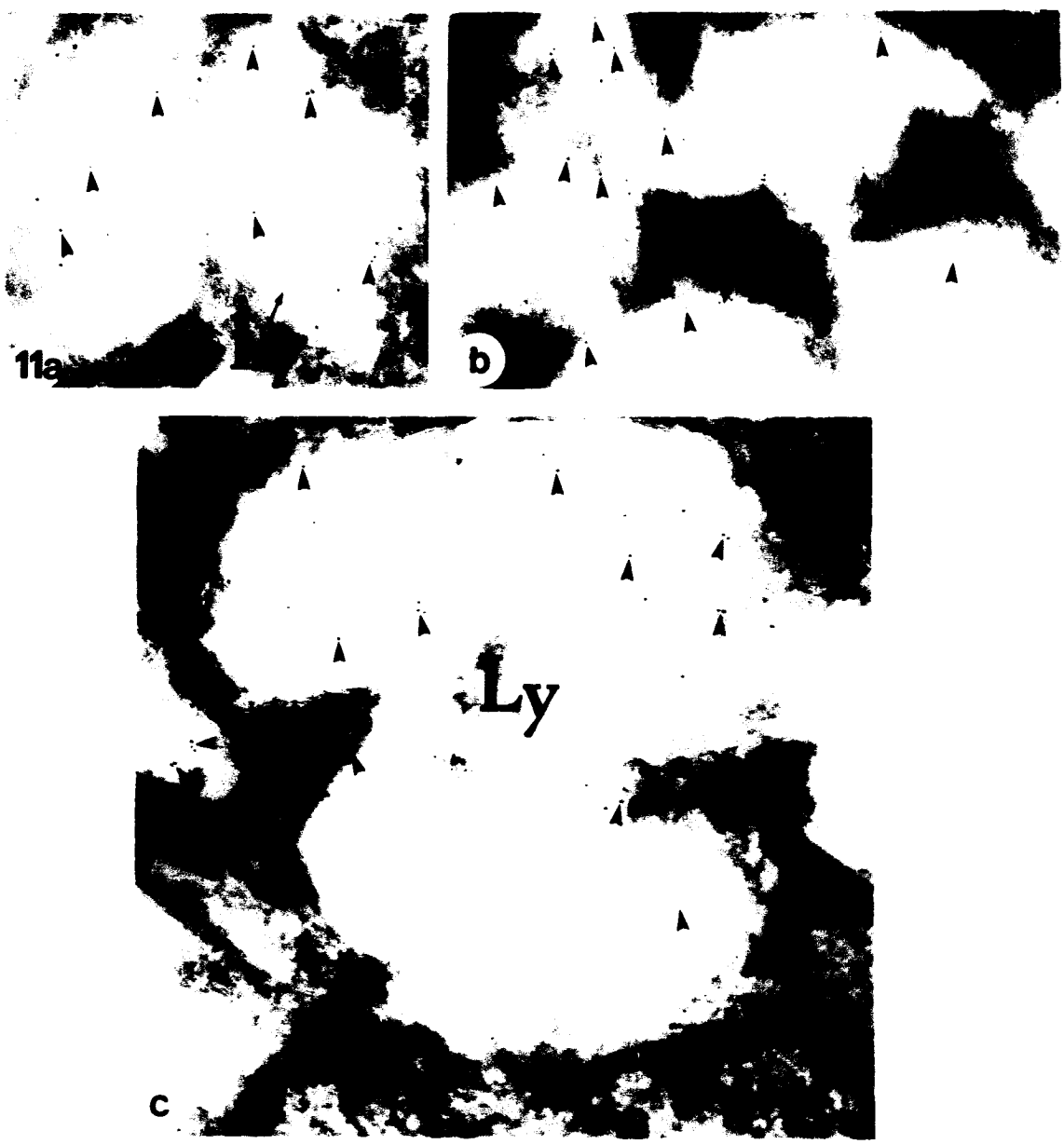

11a
b
Ly
c

Fig. 12: Electron micrograph of a highly vacuolated cell at the base of the epithelium of a 6 month old PPCA-/- mouse. This cell is in contact with the basement membrane (bm), but does not reach the lumen. The left side of the cell contains one gigantic lysosome (Ly) continuous with a smaller one (slanted arrow), while the right side contains numerous smaller lysosomes (Ly) that also merge with each other. The nucleus (N) is small and pyknotic, suggesting that this cell is degenerating. The cytoplasm of the cell in some areas forms a thin layer that is barely detectable (long arrows). Neighboring principal cells (P) contain pale lysosomes (Ly) and a large spherical nucleus (N). My, myoid cell; M, macrophage. X 8250.

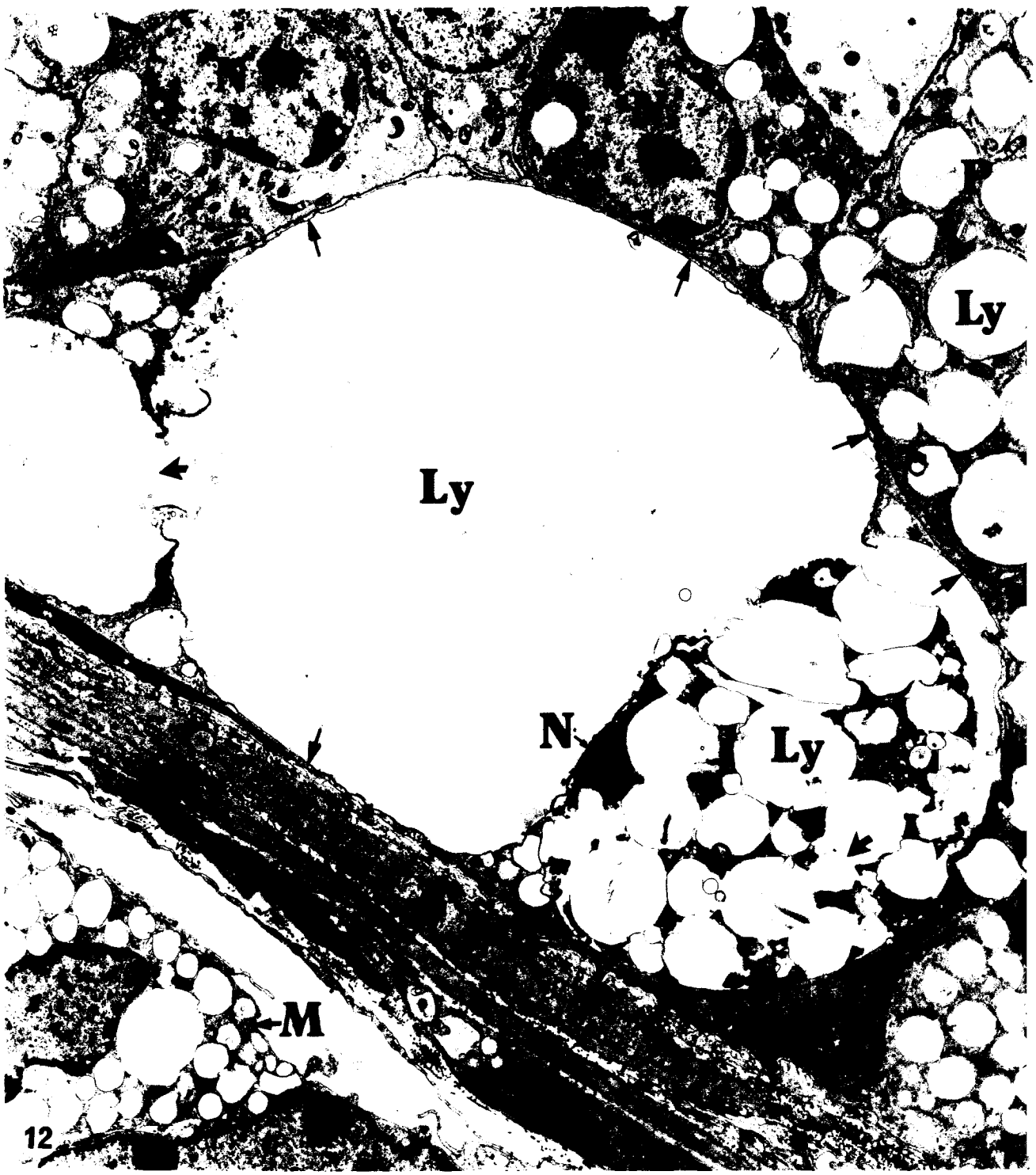
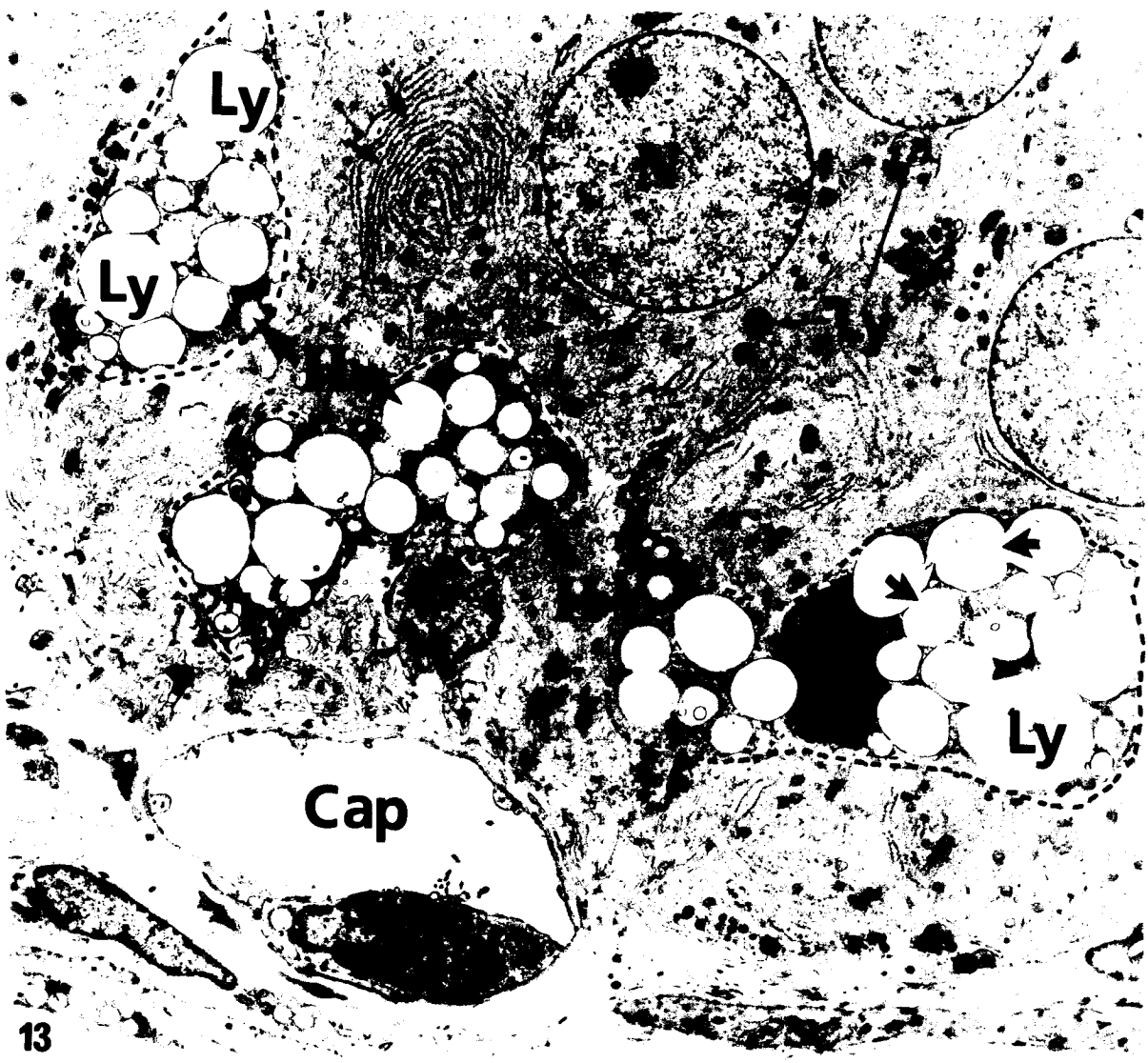

Ly
Ly
N
Ly
M
12

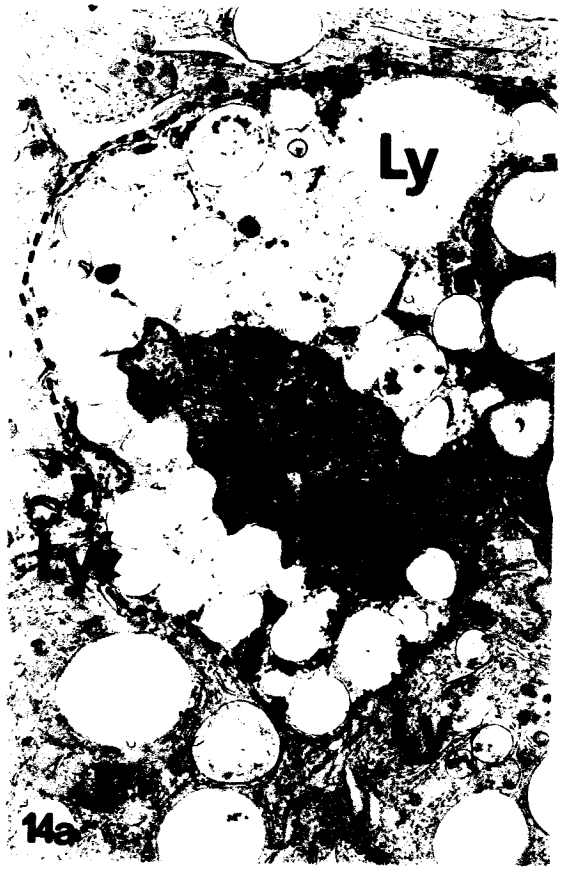
Fig. 13: Electron micrograph of the initial segment of the epididymis of a PPCA-/- mouse at 8 months of age. The basal region of principal cells shows numerous concentric cisternae of ER (arrows) and a large spherical nucleus (N). There are several small dense lysosomes (Ly) in this region of the cell. Note several well-defined irregularly shaped cells showing an abundance of pale lysosomes (Ly). Such cells are surrounded by principal cells, do not contact the lumen, and do not possess organelles characteristic of principal, narrow or clear cells; their small size and the fact that they do not contact the basement membrane excludes them as basal cells. They are considered to be halo cells (H). Cap, capillary. X 7095.



13

Figs. 14a, b: Halo cells in the corpus epididymidis of a PPCA-/- mice at 10 months of age. In (a), the halo cell contains numerous pale lysosomes (Ly) that fill the entire cytoplasm and deeply indent the irregularly shaped nucleus (N). In (b), the halo cell appears more normal looking, as does the more-or-less spherical nucleus (N), revealing several organelles, in addition to a few small pale lysosomes (Ly), in a large expanse of cytoplasm. Neighboring principal cells (P) surround both halo cells. a, X 8,745; b, X 12,255.

Fig. 15: Macrophages in the intertubular space (IT) of the epididymis of a 6-month old PPCA-/- mouse. Identified as such by the macrophage-specific marker F4/80, these cells are larger than halo cells and completely occupied with pale lysosomes (Ly). Cap, capillary; My, myoid cell; N, nucleus. X 7,260.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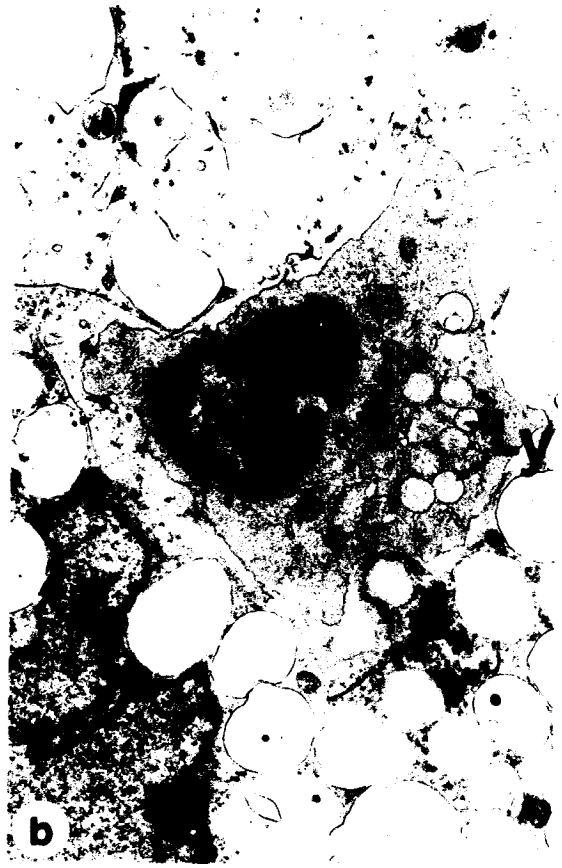
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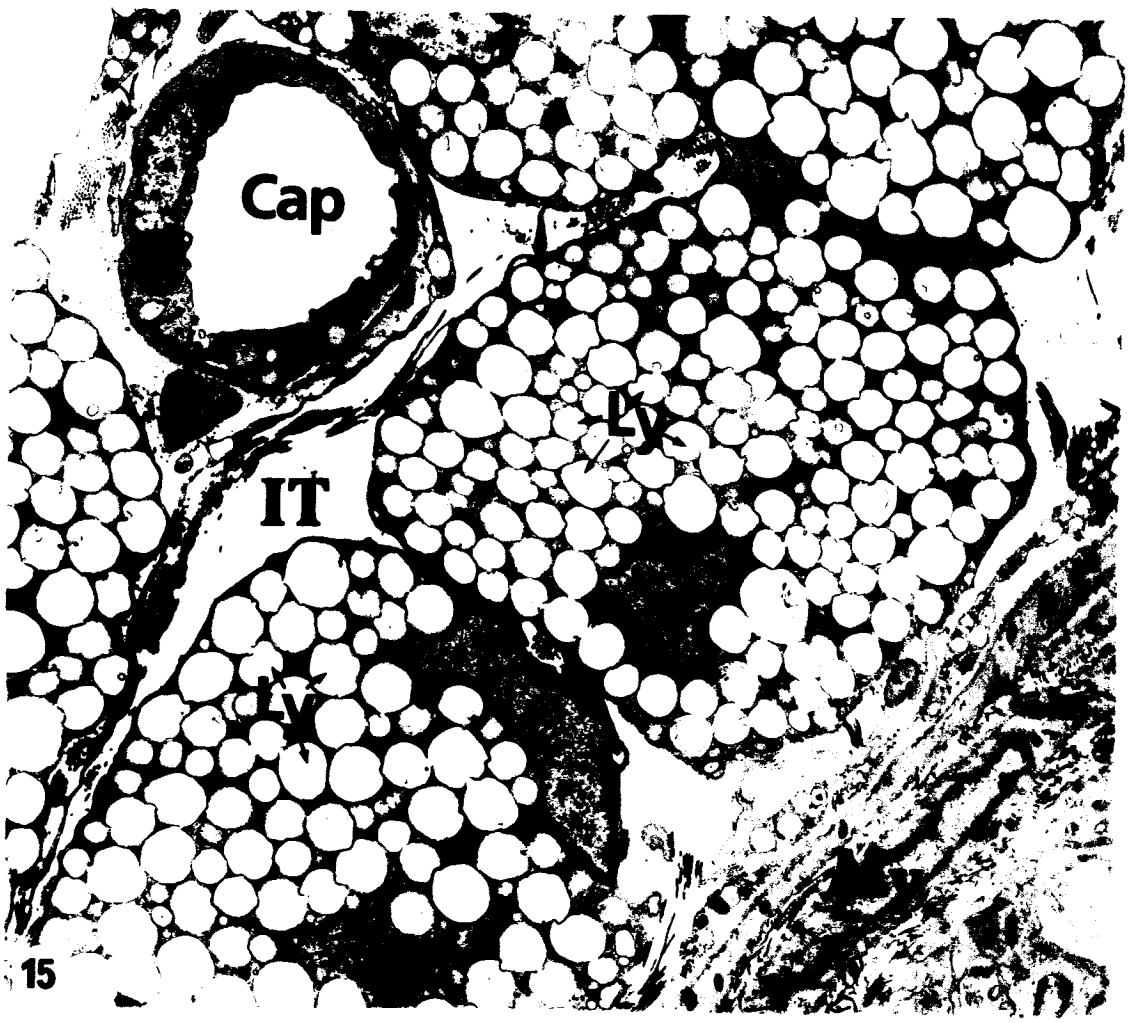
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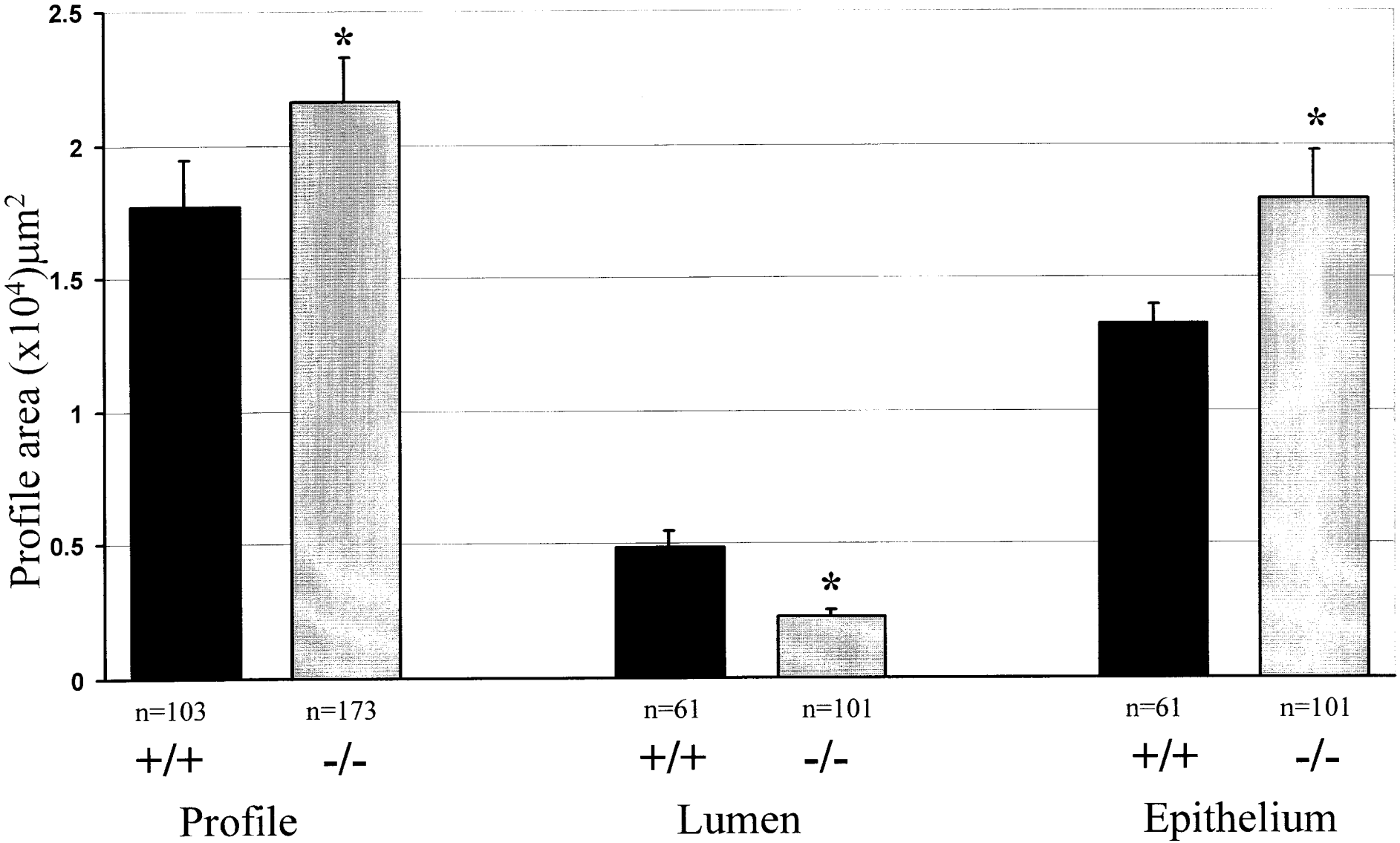
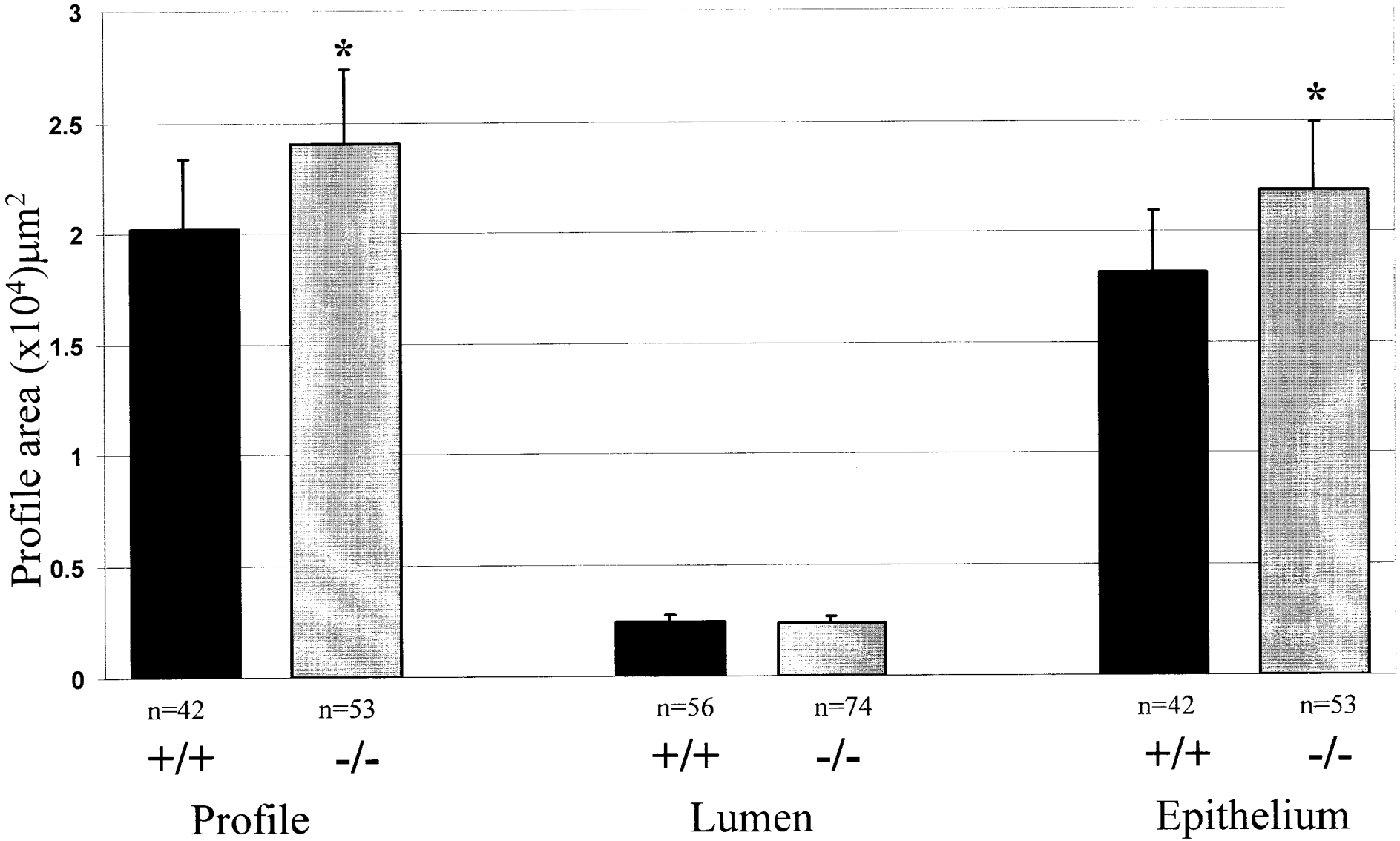
Graph 1: Mean profile area of caput/corpus epididymal tubules of wild type (solid bars) and PPCA -/- mice (grey bars). There is a significant increase ($p<0.05$) in the mean profile area and epithelial area in the PPCA -/- mice, while a significant decrease ($p<0.05$) is noted in the luminal profile area between wild type and PPCA-/- mice. All three parameters showed significance using both the Mann-Whitney U test and the Kolmogorov-Smirnov test.

Mean profile areas of caput/corpus epididymal tubules of wild type and PPCA -/- mice



Graph 2: Mean profile area of the initial segment epididymal tubules of wild type (solid bars) and PPCA -/- mice (grey bars). There is a significant increase ($p<0.05$) in the mean profile area and epithelial area in the PPCA -/- mice, while no change is noted in the luminal profile area between wild type and PPCA-/- mice. Significance was determined using both the Mann-Whitney U test and the Kolmogorov-Smirnov test.

Mean profile areas of initial segment epididymal tubules of wild type and PPCA -/- mice



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CHAPTER IV

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Summary

We have demonstrated that in PPCA deficient mice, despite a structurally normal-looking seminiferous epithelium in the testis, the profile area of these tubules differed significantly with age.

We showed that with age, the size and number of macrophages in the interstitial space of PPCA -/- mice increases significantly. Furthermore, these cells become congested with pale lysosomes.

We also demonstrated that Leydig cells showed lysosomal accumulation in PPCA -/- mice.

We have provided circumstantial evidence that the recruitment of macrophages into the testis is probably a reflection of the affected Leydig cells, as these two cell types have an intimate and functional relationship. This does not dismiss, however, the possibility that the changing seminiferous tubules could also be the reason for an increase in the number of testicular macrophages.

We illustrated that region-specific abnormalities occur in the epididymis of PPCA -/- mice, with little effect on the efferent ducts and initial segment, while the caput and corpus epididymides were the most affected. Since PPCA is expressed throughout the epididymis, the region-specific abnormalities suggest that different enzymes may compensate within the lysosomes of the less affected areas. On the other hand, different substrates found along the epididymal duct could also explain why some regions are more affected by the absence of PPCA than others.

Furthermore, halo cells became engorged with lysosomes throughout the epididymal epithelium, including unaffected regions such as the efferent ducts. Thus we

postulate that halo cells serve a vital role in the epididymis of wild type and PPCA -/- animals.

We noted that macrophages appeared in the intertubular space of the epididymis, and hypothesized that the degenerating epithelium and inefficiency of abnormal halo cells caused the recruiting of macrophages into the epididymis. The numerous macrophages in the intertubular space might reflect the type of substrates emanating from the epithelium in PPCA -/- mice.

In conclusion, it is evident that the lack of PPCA results in major abnormalities to the testis, efferent ducts and epididymis in a cell- and region-specific manner. This is concomitant with the idea that cells of specific regions along the male reproductive tract are involved in establishing a complex and changing microenvironment within the lumen in order to produce and mature sperm. The absence of PPCA and its other lysosomal enzymes in the degradative pathway of glycoproteins and glycolipids leads to the accumulation of unmetabolized substrates, resulting in the lysosomal storage disorder galactosialidosis. With many organs of the PPCA knockout mouse showing extensive vacuolation, including the testis and epididymis, it is no wonder many important body functions deteriorate with age. Decreased frequency of pregnancies and poor mating habits were noted in an earlier study, and the data of this study suggest that sperm in the lumen may be reduced in numbers, as judged by the reduction in seminiferous tubular diameter, and may not acquire their full maturational state in the epididymis, where major abnormalities were noted to the epithelium.

Future studies are needed to confirm some of the results, such as motility and fertility tests in order to determine the state of sperm found in the cauda epididymidis. In

addition, lanthanum studies would verify the state of the blood-testis and blood-epididymis barrier, while measurements of testosterone levels of PPCA -/- mice in comparison to wild type counterparts would broaden any hypotheses about the testis and sperm production in PPCA deficient animals.

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