NOTE TO USERS

Structural and functional alterations within the Testis and Epididymis of the Follitropin Receptor Knockout (FORKO) Mouse

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It all started with a man named Leo Yaffe
and a wine and cheese in his honour.
The rest is history…

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In keeping true to my procrastinatory nature I've decided to leave this section, what I feel to be the most important, to the very end i.e. the day before I submit my thesis. It is with great pleasure that I now take the opportunity to embrace and convey my deepest appreciations and thanks to all those who have been instrumental in allowing me to submit this thesis. Whether it was through technical advice, or just friendship, or staying till 2am to complete an immuno with me while finishing a bottle of wine, Ricardo; or skip out to lunch with *Lozenge* at Place Milton, Nadine; or even helping me to steal a stuffed rooster, Louis; that you all took the time to be there for me during my times of need puts me forever in your debt.

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ABSTRACT

Follicle stimulating hormone (FSH) acting on Sertoli cells of the testis plays important roles during reproductive development. FSH-R knockout (FORKO) mice provide a model to examine alterations in testicular and epididymal structure and function in its absence. Examination of the FORKO testis revealed a gross alteration of Sertoli cell structure indicative of a fluid imbalance. Functional parameters, such as ABP secretion were also significantly reduced in FORKO testis. Morphometry revealed quantitative reductions in seminiferous tubule size. The epididymal epithelium, appeared abnormal and morphometry revealed that epididymal tubule size was reduced in the knockout. Computer Assisted Sperm Analysis on sperm from the cauda epididymidis revealed significant alterations in parameters corresponding to sperm motility as well as sperm counts. These data suggest an important role for the FSH receptor on Sertoli cell structure and functions and on epididymal epithelial size and functions in relation to sperm motility.

RÉSUMÉ

L'hormone folliculostimulante (HSF), qui agit sur les cellules Sertoli dans les testicules, joue un rôle important durant le développment du système de reproduction. Les souris HSF-R knockout (FORKO) fournissent un modèle pour l'analyse des changements structurels et fonctionels des testicules et de l'épididyme en l'absence de ces récepteurs. L'étude des testicules FORKO a révélé de grosses altérations structurelles de la cellule Sertoli, indiquant un déséquilibre des fluides. Des paramètres fonctionnels, tel que l'expression de l'Androgen Binding Protein (ABP), étaient significativement diminués dans les testicules des souris FORKO. Les analyses morphométriques ont révélé une réduction de la taille des tubules séminifères. D'autre part, l'épithélium de l'épididyme avaient une apparence anormale et des études morphométriques ont démontré une réduction au niveau de la taille du canal de l'épididyme chez le knock-out. L'Analyse de Spermatozoïdes Assistée par Ordinateur a démontré des altérations significatives dans de nombreux paramètres correspondant à la mobilité des spermatozoids ainsi q'à leur nombre. Les résultats mettent en évidence l'importance du récepteur FSH sur le maintient des fonctions et de la structure des cellules Sertoli ainsi que sur les dimensions et les fonctions de l'epithélium de l'épididyme en relation à la mobilité du sperme.

CHAPTER I

INTRODUCTION

Charles Darwin named his greatest work *The Origin of Species*. A species can be defined as being reproductively isolated from every other species. The continuity of a species depends on the capacity of a living organism's ability to reproduce. Within the male, reproductive capacity is dependent on three major processes; first, spermatogenesis i.e. the production of sperm within the testis; second, the act of copulation; and lastly, the regulation of both processes by hormonal control within the organism [1]. Ethical considerations pose limitations when wanting to carry out clinically related studies in human reproduction. As a solution, the use of various animal models provides scientists with the resources to carry out experimental research. There is considerable homology between mice and humans, and given that the mice genome has been cloned and can be manipulated, studies involving gene knockouts and/or transgenic mouse models have opened the door to a new realm of discovery. The following research was gathered using a transgenic murine model.

The anatomy of the male reproductive system consists of two testes, two epididymides each with its own vas deferens and the male accessory glands. The male gonad, the testis, is the site for the production of haploid germ cells (gametogenic role); and the important steroid hormone testosterone (endocrine role). The testis is also the site of action of two pituitary gonadotropins namely Follicle Stimulating Hormone or Follitropin (FSH), and Lutenizing Hormone or Lutropin (LH) [2]. Testosterone produced within the testis exhibits both paracrine and endocrine effects within the body and is essential to the development of the epididymis. While the testis is the site of production of spermatozoa, the epididymis is the site of maturation where sperm acquire their fertilizing capability and motility. The vas deferens is responsible for the ultimate transport of sperm to the urethra and final delivery out of the body [3;4].

HISTOLOGY OF THE TRACT

While advances in molecular biology have introduced today's researcher to a new realm of scientific techniques, and while such techniques have been instrumental in the further understanding of the function of the male reproductive tract; the wealth of knowledge that exists on the structure and histology of the male reproductive tract is the result of the many meticulous morphological observations recorded via light and electron

microscopy. Without an understanding of structure or morphology, one cannot progress to understand function or physiology.

TESTIS

The testes are ovoid or walnut-shaped bodies that have the organization of compound tubular glands. Each testis is enclosed in a dense fibrous capsule, the tunica albuginea, underneath which there is a looser layer of connective tissue rich in blood vessels, the tunica vasculosa [5-7]. On its posterior surface, dense connective tissue extends a short distance inward from the tunica albuginea forming the *mediastinum testis* through which the blood vessels enter and its ductili efferentes leave the organ. Radiating from the mediastinum are thin fibrous septa which subdivide the testis into approximately 250 pyramidal compartments, the lobuli testis. Each lobule contains one to four extensively folded seminiferous tubules which form a double-open-ended loop. These are 150-250 μm in diameter, 30-70 cm long and extremely tortuous forming highly convoluted loops [8]. Both ends of each tubule open into an anastomozing network of tubules within the mediastinum, the *rete testis.* The testicular fluid and spermatozoa enter the rete testis and from there subsequently exit via the ductili efferentes, or efferent ducts; which conduct the spermatozoa to the epididymis. A richly vascularized loose connective tissue intimately surrounds each tubule within the interstices. The surrounding connective tissue contains perivascular mesenchymal cells, fibroblasts and a few macrophages within a meshwork of fine reticular fibers [8]. Within the stroma, there are clusters of Leydig cells occupying the angular interstices between the seminiferous tubules.

The seminiferous tubules are lined by a complex stratified epithelium containing spermatogenic cells and non-germinal supportive Sertoli cells [7;9;10]. The spermatogenic cells include: the stem cells, spermatogonia; the cells of meiosis; primary and secondary spermatocytes; the round and elongated spermatids; as well as the spermatozoa.

SERTOLI CELL

Histology

The Sertoli cells constitute the only non-germinal cells within the seminiferous epithelium [11]. First described by Enrico Sertoli in 1865, the Sertoli cell is also known

as the sustentacular, supporting or nurse cell [12]. The Sertoli cell is a tall (75-100μm) simultaneously columnar and stellate cell with a base attached to a basement membrane, an apex projecting towards the lumen, and numerous lateral and apical veil-like processes extending between and around every germinal cell [13]. Also present are deep apical invaginations into which the more mature germ cells are embedded [12;14].

The Sertoli cell ultrastructure is unique from all other cells and as a result does not fit into any of the traditional classification of cell types typically presented in histology texts. The surfaces of the Sertoli cell are designated to be basal, lateral and apical [12;14]. The basal surface is that which is in intimate contact with the basal lamina of the basement membrane. The most extensive surface is the lateral surface and can be subdivided into surfaces that line germ cells of the basal compartment, germ cells of the adluminal compartment, and those surfaces that relate to other Sertoli cells at the level of the Sertoli-Sertoli cell barrier. The apical surface faces the lumen and is associated with germ cells positioned deep within the apical crypts or invaginations [12;14;15]. Oriented radially within the round profile of a cross sectioned seminiferous tubule [12], Sertoli cells possess a basally situated nucleus which is characteristically identifiable in light microscopic preparations [12].

In virtually all mammalian species, the Sertoli nucleus is basally located and is distinctive in comparison with that of other cell types [12]. Its irregular shape is described as being ovoid or pyramidal [12]. The nuclear envelope is infolded producing clefts where the cytoplasm appears to indent the nucleus deeply; giving the impression that the nucleus is lobulated [10;12;16]. The nucleus is homogenous in appearance, a result of the euchromatin present [12]. The nucleolus can be distinguished easily and its tripartite structure characteristically define the mouse and rat Sertoli cell nucleus [10;12].

Cytoplasmic organelles, most of which are represented within the Sertoli cell, display compartmentalization or polarization [12;15]. This distribution caters to the regional physiological needs of the various germ cell types associated with the Sertoli cell; as well as provides support for the notion of polarized function/secretion of the Sertoli cell [12;17].

While the Sertoli cell remains the only non-germinal cell within the seminiferous epithelium, it is also a terminally differentiated somatic cell which no longer divides in

the adult [18]. The mitotic activity of Sertoli cells is most pronounced during the fetal and early post natal period, yet ceases between the twelth and fifteenth day after birth in the rat [11;19]. The unique cytoarchitectural and nutritional functions of each Sertoli cell dictate that each cell can support the development of only a limited number of germ cells [11;20-23]. Thus the total complement of Sertoli cells, in the adult testis, dictates in part, fertility because it is generally recognized that the density of sperm in an ejaculate is a significant parameter of fertility [20;24].

Function

While Sertoli cells are histologically unique, it is their functional character that makes them stand out as one of the truly incredible cells within the body. Sertoli cells carry out a multitude of specialized functions, many of which occur simultaneously. Some of the most relevant ones will be mentioned here.

The most obvious role is that of a supporting or sustentacular cell for germ cells [13]. Like the scaffolding of a tall sky scraper supports the various offices and units within, the Sertoli cell provides the "scaffolding" support of the various generations of germ cells within the seminiferous epithelium. While this role is accepted as evident, it remains to be explained at the molecular level [13].

In addition to its structural role, the Sertoli cell carries out numerous other functions that are instrumental to maintaining spermatogenesis. A second function is the Sertoli cell's capacity to internalize or phagocytose and eliminate residual cytoplasmic bodies that detach from late spermatids at the time of spermiation [13]. Along with this phagocytic activity, Sertoli cells demonstrate fluid phase pinocytosis at the luminal aspect and receptor mediated endocytosis along the basal surface [13].

Through studies utilizing electron microscopy, a third and important function of Sertoli cells has been elucidated. Adjacent Sertoli cells in contact with one another constitute what is known as the *blood-testis barrier*; by means of intercellular tight junctions [25]. This allows for compartmentalization of the seminiferous epithelium into an adluminal compartment containing meiotic spermatocytes and spermatids at various steps of spermiogenesis; and a basal compartment containing spermatogonia and early spermatocytes. While the barrier protects the germinal haploid cells from immune attack, it also implicates the Sertoli cell as the sole conveyer of signals and messages from the

basal systemic side to the adluminal germinal side. The Sertoli cell is crucial for the transport of many substances from the basal to the adluminal compartment, for instance, iron via transferrin or copper via ceruloplasmin [13;17;26;27].

Sertoli cells have an obvious nutritive role as well. There is a close association between every germinal cell and a given Sertoli cell. It is therefore plausible to consider that Sertoli cells are instrumental in the delivery of nutritive substances such as sugars, amino acids, lipids, metallic elements etc., to these very germinal cells they are in close contact with [13]. Furthermore, adluminal germ cells such as meiotic spermatocytes and spermatids rely strictly on their contact with solely Sertoli cells for their nutritive requirements. Various labeling studies and subsequent radioautography have demonstrated the incorporation of various substances by germ cells after intratesticular or vascular administration [28-30].

It is well recognized that a major function of Sertoli cells is secretion. Sertoli cells massively secrete a wide variety of proteins as well as ions and water, both apically and basally [13]. Sertoli-Sertoli junctional complexes sequester the meiotic and post-meiotic germ cells in an adluminal compartment which is inaccessible to macromolecules from the serum or lymph [17]. The multiple secretion products of Sertoli cells are what determine this local microenvironment and can, as a result, influence meiosis as well as spermatid and spermatocyte development. While the most studied secretion products are hormones and proteins such as inhibin, SGP-2 [31;32] and androgen-binding protein (ABP) [33], Sertoli cells have a very prominent role in the secretion of fluid within the tubule lumen as well [34;35].

Inter- and intracellular communication within the testis is not only a very complex and intricate system; but also one of great bewilderment. At the centre of this elaborate communicative network lies the Sertoli cell. Conveying signals to germ cells, to Leydig cells, from Leydig cells to germ cells, from the interstitium to germ cells, the Sertoli cell is like a traffic officer directing the flow of information within and throughout the testicular compartment [36]. This interaction among the gametogenic and androgenic compartments is essential to maintaining the efficiency of the spermatogenic process. Similar to the dependence of germ cells on its sustentacular partner, the Sertoli cell; Leydig cell activity function and survival are also dependent on the continued presence of

Sertoli cells [37-39]. It is plain to see that regulation and maintenance of optimal Sertoli cell proliferation, activity and function throughout development is crucial for normal male fertility.

LEYDIG CELL

The Leydig cells constitute the major cell type found in the testicular interstitium [40;41]. Their primary function is the biosynthesis of androgens, namely testosterone, in response to direct stimulation by the pituitary gonadotropin luteinizing hormone (LH) [42;43]. The testosterone then travels to the Sertoli cell where it has one of two fates: association with the Sertoli cell synthesized Androgen Binding Protein (ABP); or migrating to the nucleus to interact with its cognate receptor stimulating both genomic and non-genomic actions [44].

Leydig cells are ovoid in shape with an eccentrically located nucleus that is sometimes irregularly shaped. Clumps of Leydig cells often aggregate around the many blood vessels of the intertubular space to have easy access to circulating cholesterol. Within the cells are numerous lipid inclusions in the cytoplasm, storage sites for cholesterol, which provide the substrate for steroidogenesis [40]. Also abundant within the Leydig cell is its smooth endoplasmic reticulum, home to the numerous enzymes of the steroid biosynthetic pathway.

SPERMATOGENESIS

Spermatogenesis is a complex, cyclic process that involves germ cells undergoing mitotic divisions, meiosis and terminal differentiation, and interacting over time and space within the seminiferous tubules. As the cells mature, they transcend towards the lumen. Their vacated positions are simultaneously filled by germ cells of an earlier stage of development. At any location within a seminiferous tubule, 1-2 generations of spermatogonia, 1-2 generations of spermatocytes, and 1-2 generations of spermatids are present [9]. In the seminiferous epithelium, the different generations of spermatogenic cells form associations with constant composition. At the base of the epithelium, Sertoli cells and mitotically proliferating spermatogonia have a direct contact with the basal lamina. Spermatocytes in meiosis form the next layer and haploid spermatids are found more apically within the epithelium. It is these differing cellular associations, seen within the seminiferous epithelium, which define the Stages of the Cycle of the Seminiferous

Epithelium. The stages are usually most accurately defined by the morpohology of the developing acrosomes and of the nuclei of the early spermatids [9;45]. The number of stages per cycle varies with species. The mouse for example consists of 12 stages in the cycle, while spermatogenesis within the rat seminiferous epithelium is comprised of 14 stages. Along the seminiferous tubules, the stages follow each other in a wave-like fashion [46]. Sertoli cells have a cyclic function that depends on the stage of the cycle of the seminiferous epithelium [47].

Spermatogenesis in the seminiferous epithelium comprises three main phases: spermatogonial multiplication, meiosis, and spermiogenesis [47]. During the first phase spermatogonia divide by mitosis to renew themselves and constitute the pool of cells destined for meiosis. Daughter cells of the latter remain connected via a cytoplasmic bridge due to incomplete cytokinesis. The cells differentiate along the way giving rise to spermatogonia type A, Intermediate and Type B, identifiable based on nuclear morphology. All spermatogonial cell types reside in the basal compartment [25]. Division of the last generation of spermatogonia produces primary spermatocytes, or the germ cells of meiosis.

In the second phase of spermatogenesis, meiosis, the spermatocytes undergo a series of two cell divisions reducing the chromosome number in half. The primary spermatocytes prepare for, and execute, the reductional division to produce the secondary spermatocytes which proceed with the second, mitotic-like, equatorial division producing haploid round spermatids. Primary spermatocytes exhibit an extended prophase of meiosis. On this basis, prophase I can be further subdivided into sequential phases used to distinguish the stages of spermatogenesis: pre-leptotene spermatocytes, leptotene spermatocytes, zygotene spermatocytes, pachytene spermatocytes and diplotene spermatocytes. Once spermatogonia differentiate into early spermatocytes, the Sertoli cells project cell processes beneath the cell in an effort to move the cell towards the lumen and establish a new tight junction below the cell, thus putting the germ cell within the adluminal compartment [12;15;48;49]. This lifting function of the Sertoli cell causes zygotene and subsequent spermatocytes to exist within the adluminal compartment; and pre-leptotene and leptotene spermatocytes to exist solely within the basal compartment.

The final stage of spermatogenesis, spermiogenesis, involves a sequence of events that transform round spermatids into spermatozoa. The changes include the formation of an acrosome, nuclear compaction, development of a flagellum, and reorganization of the cytoplasm and organelles [22]. Spermiation is the term referring to the release of the spermatozoa from the seminiferous epithelium. In their final stages of development, the tails of the spermatozoa project into the lumen. The acrosome capped nucleus and an appendage of excess cytoplasm occupy a conforming recess in the apical surface of Sertoli cells. During the release of a spermatozoon, the head is actively extruded by the Sertoli cell and the appendage of cytoplasm, the residual body, is pinched off and subsequently phagocytosed by the Sertoli cell [50].

EPIDIDYMIS

The epididymis and vas deferens possess a pseudostratified columnar epithelium with stereocilia, which are microvilli. The epithelial characteristics vary along the length of the duct, however the cell types are relatively consistent between regions [3;4].

CELL TYPES AND FUNCTION

Principal Cells

The principal cell is the major cell type found throughout the epididymis and vas deferens. They are columnar cells with a basally located nucleus displaying a distinct nucleolus. Apically, the cell surface possesses a brush border made of numerous microvilli. In addition, there is an extensive endocytic compartment, a well-developed Golgi apparatus and numerous cisternae of rough endoplasmic reticulum in its cytoplasm [3;4]. The functions of this cell varies from region to region within the epididymis and subsequently, some of its histological features may differ between cells dependent on their localization. For example, lipid droplets are found only in principal cells of the corpus epididymidis [4].

Analagous to the testis, a series of tight junctions are found between adjacent principal cells at the apical region of their lateral membranes, forming the blood-epididymis barrier [51]. Like the blood-testis barrier, the blood-epididymis barrier enables the germ cells to exist in an immune privileged compartment.

Principal cells also have a very important secretory function. These cells secrete numerous substances into the tubular lumen, such as sulfated glycoprotein-2 (SGP-2),

androgen binding protein (ABP) and immobilin, which interact with the sperm; thus altering their composition and establishing the luminal microevironment necessary for the acquisition of fertilizing capability [50;52-56]. Principal cell secretion varies depending on epididymal region, and as a result the sperm are always exposed to a dynamic microevironment along its course throughout the epididymis [57-59]. The sheer number of different secretions and the variability in the different regions of the epididymis truly contributes to the complex synchronization and organization of this very important organ. Lastly, endocytosis of luminal contents by the principal cells in a region specific manner also aids in creating this gradually changing microenvironment [3;54].

Narrow Cells

Only found in the initial segment [60], these cells are tall slender columnar cells with a narrow base and wider apex. They posses an elongated nucleus located in the upper half of the cell, surrounded by a darkly staining cytoplasm. The apex is filled with numerous cup shaped vesicles that have been shown to be involved in endocytosis and secretion of H^+ ions into the epididymal lumen [61]. Similar to the principal cells, narrow cells also exhibit a regional specificity, within the intial segment, with regards to expression of certain proteins [62].

Clear Cells

Absent from the initial segment and vas deferens, clear cells are found only in the caput, corpus and cauda epididymides [4;63]. These pale staining cells have basally located nuclei which too are pale and round in appearance. The cytoplasm displays numerous apical vacuoles and dense bodies, among which many lipid droplets are frequently found [63;64]. A few microvilli can also be found at the apical surface [65]. These cells are highly endocytic, more so than principal cells [66;67]. Their role is to take up the remnants of the cytoplasmic droplets from sperm and endocytose various proteins from the epididymal lumen, also in a region specific manner [55;68;69].

Basal Cells

Basal cells are found throughout the entire tract between principal cells. They are small cells, located at the base of the epithelium, that do not reach the lumen of the tubule. They are round or elongated in shape, possessing a large nuclei with thin rim of cytoplasm; and have been noted to interdigitate with the lateral plasma membranes of

principal cells [4]. They appear to secrete as well as endocytose substances derived from principal cells or the blood [70].

HORMONAL CONTROL OF TESTICULAR FUNCTION

The testis is under the hormonal control of the pituitary gonadotropins, namely Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH). Both are produced in the anterior pituitary and in addition to Thyroid Stimulating Hormone (TSH) and human Chorionic Gonadotrophin (hCG) all share a common alpha (α)-subunit [2]. Each hormone has a distinct beta (β)-subunit, which confers hormone specificity. The two gonadotropins, which are under hypothalamic control via gonadotropin releasing hormone (GnRH), have been demonstrated to affect testicular growth and maturation; and exert their effects within the testis directly. In the testis, the responses of both FSH and LH ultimately converge onto Sertoli cells, which are the only cells within the seminiferous tubules that express receptors for FSH [71-75] and the androgens synthesized in response to LH [76].

While Sertoli cells are restricted to the tubular compartment of the testis, Leydig cells are present in the interstitial space. FSH receptors are expressed exclusively on Sertoli cells and LH receptors are correspondingly expressed on Leydig cells. Thus these two receptors are mutually exclusive. LH interacts with its cognate receptor and upon binding stimulates the production of androgens from the Leydig cells, which is essential for the development and maintenance of the male reproductive tract and spermatogenesis [77]. FSH however, acts exclusively on Sertoli cells to moderate a multitude of functions within the testis (see FSH and FSHR below).

The importance of the gonadotropins in spermatogenesis and testicular development has long been observed. When gonadotropins are withdrawn from the adult animal sperm production is abolished [78]. This outcome can be achieved by hypophysectomy [78], gonadotropin immunization [79], GnRH immunization [80], GnRH analogue treatment [81] and the suppression of gonadotropins by exogenous androgens [82].

FOLLICLE STIMULATING HORMONE (FSH)

From Gene to Protein

With regards to the two gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH), FSH is often considered more elusive, whereas the functions of LH are quite straightforward and well understood. FSH, a dimeric pituitary glycoprotein hormone composed of an alpha (α)-subunit and a β-subunit, is produced solely in the pituitary gland [83-85]. The common α-subunit non-covalently joins the β-subunit to form the biologically active hormone.

Fiddes and Goodman showed that the human α-subunit gene is 9.4 kb, localised to chromosome 6q and is composed of 4 exons. The gene encodes a 92 amino acid protein that is N-linked glycosylated at positions 52 and 78 [86]. The 10 kb human FSHβ gene localised to chromosome 11p, contains three exons and encodes a 111 amino acid subunit with N-linked glycosylation at positions 7 and 24 [87]. Unlike LH, FSH has a long circulatory half-life as a result of the differences in the number and composition of its carbohydrate side chains [84].

Following individual subunit transcription, translation and glycosylation, dimer formation occurs. Recently, the crystal structure of a βThr26Ala mutant of human follicle stimulating hormone (hFSH) has been determined to a 3.0 Å resolution. The βThr26Ala mutation results in elimination of the βAsn24 glycosylation site, yielding a protein more suitable for crystallisation without affecting the receptor binding and signal transduction activity of the pituitary glycohormone [88].

The synthesis and secretion of FSH is partly under the positive control of the hypothalamic gonadotropin-releasing hormone (GnRH), but gonadal activin/inhibin peptides and steroid hormones also participate in the regulation of FSH secretion [84].

Protein Function

Studies over a number of years have shown that FSH can act to regulate Sertoli cell function. Hormone function studies using hypophysectomized rats, as well as subsequent cell culture experiments have shown that FSH could stimulate aspects of Sertoli cell function [39;89;90]. Numerous studies have reported that both FSH and testosterone are required for quantitatively normal spermatogenesis [22;91;92]. While FSH has a role in the development of the immature testis [93], there remains considerable uncertainty regarding its precise involvement in spermatogenesis; namely the specific circumstances under which it is required and what it specifically does. This notion is

supported given recent clinical observations of an infertile man with small testes and azoospermia who was found to have an inactivating mutation in the FSH β-subunit gene [94] and another man with complete gonadotropin deficiency yet, in whom an activation mutation of the FSH-receptor (FSHR) led to a remarkable preservation of testicular volume, spermatogenesis and fertility [95]. In addition, reports exist of men with an inactivating mutation of the FSHR that are still fertile [96]; adding more confusion and uncertainty to the role of FSH and its subsequent signaling.

Reports of FSH being required for the initiation of spermatogenesis in immature rats have also been documented [22]. However, an important recent study reported that FSH-deficient male mice, though their testes were small, were fully fertile [97], thus arguing against the necessity for FSH even for the initiation of spermatogenesis.

Further controversy regarding the need for FSH in adulthood stems from the observation that spermatogenesis continues to proceed upon re-administration of testosterone to rats made gonadotropin deficient by immunization against GnRH [80;81]. Similar classical approaches to determine gonadotrophin function *in vivo* have also involved hypophysectomy followed by replacement of exogenous FSH or LH/androgen [98;99]. While extremely useful, both these approaches to study the *in vivo* role of FSH posses an inherent limitation compromising the utility of these mice as experimental models; namely that in all these previous studies, the complete lack of gonadotrophins is restricted to postnatal development, and these studies cannot account for any specific gonadotrophin dependent changes observed in utero. This limitation has been remedied given recent advances in targeted germline modifications through modern molecular techniques. The generation of the hypogonadal (*hpg*) mouse is one such example [100]. Extensive work involving the *hpg* mouse, which is functionally deficient in both FSH and LH/androgen activity because of naturally occurring germline deletion in the GnRH gene, has shed further light on the role of FSH *in vivo*. Research using this mouse background provided definitive evidence that androgens alone, in the absence of FSH activity, were sufficient for *qualitatively* normal spermatogenesis [101].

Recent studies have postulated that FSH may be important in the early stages of spermatogenesis and Sertoli cell development. Namely that FSH may stimulate early events such as spermatogonial proliferation and meiosis; as well as increasing the

proliferative capacity of Sertoli cells before they reach their end-dividing point of approximately day 15 postpartum [102;103].

Sertoli cells proliferate in the mouse through both the fetal and neonatal period. In mice lacking FSH stimulation however, the normal Sertoli cell number at birth and during the early postnatal period indicates that FSH is *not* required for the determination of Sertoli cell number during fetal and neonatal periods and only becomes necessary during the final establishment of Sertoli cell number [39;104]. Interestingly, Sertoli cell number in adult testicular feminization (*tfm*) mice i.e. those lacking the Androgen receptor and subsequent androgen signaling, was significantly lower on d20 old mice compared to wildtype and *hpg* mice, suggesting that androgens may be required to maintain initial Sertoli cell number [39].

In addition to its role in spermatogenesis and Sertoli cell development, FSH has a multitude of other functions within the testis. One such example is its role in fluid balance. Sertoli cells are recognized as the cells responsible for fluid secretion within the seminiferous tubules [34]. Serving as a vehicle for moving sperm from the testis to the epididymis, water is transported from the interstitial space to the lumen via the Sertoli cells [105]. Jegou et al. [35] first demonstrated a selective action of FSH, proposing that this hormone is involved in fluid absorption/secretion; however the cell type involved or the mechanism of action was not evaluated. Other investigators have also shown a correlation between increased tubular fluid production and FSH administration [106;107]. In addition, the onset of tubular fluid production at 20-35 days post partum coincides with a prepubertal rise in FSH [35]. Aquaporins are water selective channels which are essential for regulating water homeostasis and for providing sustained and rapid movement of fluid across a membrane [108;109]. Various aquaporins have been localized within the testis, specifically the Sertoli cell of the rat [110]. It has also been demonstrated in the female equivalent of the Sertoli cell, the granulosa cell, that aquaporin-8 expression is regulated by FSH [111].

FSH-RECEPTOR (FSHR)

From Gene to Protein

The biological action of FSH is initiated upon interaction with its specific receptor. The human FSH receptor (FSHR) is a glycosylated, heptohelical G_s-protein

coupled receptor, with a large extracellular domain, a seven transmembrane domain, three intracellular and three extracellular loops and an intracellular cytoplasmic tail [112-114]. The FSHR is a 695 amino acid glycoprotein and is encoded by a 54 kb gene localised to chromosome 2q. It is a protein of approximately 80 kDa in mass [115]. The gene contains ten exons, the first nine of which encode the large extracellular domain, while the last exon encodes the entire transmembrane and intracellular domains of the receptor [95;112;113]. To date, the FSHR has been found exclusively, in the male, on Sertoli cells [71-75]. Specifically, on the basal surface. Upon binding of FSH, which is found in the testicular lymph, the predominant second messenger cAMP is generated and activates downstream events, end-resulting in the production of protein kinase A (as described subsequently) [89;116;117].

FSHR Signalling within the Sertoli cell

The spermatogenic developmental program is under tight control, indirectly, of the hypothalamus, which regulates the level of pituitary gonadotropins (FSH and LH). Hormonal stimulation, rather than acting directly on germ cells, is mediated by the Sertoli and Leydig cells. The mechanism of FSH action on Sertoli Cells is via the cAMP Mediated Pathway [89]. As previously mentioned, the FSH receptor belongs to the family of G-Protein Coupled Receptors (GPCRs). They consist of a large extracellular N-terminal segment, 7-transmembrane (TM) spanning domains, and a cytoplasmic C-terminal segment [115]. As with most glycoprotein hormones (≅30 kDa), the ligand binds exclusively to the large N-Terminal segment [115]. The liganded-N-terminal then interacts with the exoloops to generate a signal. The 7 transmembrane receptor then interacts with the heterotrimeric G protein. The G-Protein is comprised of α, β and γ subunits.

In its inactive state, the α-subunit is bound to a GDP molecule. Upon ligand (FSH) binding, and signal transduction to the G-Protein, there is an exchange of GDP for GTP on the α-subunit. This causes the $\beta\gamma$ subunit to then dissociate. The active α_S-subunit, bound to the 7TM receptor, interacts with adenylate cyclase, also in the plasma membrane, activating it. RGS (Regulators of G Protein Signalling) increases the intrinsic GTPase activity of the α-subunit, resulting in the hydrolysis of GTP into GDP. This shift

in equilibrium favours the reassociation of all three subunits, and the protein is ready to be activated once again [118].

Adenylate cyclase facilitates the conversion of ATP into cyclic AMP (cAMP). Intracellular levels of cAMP are regulated primarily by adenylate cyclase [118]. The increased cAMP levels, as a result of GPCR activation, directly affect the function of the tetrameric protein kinase A (PKA) complex [119]. Binding of cAMP to two PKA regulatory subunits releases the catalytic subunits enabling them to phosphorylate target proteins [118]. These catalytic subunits are translocated from cytoplasmic and Golgi complex anchoring sites and phosphorylate a number of cytoplasmic and nuclear proteins on serine residues [120;121].

In the nucleus, PKA-mediated phosphorylation of various transcription factors ultimately influence the transcriptional regulation of various genes through distinct, cAMP-inducible promotor responsive sites [120;122]. Of these cAMP-inducible promoter sites, the best characterized is the CRE, cAMP Response Element. A consensus CRE site constitutes an 8-bp palindromic sequence (TGACGTCA) [123].

Several CRE-binding factors, such as cAMP response element binding protein (CREB) or cAMP response element modulator (CREM) have been identified. All these proteins belong to the bZip transcription factor class [121]. The CRE-binding proteins may act as both activators and repressors of transcription. The activator proteins CREB, CREM, and ATF-1 mediate transcriptional induction upon their phosphorylation by PKA [121].

In addition to being present in Sertoli cells, CREM proteins have been localized to germ cells. In the haploid spermatids, CREM proteins activate a number of cellular genes expressed specifically during spermatid maturation [124]. Several genes containing a CRE motif in their promoter region have been identified. These genes encode mainly structural proteins required for spermatozoa differentiation. Genes such as RT7 [124], transition protein-1 [125], angiotensin converting enzyme [126], CYP51 [127] and calspermin [128] have been shown to be CREM targets by various experimental approaches [129].

It should also be noted that the actions of FSH on the Sertoli cell are pleiotropic stimulating growth, differentiation, steroidogenesis, and calcium influx [130;131];

prompting the question as to whether or not the cAMP/PKA is the sole signaling pathway of the FSHR. Within the female, several events such as the increase in synthesis of inhibin B and cytochrome P450 aromatase by FSH occur well before the expression of the G_s-coupled FSHR, suggesting the possibility of other FSHR motifs being present during development. In addition, in testicular target cells isolated from animals, not all the actions of FSH and other glycoprotein hormones are fully reproduced by cyclic AMP analogs or nonhormonal activators of adenylate cyclase prompting the speculation about the involvement of other second messengers and signaling pathways [130;132-134]. Given that extensive alternative splicing of the FSHR gene has been recorded in many species [135-138], the notion of alternatively spliced FSHR transcripts seems more of a reality. In fact, at least three other FSHR isoforms with different structural motifs that might be of biological significance have been cloned [134;136;139-142]. One of these isoforms, designated FSHR-3, has been demonstrated to be a single transmembrane growth factor type I receptor; and upon FSH binding, stimulates DNA sythesis [134] and enhances calcium signaling [142].

The presence of other signaling pathways within the Sertoli cell such as the protein kinase C pathway [143;144], or the AKT/protein kinase B (PKB) pathway [145;146] have been reported. The effect of FSH on the PKC pathway remains unclear [115]; however it has been demonstrated that FSH increases phosphorylated PKB levels in a phosphatidylinositol 3-kinase (PI3K) dependent and PKA-independent manner in rat Sertoli cells [145]. Thus it has been demonstrated that signal transduction pathways for an individual hormone vary with the same cell type; and possibly with the maturation state of the cell.

CLINICAL SIGNIFICANCE OF STUDYING FSH AND ITS RECEPTOR

Recent reports on human mutations have begun to draw attention to the role of FSH and/or its receptor to maintaining optimal testicular function conducive to normal spermatogenesis. It is through the identification of several human mutations and by the generation of genetically engineered mice with various alterations of the endogenous genes for the FSH ligand and FSH receptor (as described subsequently) that has greatly enhanced our knowledge of the FSH/FSHR signaling system. Human males with a mutation of the FSHβ gene mutation display normal pubertal development with small

testes, normal adult testosterone levels, high LH and low FSH [94]. Others, with a more severe mutation, have displayed no pubertal development with very small testes, low testosterone, elevated LH and low FSH [147]. In both cases, the mutation of the FSHβ gene also resulted in azoospermia suggesting the necessity of FSH in men for androgen and gamete production.

FSHR mutations are rare in most parts of the world except Finland, where the prevalence of a gene mutation in the FSH receptor is higher than expected [148]. Described as the Finnish mutation, five men homozygous for the Ala189Val FSHR gene mutation have been identified to date [96]. While displaying normal puberty, these men exhibited small testes, but normal testosterone levels. FSH levels were elevated, while LH remained normal or slightly elevated. All men had abnormal sperm parameters such as decreased sperm counts; however, all remained fertile [96]. The authors thus suggested that FSH function in human males may not be essential for spermatogenesis, since fertility occurred in some men despite oligospermia.

Follitropin Receptor Knockout (FORKO) Mouse

The role of FSH in the male testis of adult mammals is not yet clearly defined but the importance of FSH in testicular development has been suggested. In order to effectively study the role of this gonadotropin in mammals, various mutations in the FSH Receptor (FSH-R) and/or FSH itself have been generated and studied. The development of mice deficient in the FSH receptor (FORKO) provides a model to examine alterations in testicular structure and function in its complete absence [149]. Mutant adult mice display smaller testes, delayed sexual maturity and a reduced fertility [149-151]. In addition, serum FSH levels are elevated [149] and testosterone levels are reduced [149;152] amid normal circulating levels of LH [152]. Female mice homozygous to the mutation are completely sterile due to a block in follicular maturation; and show disturbances similar to the changes observed in postmenopausal women [153].

Aims of Project

While initial studies have been carried out and several organs have been examined in the FORKO mouse, little attention has been paid to changes within cells of the testis and epididymis. Specifically, details on the structure and functions of these tissues of FORKO mice at early and later ages have not been studied in any detail. The aims of this

project were to perform an in depth structural and functional comparison of Sertoli cells of both wild type and FORKO mice using techniques including light and electron microscopy, morphometry, immunocytochemistry and western blot analyses. Subsequently, the second objective was to perform a similar analysis comparing the epididymides of both wild type and FORKO mice using light and electron microscopy, morphometry, immunocytochemistry, and computer assisted sperm analyses (CASA).

HYPOTHESIS

It is hypothesized that the phenotypic differences seen in the FORKO mouse, specifically reduced fertility, may arise because of deficient Sertoli cell function due to the loss of FSH-R signaling; and subsequently, phenotypic alterations to the epididymis affecting sperm maturation are also indirectly due to the loss of FSH-R signaling.

Since FSHR signaling is crucial in both mouse and human reproduction, it is hoped that our research into understanding the importance of FSHR signaling in testicular development, spermatogenesis and sperm maturation will eventually generate renewed interest in the development of a new type of male contraceptive and/or therapeutic drugs directed towards infertility, both of which may involve targeting of the follitropin receptor (FSHR).

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CHAPTER II

Structural and Functional Modifications of Sertoli Cells in the Testis of Adult FORKO Mice

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ABSTRACT

Follicle stimulating hormone (FSH) plays important roles during testicular development and in the maintenance of spermatogenesis in the adult. However, the cellular events or pathways that FSH regulates to achieve these effects in Sertoli cells, where the FSH-Receptors (R) are located, is still not fully elucidated. The development of FSH-R knockout (FORKO) mice provides a model to examine alterations in testicular structure and function in its absence. To this end, light (LM) and electron microscopic (EM) analyses of perfusion-fixed testes of wild type and FORKO mice of different ages were performed. Under the LM, a significant reduction was noted in the profile area of seminiferous tubules of FORKO mice compared to their wild type counterparts at different ages. In addition, FORKO testes revealed large irregularly shaped spaces within the seminiferous epithelium, extending from the base to the lumen. Such spaces were often separated by anastomotic cords of spherical germ cells or completely surrounded elongating spermatids. This phenotype was restricted to half or less of the circumference of only some tubules, but was seen at all stages. EM analyses revealed that the spaces corresponded to an apparent accumulation of fluid in the Sertoli cell cytoplasm, coincident with an absence of the fine flocculent ground substance seen in wild type mice. However, the Sertoli organelles, while less prominent, appeared intact and to be floating in the enlarged fluid filled cytoplasm. Functionally, androgen-binding protein (ABP), a major secretory protein of Sertoli cells, was dramatically reduced in FORKO mice. These results suggest that FSH-R signaling normally maintains water balance in Sertoli cells, in addition to regulating ABP production.

INTRODUCTION

Follicle stimulating hormone (Follitropin, FSH), a heterodimeric pituitary glycoprotein hormone considered essential for mammalian fertility, interacts specifically with its cognate receptor (FSH-R) localized in Sertoli and ovarian granulosa cells. The FSH-R, derived from a single gene, is produced as a Gs-protein coupled, seven-transmembrane receptor, which activates several signaling pathways to integrate target cellular activities [1-3]. In the testis, FSH has a differential effect on Sertoli cells in accordance with the different stages of the cycle of the seminiferous epithelium. While the hormone is maximally bound at stage I [4-5], FSH stimulated cAMP production peaks at stage IV, with CREB mRNA levels peaking at stage VII of the cycle [6-7]. In the adult testis, the non-dividing population of Sertoli cells performs diverse functions. In addition to anchoring and nourishing germ cells, they form the blood testis barrier, phagocytose residual bodies, release sperm at the time of spermiation, and participate in secretion and endocytosis of various substances, including ions and proteins [8-11].

Also important is the fact that Sertoli cells transport water from the interstitial space into the lumen, serving as the vehicle for moving sperm from the testis to the epididymis [12-13]. In addition to basolaterally located ion channels, recent studies have revealed that aquaporins (water channels) are abundant in the testis, with some being localized to Sertoli cells [14-16]. Interestingly, various members of the aquaporin gene family contain CRE motifs (CREB binding regions) and are under cAMP regulation [17-20], a second messenger that is activated upon FSH-R signaling [1]. In the testis, Jegou et al. [21] first demonstrated a selective action of FSH proposing that this hormone is involved in fluid absorption/secretion, however, the cell type involved or the mechanism of action was not evaluated.

In addition to fluid regulation, Sertoli cells are responsible for the secretion of numerous proteins into the seminiferous tubular lumen, such as the glycoprotein androgen binding protein (ABP). ABP binds androgens with high affinity and transports them to the epididymis [22-24]. Regulation of ABP by FSH and testosterone has been demonstrated [25], although whether one or both are required for complete function remains to be resolved [22]. ABP also displays a stage specific expression pattern within the seminiferous epithelium and its secretion has often been regarded as an index of Sertoli

cell function [22, 26]. Recent work with transgenic mice over expressing rat ABP has shed more light on the role of ABP in spermatogenesis. The predominant phenotypic anomaly observed in these mice is a decrease in fertility suggesting that altered levels of ABP are in fact associated with impaired fertility [22, 27, 28].

While Sertoli cells respond to FSH stimulation, the nature of the response itself varies depending on the developmental status of the animal. Early studies demonstrated that FSH acts as a Sertoli cell mitogen in the prenatal and newborn animal and is instrumental in determining the final spermatogenic capability of the testis [29]. In immature mice, it has been shown that FSH-R signaling appears to be essential for testicular development, the initiation of the first wave of spermatogenesis, and sexual maturity [30, 31]. The critical role played by FSH/FSH-R signaling has been further illustrated through newly uncovered human mutations in the FSH β subunit [32-35]. Such men display hypogonadism, obstructed pubertal development, small testis size and azoospermia [32-34]. Interestingly, mice that lack the FSH β subunit [35] are fertile, as are some Finnish men with an inactivating FSH-R mutation [36]. In both these examples testis size and sperm production levels are reduced [35, 36].

The development of mice deficient in the FSH-Receptor (FORKO) provides a model to examine alterations in testicular structure and function in its complete absence [37]. Mutant mice at 2 months of age have smaller testes, delayed sexual maturity and reduced fertility [30, 37, 38]. In addition, serum FSH levels are elevated [37], and testosterone levels are reduced [37-39] amidst normal circulating levels of LH [39]. However, details on the structure and functions of the cell type(s) affected in the testis of FORKO mice at early and later ages have not been studied in any detail. This investigation was designed to shed more light on the structural alterations in Sertoli cells of the mutants and their potential impact on functions.

MATERIALS AND METHODS

Animals

This investigation was approved by the ethics committee of the Clinical Institute of Montreal and McGill University and was conducted according to accepted standards of animal experimentation. The FORKO mice were generated by homologous recombination as described by Dierich et al. [37]. This alteration eliminates the entire repertoire of FSH-R forms, producing complete loss of hormone signaling. Breeding F2 heterozygous males and females produced mice of all three genotypes in the SV129 background. The animals were maintained under well-controlled conditions of temperature (22°C) and light (12L: 12D), with food and water provided ad libitum. The primers and amplification conditions used for the multiplex polymerase chain reaction (PCR) to identify the phenotypes have been described in detail elsewhere [40]. According to this protocol, a single PCR performed on each sample allowed unambiguous identification of +/+, +/-, and -/- mice.

Routine Light and Electron Microscopic Methods

A total of 16 mice at 3 and 6 months of age (wild type, n=4; FORKO, n=4, for each age group) were used for detailed ultrastructural analyses of their testes. The mice were anesthetized by an intraperitoneal injection with sodium pentobarbital (Somnitol, MTC Pharmaceuticals, Hamilton, ON, Canada). Prior to perfusion, a hemostat was placed over the testicular vessels entering the left testis of each animal; this testis was removed, immersed in Bouin's fixative and after 10 minutes cut in half and left in Bouin's for 24 hrs. Thereafter, all the left testes were dehydrated in alcohol and subsequently embedded in paraffin.

The remaining right testis of each animal was kept intact and immediately fixed by cardiac perfusion with 2.5% or 5% glutaraldehyde buffered in sodium cacodylate (0.1M) containing 0.05% calcium chloride (pH 7.4). After 10 minutes of perfusion, the right testes were removed from the scrotum, cut into small 1mm cubes and placed in the same fixative for an additional 2 hr at 4°C. Thereafter, the tissues were thoroughly rinsed three times in 0.1M sodium cacodylate buffer containing 0.2M sucrose and left in this buffer overnight. On the following day, the testes were post fixed in ferrocyanide-reduced osmium tetroxide for 1 hr at 4°C, dehydrated in a graded series of ethanol and propylene

oxide, and embedded in Epon. Thick sections (0.5μm) were cut with glass knives and stained with toluidine blue and observed by light microscopy. Thin sections (grey to silver interference colour) 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 (2 minutes). Sections were examined with a Philips 400 electron microscope.

Quantitative Analyses

The left testes from the 3 and 6 month old wild type and FORKO mice that had been fixed in Bouin's fluid were utilized for quantitative analyses, along with other testes obtained from 12 month old wild type (n=4) and FORKO (n=4) mice that had been prepared and fixed in Bouin's fluid for a different study. Scaled digital images of 5 μm thick paraffin sections of seminiferous tubules from these animals were captured on a Zeiss Axioscop 2 equipped with an AxiocamMR camera, and the peripheral outline of selected tubules was traced and the profile areas determined using the appropriate measurement tool available in Version 3.1 of the Axiovision Imaging Software (Carl Zeiss Canada Ltd., Montreal, QC). In all cases, only those seminiferous tubules showing a near perfect transverse plane of section were measured, and a minimum of 150 tubular cross sectional profiles were outlined in sections from animals of a given age and treatment group. Preliminary analyses of the data indicated that profile areas of the seminiferous tubules were not distributed along a normal curve in either the wild type or the FORKO mice, and $\log_{10}$ transformations of raw data had to be done in order to obtain normal distributions. These transformations and subsequent Univariate Factorial ANOVA test and Post-hoc unequal N HSD *t*-tests were done using Version 6.1 of STATISTICA for Windows (Statsoft, Inc., Tulsa, OK); p values < 0.05 were considered significant.

LM Immunocytochemistry

The following affinity purified polyclonal antibodies were used at 1:100 dilution (v/v) for routine peroxidase immunostaining: (1) anti-prosaposin antibody, provided by Dr. C.R. Morales, McGill University, Montreal, Canada (purified and characterized as described previously [41]), and (2) rabbit anti-ABP antibody prepared against a GST-ABP fusion protein (42) was provided by Dr. G.L. Hammond, University of Western Ontario, London, Canada.

For the anti-prosaposin antibody, 5 μm thick paraffin sections of Bouin's fixed testes were deparaffinized in Histoclear (Diamed Lab Supplies Inc, Mississauga, ON, Canada) and hydrated through a series of graded ethanol solutions. During hydration, residual picric acid was neutralized in 70% ethanol containing 1% lithium carbonate, and endogenous peroxidase activity was abolished by treating the sections with 70% ethanol containing 1% (v/v) H_2O_2. Once hydrated, the sections were washed in distilled water containing glycine to block free aldehyde groups. Non-specific binding sites were blocked using 10% goat serum for 30 min. The sections were then incubated at 37°C in a humidified chamber for 90 minutes with 100μL of primary antibody diluted in Tris-buffered saline (TBS). Following washes in 0.1% Tween20 in TBS, the slides were incubated with secondary antibody (1:250; 100μL) labeled with horseradish peroxidase for 30 min at 37°C in a humidified chamber. Reactions were revealed with diaminobenzidine tetrahydrochloride (DAB). Sections were counterstained with methylene blue, dehydrated in ethanol and Histoclear and mounted with cover slips using Permount. TBS substitution for primary antibody was used as a negative control. No reactions were observed in these sections.

For the anti-ABP antibody, paraffin sections were processed for immunostaining using the ImmunoCruz ABC Staining System (Santa Cruz Biotechnology Inc., California USA). The sections were deparaffinized and hydrated as described above. Sections then were microwaved for antigen retrieval in citrate buffer (43). After boiling and cooling, the ImmunoCruz system was employed as per the suppliers' instructions. Sections were incubated with the anti-ABP antibody at a dilution of 1:100 overnight at 4°C. The sections were then washed in phosphate buffered saline (PBS) and incubated in biotinylated secondary antibody (1:250) for 30 minutes at room temperature. Sections were washed again in PBS and incubated in an avidin-biotin-horseradish peroxidase solution for 30 minutes at room temperature. Reactions were visualized by DAB. The sections were counterstained with methylene blue, dehydrated and mounted with cover slips. For negative controls, normal blocking serum was substituted for primary antibody; no reactions were detected in the epithelium.

Western Blot Analysis

A total of 6 mice at 6 months of age (wild type n=3 and FORKO n=3) were used for Western Blot analysis. One testis of each mouse was extracted and cut into 4 equal pieces. Each piece was then frozen in liquid nitrogen for subsequent protein extraction. The frozen pieces of testis were homogenized, suspended in lysis buffer containing detergent and a protease inhibitor cocktail [39], and the solution was centrifuged at 11000*g* for 15 minutes at 4°C. The supernatant was removed and quantified for protein using the Bradford Assay (Bio-Rad Laboratories Inc., Richmond, CA). Equivalent amounts of solubilized protein (30μg) in SDS sample loading buffer were boiled for 5 minutes and electrophoresed on a 10% SDS polyacrylamide gel. Subsequently, the proteins were transferred onto nitrocellulose membranes for immunoblotting. Following incubation with the anti-ABP antibody at 1:500 (v/v) dilution and secondary antibody conjugated to horseradish peroxidase (1:1000 v/v), bands were revealed by chemiluminescence using the Pierce SuperSignal® Western Blotting Kit (Pierce, Rockford, IL USA). Molecular weight markers were used to estimate the mass of the detected bands. The experiment was repeated a minimum of 3 times in duplicate. Equal protein loading was confirmed by Coomassie staining. Quantitative analysis of Western Blot was performed using Chemi-Imager 4.0 software (B&L Systems, Maarssen, The Netherlands), which measured the spot density of each protein band on the exposed film. Raw density data was subject to an unpaired sample *t*-test; p values < 0.05 were considered significant.

RESULTS

Light Microscopic Appearance of the Testis of FORKO Mice

At 3 and 6 months of age, the seminiferous tubules in the testis of FORKO mice showed smaller profile areas compared to wild type mice (Figs.1a and b). In addition, while the testis of wild type mice displayed a homogenous, compact seminiferous epithelium, where germ and Sertoli cells were closely associated with each other (Figs.1a, c), the FORKO mice presented a varying and vacuolated appearance (Figs. 1b, d, 2a, b). Although some tubules at both ages seemed normal in cross sectional profile (approx. 40%), nearly one half of the circumference of seminiferous epithelium of other tubules in the FORKO mice appeared disrupted showing large dilated spaces between the epithelial cells (Figs.1b, d, 2a, b). This semi lunar disruption pattern did not appear to be associated with a specific stage of the cycle of the seminiferous epithelium (Figs.1b, d, 2a, b).

In areas where the FORKO tubules were abnormal, large dilated spaces often extended from the base of the epithelium towards the lumen, and at the base, these spaces at times hovered over the nuclei of Sertoli cells (Figs. 2a, b). In the mid region of the epithelium, the dilated spaces often separated chains of round spermatids from each other. In this way, they formed cords or ribbons, which gave the epithelium an anastomotic appearance due to their extensive nature (Figs. 2a, b). The large dilated spaces of varying sizes also surrounded elongating spermatids, which they often completely enveloped (Figs. 1b, d, 2a, b). The interior of the dilated spaces often contained membranous profiles of varying sizes and spherical particulate material (Fig. 2b). The various generations of germ cells did not display any signs of structural abnormalities or spaces in their cytoplasm (Figs. 1b, d, 2a, b). In the interstitial spaces of FORKO mice that seemed to be enlarged, Leydig cells, macrophages and blood vessels were structurally similar in appearance to those seen in the wild type mice. There was no evidence of any increase in macrophage number or infiltration of neutrophils in the interstitial spaces of FORKO mice.

Immunocytochemical Analyses

Anti-prosaposin antibody revealed Sertoli cells spanning the circumference of each tubule in a regular and spoke-like manner (Figs. 3a and b). The staining pattern radiated from the base of the epithelium towards the lumen over a distance that appeared

shorter in the FORKO mice due to the smaller profile areas of tubules in these animals compared to wild type mice (Figs. 3a and 3b).

An intense anti-androgen binding protein (ABP) reaction was noted over the cytoplasm of Sertoli cells at all stages of the cycle of the seminiferous epithelium of wild type mice; the reaction extended from the base of the epithelium to the lumen (Figs. 4a, c, e). No staining was observed over germ cells. In FORKO mice, the staining over Sertoli cells at all stages of the cycle appeared weak and in some areas completely missing (Figs. 4b, d, f). A non-specific staining of Leydig cells was evident in the interstitial spaces inherent to the anti-ABP protein construct (see Materials and Methods), and the fact that Leydig cells express GSTs [44]. Control sections showed no staining over the epithelium (Fig. 4a inset).

Electron Microscopic Appearance of the Testis of FORKO Mice

While the nucleus of the affected Sertoli cells in FORKO mice was intact showing a pale stained uncondensed chromatin pattern and conspicuous nucleolus and satellite bodies as seen in wild type mice (Fig. 5a), their cytoplasm was grossly disrupted; this was especially evident in the mid and apical areas of their cytoplasm (Figs. 5b, 6, 7b, 8). In wild type mice, the organelles in the expansive Sertoli cell cytoplasm were embedded in a compact finely flocculent ground substance, with adjacent Sertoli cells being tethered together by a conspicuous Sertoli-Sertoli blood testis barrier (Fig. 5a). Sertoli and germ cells were closely associated with each other with very small intercellular spaces positioned between the two. However, in FORKO mice, the seminiferous epithelium showed large dilated spaces surrounding nearby germ cells (Fig. 5b, 6). The basal Sertoli cell cytoplasm contacted the basement membrane and displayed various organelles such as lysosomes, mitochondria, endoplasmic reticulum (ER) and the Golgi apparatus, embedded in a finely flocculent ground substance (Figs. 5b, 6). However, large dilated spaces not connected to basal areas of Sertoli cell cytoplasm, due to the plane of section, were often observed in mid and apical regions of the epithelium (Fig. 5b). The fact that these dilated spaces were delimited by a plasma membrane and contained organelles indicated that they were territories of cytoplasm. Indeed, in appropriate planes of section, the large dilated membrane bound spaces were confluent with the intact basal areas of Sertoli cell cytoplasm (Fig. 6). It was thus concluded that the large dilated spaces

corresponded to extensive dilations of the Sertoli cell cytoplasm. In contrast, the cytoplasm of the neighboring spermatocytes or early round spermatids was not disrupted, and on no occasion did it ever appear dilated (Figs. 5b, 6). In the intact basal areas of the Sertoli cell cytoplasm, the Sertoli-Sertoli blood testis barrier of FORKO mice appeared to be intact. As in wild type mice (Fig. 5a), bundles of filaments and ER cisternae closely approximated the expansive areas of tight junctions between the plasma membranes of adjacent Sertoli cells (Figs. 5b, 6).

In the mid and apical regions of the seminiferous epithelium of wild type mice, the heads of elongating spermatids were deeply embedded in niches of Sertoli cell processes (Fig. 7a). The cytoplasm of the latter contained mitochondria, lysosomes and ER cisternae, all embedded in a finely flocculent ground substance. In addition, bundles of filaments overlaid by ER cisternae, and forming the so-called ectoplasmic specializations, were closely applied to the spermatid heads (Fig. 7a). In the semi lunar affected areas of the seminiferous epithelium of FORKO mice, a gross enlargement and dilation of the apical Sertoli cell cytoplasm was evident (Fig. 7b). In some planes of section, the dilated apical Sertoli cell processes, of enormous sizes, contained few organelles, other than membranous profiles of various sizes (Fig. 8a). However, in other cases, such processes contained numerous organelles such as mitochondria, ER cisternae, lysosomes and small vesicular profiles (Fig. 8b). The ectoplasmic specializations of the Sertoli cell processes also appeared to be affected. They showed fewer filaments and occasional swellings of their ER cisternae (Figs. 8a, b). The dilated processes did not appear to be continuous with the lumen of the tubule, as they were consistently delimited by a plasma membrane (Figs. 8a, b).

Quantitative Analyses

Quantitative measurements indicated that the mean cross sectional profile area of seminiferous tubules in FORKO mice was significantly lower compared to wild type mice at all ages examined (Fig. 9, Table 1). In addition, both groups showed significant changes in mean profile areas of the tubules as the age of the animals increased (Fig. 9, Table 1). The mean profile area of tubules in wild type mice rose by about 5,700 μm^2 per tubule from 3 months and 6 months and then increased by an additional 14,300 μm^2 per tubule between 6 months and 12 months of age (Fig. 9). The mean profile area of tubules

in FORKO mice, however, decreased by about 3,500 μm^2 per tubule from 3 months and 6 months and then increased by 13,900 μm^2 per tubule, roughly the same increment detected for wild type mice, between 6 months and 12 months of age (Fig. 9). These within group changes in cross sectional profile areas of the seminiferous tubules were significant across all ages (Table 1).

Western Blots

Western blots of testicular extracts from wild type and FORKO mice probed with the anti-ABP antibody showed the presence of reactive bands, as expected, near 75 kDa. The reactive bands appeared more intense in the extracts from the wild type mice compared to the FORKO mice at equivalent total protein loads per lane (Fig. 10; density data). Another set of reactive bands near 30 kDa was also observed in these blots, which were non-specific in nature and caused by a anti-GST immunoreactivity simultaneously present in the anti-ABP antibody (the antigen used to elicit the antibody was a recombinant GST-fusion protein) (data not shown).

DISCUSSION

The most striking aspect of the present study was the finding of large irregularly shaped translucent spaces situated between germ cells in the testis of FORKO mice. For a variety of reasons, it was concluded that these spaces within the epithelium were territories of the Sertoli cell cytoplasm that were highly dilated and filled with fluid. In appropriate planes of section, it was noted that the basal cytoplasm of Sertoli cells at times appeared intact, showing a nucleus and organelles embedded in a finely flocculent ground substance (Fig. 11). These intact areas were confluent with the highly dilated space that extended towards the lumen. The fact that such spaces were always delimited by a plasma membrane indicated that they were of cellular origin. Indeed such spaces contained membranous profiles of varying sizes and organelles such as mitochondria, ER and lysosomes characteristic of those normally found in Sertoli cells (Fig. 11). On no occasion were the membrane bound dilated spaces continuous with the lumen. Furthermore, the epithelium and lumen of the efferent ducts appeared intact, as did that of the epididymis, suggesting that fluid was not backing up into the lumen of seminiferous tubules. In contrast, in ERKO mice, the epithelium of the efferent ducts is affected, resulting in fluid accumulation in the seminiferous tubular lumen and disruption of the epithelium (45, 46). More apically, the dilated spaces surrounded the heads of elongating spermatids (Fig. 11). Being membrane bound such spaces corresponded to the cytoplasm of the apical processes of Sertoli cells that are known to envelop the heads of spermatids in various mammals at different stages of the cycle [47, 48]. There was no evidence of dilations of the cytoplasm of any generation of germ cells. Thus in the absence of the FSH-R, Sertoli cells show dilations of their cytoplasm, with apparent accumulation of fluid and concurrent loss of ground substance (Fig. 11).

For several reasons we believe that observations on dilated spaces in FORKO testes are real and not artifacts. First, the testes used for EM analyses were always fixed by cardiac perfusion with either 2.5 or 5% glutaraldehyde. In both cases, the dilated spaces were consistently found in FORKO mice, but never in their wild type counterparts. Second, concurrent perfusions of mice from many other knockout models studied in our laboratory by identical procedures never resulted in dilated spaces of the type seen in the FORKO mice, indicating that these spaces are not related to the fixative or the method

used to perfuse FORKO mice [49, 50]. Third, dilated cytoplasmic areas in different generations of germ cells were never observed in the FORKO mice. The dilations were exclusively restricted to cytoplasm of Sertoli cells suggestive of something specific about the relationship of the knockout to these particular cells. Fourth, in all cases, there were no signs of bloating of the mitochondria, Golgi apparatus or lysosomes of germ cells or Sertoli cells, perturbations that are commonly seen in poorly fixed immersed testes. Thus the dilations of the Sertoli cell cytoplasm appear to be a direct consequence of the absence of FSH-R signaling in these cells. The fact that these spaces appear to be filled with fluid, as indicated by the absence of the finely flocculent ground substance seen in wild type mice (Fig. 11), suggests that FSH-R signaling could potentially regulate water balance in the Sertoli cell.

An interesting observation in FORKO mice was the finding that not all seminiferous tubules appeared affected to the same degree as only about 60% of tubules present in any random section of testis showed the characteristic dilated phenotype in the cytoplasm of Sertoli cells. In addition, the tubules that were affected usually displayed a semi lunar disruption, i.e. only one-half of the tubule was affected in a cross section. Affected tubules were found at all stages of the cycle of the seminiferous epithelium suggesting that this phenomenon is not stage specific. The quantitative data indicated very clearly that while focal areas of Sertoli cell cytoplasm appear swollen this effect does not extend globally to the entire tubule itself which actually is reduced by 16%, 35% and 30% in profile area at 3, 6, and 12 months, respectively in the FORKO mice relative to the wild type mice. It is unclear at this time if this shrinkage in cross sectional area of tubules in the knockout animals is caused by a decrease in the number of cells lining the walls of the tubules, a condensation of all cells lining the tubules, or a decrease in internal fluid pressure or amount that normally keeps the tubules in a more expanded state. Our previous studies [38] have shown that Sertoli cell numbers are compromised in the absence of FSH-receptors with a concomitant inability to support a full complement of germ cells [30, 37].

The finding that some tubules in a cross section appear normal, while others show varying degrees of altered epithelium is apparently not unique to the FORKO testis and has also been reported for other knockout mouse models [49, 51]. While the underlying

reasons for such a phenomenon are still not fully understood several speculations can be advanced. It may be that the protein(s) regulated by the FSH-R cascade and which are affected in FORKO mice may reside in only some Sertoli cells and not in others. It is also possible that not all Sertoli cells encompassing a given tubule express the FSH-Receptor, or equal amounts of it, at the same time. Nevertheless, the reason why Sertoli cells have evolved such complex mechanisms of protein regulation is unclear, but the data suggest that the testis has some capacity to maintain itself in part under adverse conditions.

The question also arises as to what mechanism(s) possibly gives rise to the abnormalities found in the cytoplasm of Sertoli cells of FORKO mice. Considering the dilated nature of the cytoplasm, it is likely that these cells as noted earlier are accumulating fluid, which would account for the absence of ground substance and the transparent nature of the FORKO Sertoli cytoplasm. Among their many functions, Sertoli cells move water from the interstitial space to the lumen of the seminiferous tubule, where it serves as the vehicle for transporting sperm into the epididymis [12-13]. This occurs along a standing osmotic gradient created by Na^+K^+/ATPase, which has been localized to the basolateral plasma membranes of Sertoli cells [52, 53]. In early studies, several investigators showed a correlation between increased tubular fluid production and FSH administration [21, 54, 55]. In addition, the onset of tubular fluid production at 20-35 days coincides with a prepubertal rise in FSH [21]. Based on these observations and our findings of a dilated Sertoli cell cytoplasm in FORKO mice, it seems likely that FSH-R regulates water balance in the Sertoli cells. Recent findings reported by Haywood et al. [56] using the hpg mouse model lend support to this hypothesis. These mice lack gonadotropins, and constitutively expressing a mutated FSH-R, show increased levels of tubular fluid secretion resulting from overactive FSH-R signaling.

Recently, the expression and immunolocalization of several water channel proteins, aquaporins (AQPs), have been described in the testis of rats [14, 15, 57]. AQPs are essential for regulating water homeostasis and for providing sustained and rapid movement of fluid across a tightly sealed epithelium with minimal activation costs [58, 59]. There are 11 different members of the AQP family [16], with several containing cAMP motifs and CREB binding regions that regulate their transcription [17-20]. A

systematic effort to localize AQP's in the testis of these mice would be required to fully understand hormonal/receptor regulation of these channels.

Considering the abnormality seen in the Sertoli cell cytoplasm, the question arises whether it is possible that such cells degenerate with time thus compromising the seminiferous epithelium. In fact, at no time did we observe pyknosis of Sertoli cell nuclei, degeneration of their organelles or evidence of apoptotic figures in the seminiferous epithelium. In addition, the basal Sertoli cell cytoplasm and junctional complexes between adjacent Sertoli cells appeared intact. Furthermore, the absence of an increased number of macrophages or neutrophils in the interstitial space of the testis of FORKO mice suggests that there is no leakage of substances from the epithelium as a result of the swelling of the Sertoli cell cytoplasm or complete breakdown of the blood testis barrier. On the other hand, the ectoplasmic specializations enveloping the heads of spermatids (steps 9-16) appeared compromised. The bundles of filaments were not extensively distributed and the ER cisternae of these specializations were at times dilated (Fig. 11), unlike relationships normally seen in wild type mice [60]. However, the adhesive function of these structures appeared to be maintained, as spermatids were still closely associated with the apical Sertoli cell processes, despite the fact that the latter were grossly dilated.

One of the major proteins regulated by FSH and secreted by Sertoli cells into the lumen is ABP [22, 26]. In the present study, LM immunocytochemistry revealed that ABP was expressed intensely in Sertoli cells, but the reaction was dramatically reduced in FORKO mice, a finding confirmed by the Western blot analyses. This may be due to the dilated nature of the Sertoli cell cytoplasm and its effect on secretory organelles, such as the ER and Golgi apparatus, which appeared to be less prominent than in wild type mice and which would affect secretion. However, it is equally possible that regulation is affected at the mRNA level. As ABP is a major carrier protein delivering high concentrations of testosterone to the epididymis [61], it is also likely that the epididymis, an androgen dependent tissue, may also be compromised in FORKO mice [30, 38]. Thus aside from the apparent effect on water balance in Sertoli cells, FSH-R signaling also appears to be a main player in regulating ABP production.

ACKNOWLEDGEMENTS

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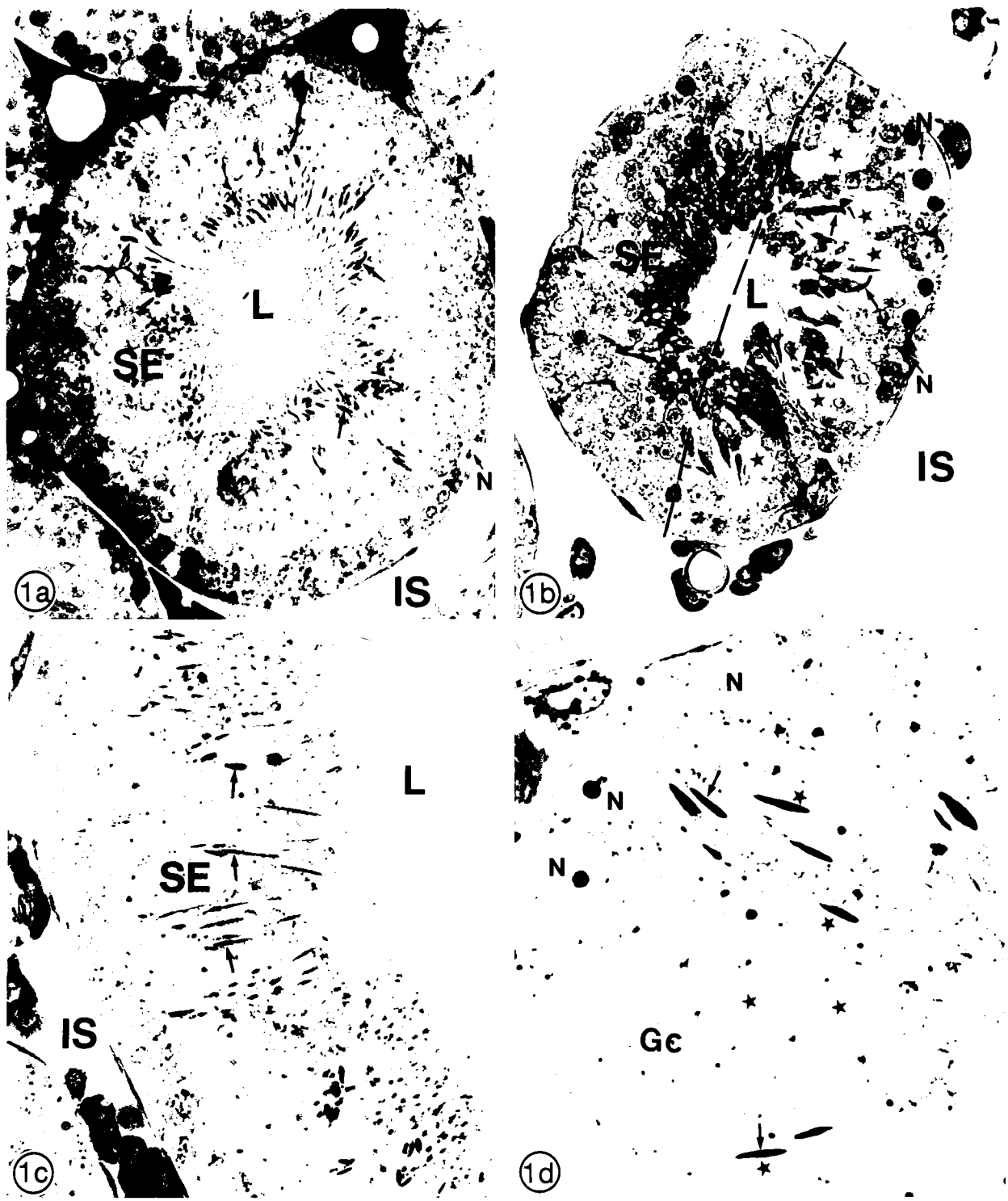
Figs. 1a-d: Light micrographs of seminiferous tubules of the testis of wild type (a, c) and FORKO (b, d) mice at 3 months of age. Note reduction in size of the tubules of FORKO mice (b) as compared to their wild type counterparts (a). In (a) and (c), the seminiferous epithelium (SE) shows a close association of Sertoli cells with germ cells. The heads of the elongating spermatids (arrows) are tightly apposed to the enveloping Sertoli cells in wild type mice (a, c). In contrast, in (b) and (d), large empty spaces appear in the epithelium (stars), which tend on the whole to be localized to half the circumference of the affected tubules. Note that the dilated spaces (stars) in the mid region of the epithelium surround the heads of elongating spermatids (arrows). Gc, germ cells; N, nucleus of Sertoli cell; IS, interstitial space; L, lumen. (a,b) 250X; (c) 390X; (d) 1000X.


L
SE
N
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IS
1a
SE
L
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N
IS
1b
L
SE
IS
1c
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N
Gc
1d

Figs. 2a-b: Low (a) and high (b) power light micrographs of seminiferous tubules of FORKO mice at 6 months of age. In (a) and (b), the highly dilated translucent spaces (stars) are situated basally in the epithelium, with some enveloping the heads of elongating spermatids (thin arrows). In (a) and (b), some spaces (thick arrows) are confluent with areas containing the Sertoli nucleus (N). The extensive nature of the dilated spaces gives rise to anastomotic cords (arrowheads) of spherical germ cells (Gc), which themselves do not appear to be dilated. Despite the extensive epithelial dilatations, there is no suggestion of pyknosis in the nuclei of Sertoli cells or of apoptotic cells. In the dilated spaces, numerous membranous profiles of varying sizes and shapes (curved arrows) are evident, as well as small particulate and granular material (square). (a) 390X; (b) 1000X.

Figs. 3a, b: Light micrographs of seminiferous tubules of 3-month old wild type (a) and FORKO (b) mice immunostained with anti-prosaposin antibody. Note reduction in the profile area of the tubules between wild type and FORKO mice. Sertoli cells (arrows) are highly reactive and extend from the base of the seminiferous epithelium (SE) to the lumen (L) in both (a) and (b); germ cells are unreactive. (a,b) 390X.

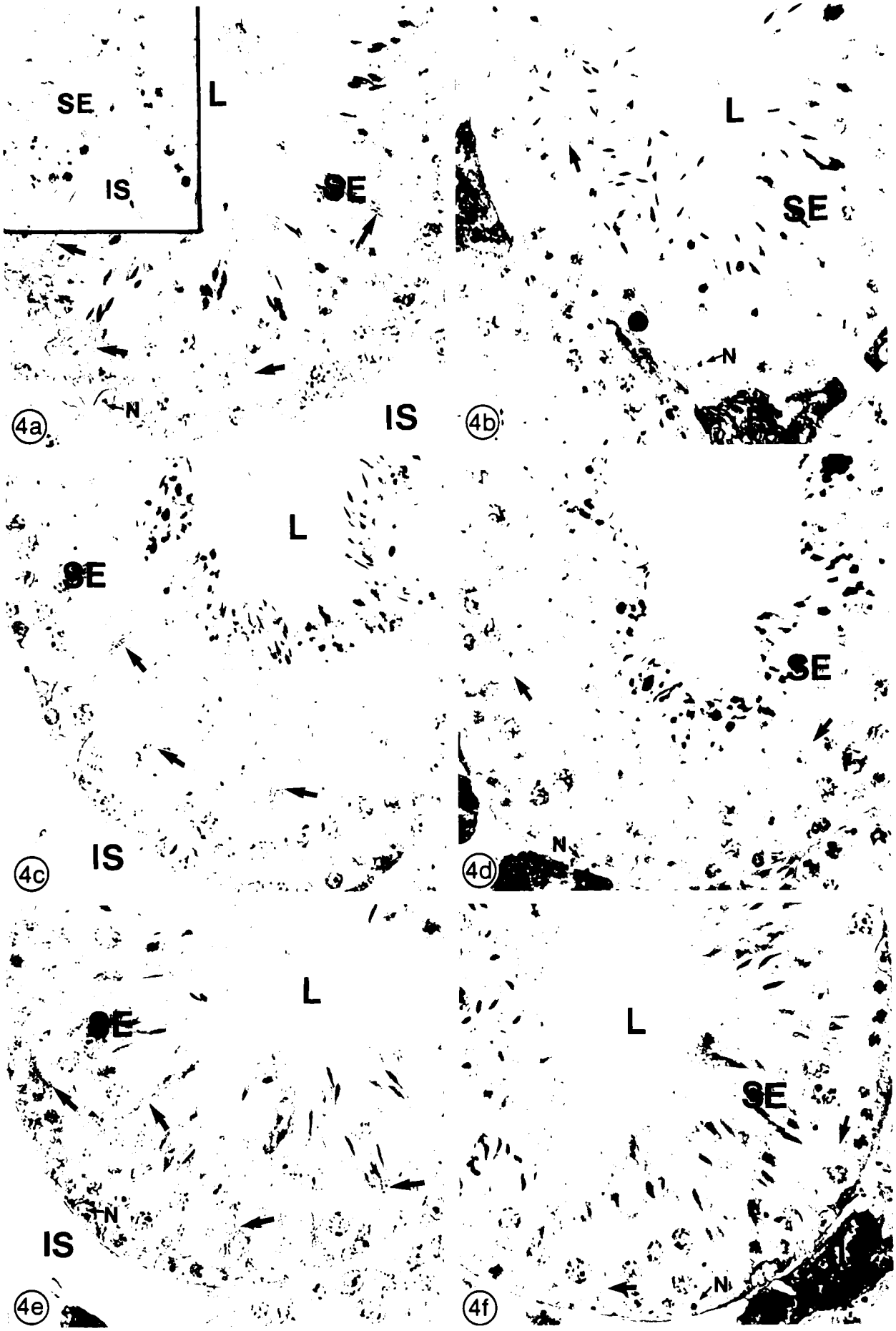
2a

2b

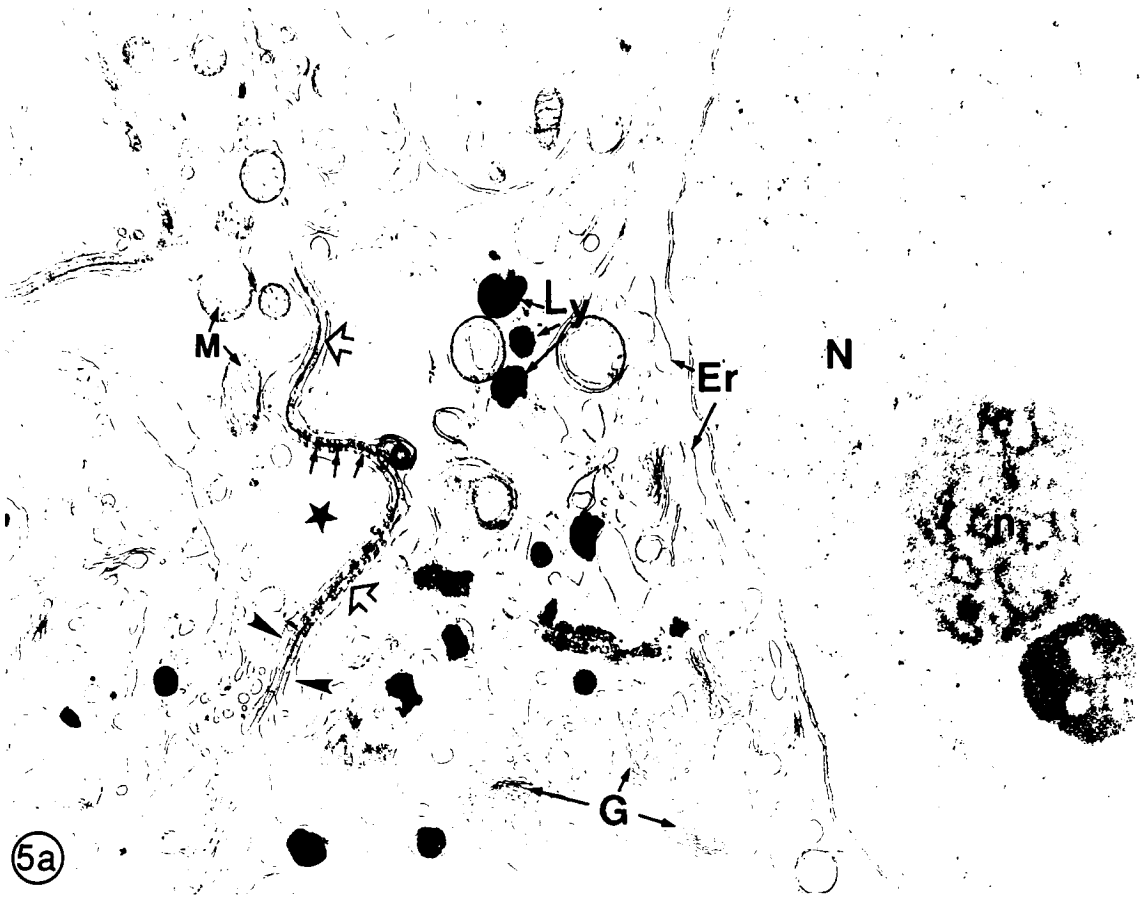
3a

3b

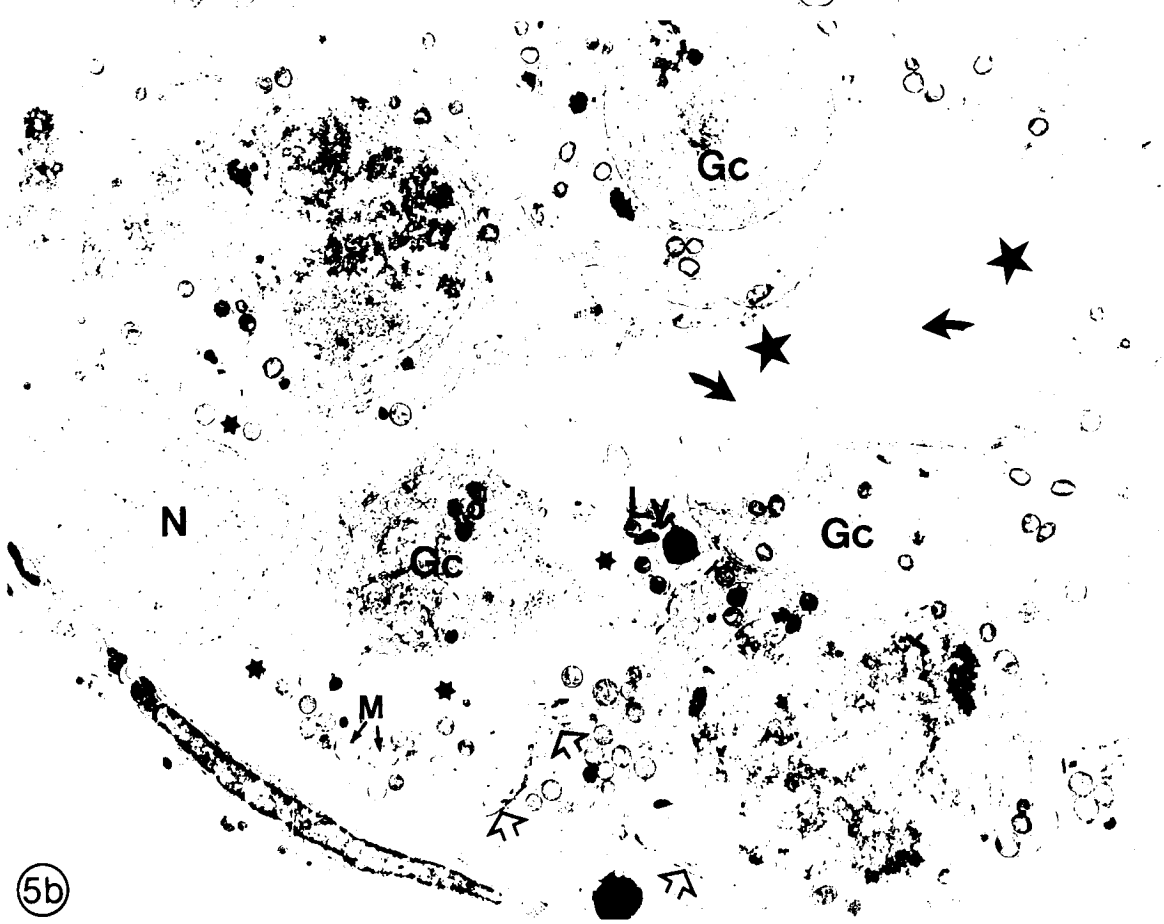
Figs. 4a-f: Light micrographs of seminiferous tubules of wild type (a, c, e) and FORKO (b, d, f) mice at 3 months of age immunostained with an anti-ABP antibody. In wild type mice at early (a, stage II-III), mid (c, stage VII) and late (e, stage XII) stages of the cycle, a reaction is evident in Sertoli cells, either basally or as distinct bands radiating across the width of the epithelium (arrows). This is in contrast to the weak staining or absence of a reaction over Sertoli cells of FORKO mice (arrows), seen at stages I (b), VII (d) and XII (f). The reaction over Leydig cells in the interstitial space (IS) is nonspecific. SE, seminiferous epithelium; L, lumen; N, nucleus of Sertoli cell. (a-f) 390X; (inset) 250X.


SE
L
IS
N
4a
4b
4c
4d
4e
4f

Figs. 5a, b: Electron micrographs of the base of the seminiferous epithelium of wild type (a) and FORKO mice at 3 months of age. In (a), organelles in the Sertoli cell cytoplasm, such as Golgi apparatus (G), lysosomes (Ly), mitochondria (M) and endoplasmic reticulum (ER), are embedded in a finely flocculent ground substance (star). The Sertoli-Sertoli blood testis barrier (open arrows) is intact and composed of bundles of filaments (small arrows) overlaid by flattened cisternae of endoplasmic reticulum (arrowheads). The Sertoli cell nucleus (N) is pale stained and shows a prominent nucleolus (n) and closely associated satellite body (s) in this plane of section. In (b), the Sertoli cell cytoplasm is dilated in the mid area of the seminiferous epithelium (stars), where large membranous whorls are evident (curved arrows). Nearer to the base of the epithelium, the Sertoli cytoplasm shows small territories enclosing spherical mitochondria (M), lysosomes (Ly) and a pale stained nucleus (N) all embedded in a finely flocculent ground substance (asterisks). The large dilated Sertoli cytoplasm surrounds spherical germ cells (Gc) that do not show any structural abnormalities. a: 14,000X; b: 6,600X.



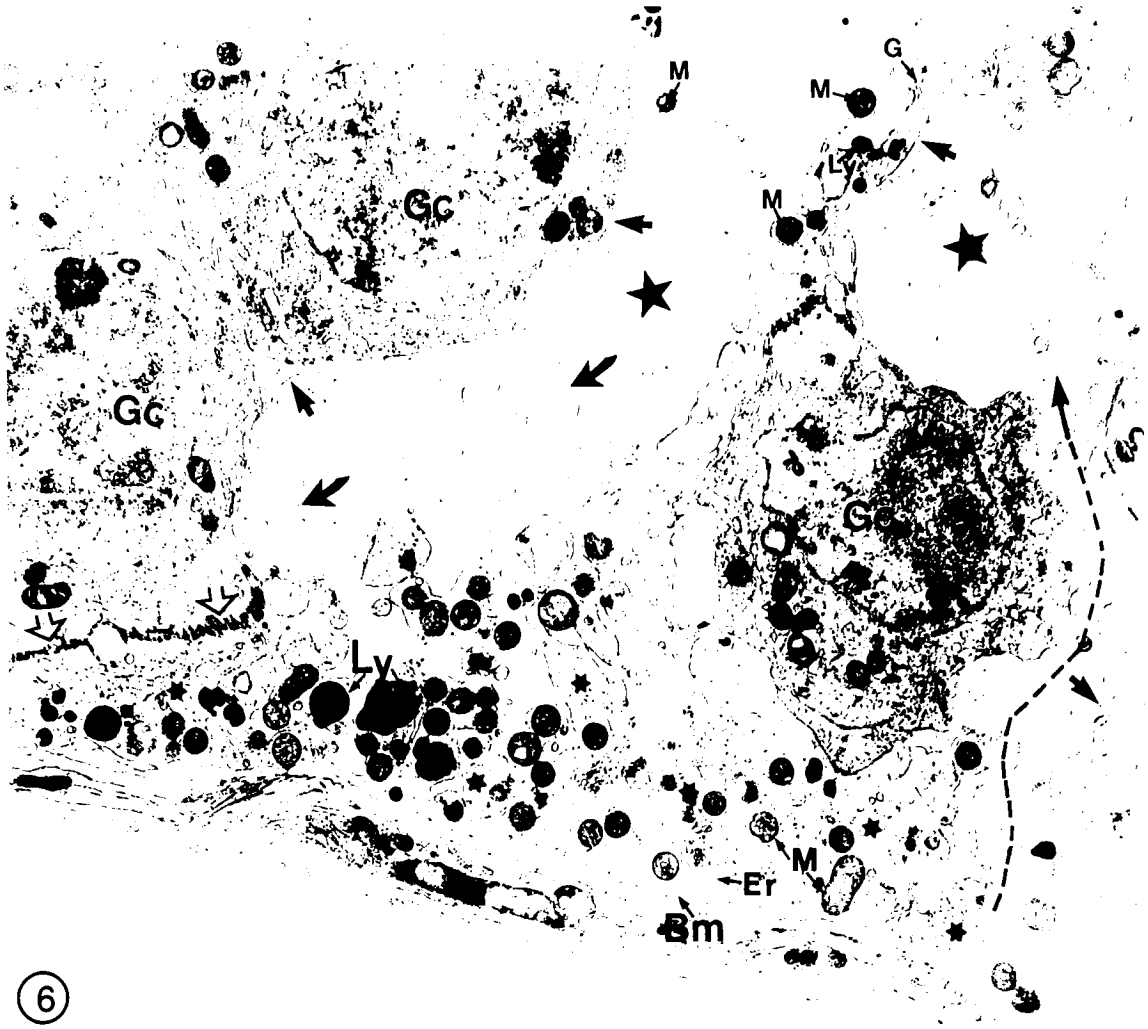
5a



5b

Fig. 6: Electron micrograph of the base and mid region of the seminiferous epithelium of a 6-month-old FORKO mouse. Areas of Sertoli cell cytoplasm (asterisks) located basally are in contact with the basement membrane (Bm) and contain mitochondria (M), lysosomes (Ly) and cisternae of endoplasmic reticulum (ER), embedded in a finely flocculent ground substance. In the mid region of the epithelium, a large dilation of Sertoli cell cytoplasm (star), containing large membranous profiles (curved arrows), is separated from the basal cytoplasm of Sertoli cell cytoplasm presumably due to the plane of section. In the upper right hand corner of this dilation, mitochondria (M), a Golgi apparatus (G) and lysosomes (Ly) are evident, with two mitochondria (M) appearing to be floating freely in the interior of this dilation. Another large dilated space (long dashed arrow) extending towards the lumen appears to be confluent with the basal area of Sertoli cell cytoplasm containing intact organelles and ground substance. As both dilated spaces contain organelles and are delimited by a plasma membrane (arrows), they are considered to be dilations of the Sertoli cell cytoplasm and not extensions of the lumen. The Sertoli-Sertoli blood testis barrier at the base of the epithelium is evident and appears intact (open arrows). Germ cells (Gc) do not show any apparent structural abnormalities of their cytoplasm, nucleus or organelles, and the organelles of the Sertoli cell themselves appear intact. a: 8,600X.

Figs. 7a, b: Heads of elongating spermatids enveloped by Sertoli cell processes in wild type (a) and FORKO (b) mice at 3-months of age. In (a), the cytoplasm of the Sertoli cell processes (S) enveloping the spermatid head contains mitochondria (M), lysosomes (Ly) and ER, embedded in a finely flocculent ground substance. The ectoplasmic specializations, comprised of thick bundles of filaments (small arrows) and overlying flattened ER cisternae (arrowheads), are closely applied to the spermatid head. In (b), the Sertoli cell process enveloping the spermatid head is greatly dilated and appears predominately organelle-free (star). The ectoplasmic specialization adhering to the spermatid head appears to be less extensive showing fewer bundles of filaments (small arrows) and at times dilated ER cisternae (arrowheads). However, the plasma membrane of the Sertoli cell process is closely apposed to the spermatid head (long arrows) and to that applied to the elongating germ cell (Gc) cytoplasm (short arrows). A, acrosomes of spermatid head; N, nucleus of spermatid heads. a: 16,100X; b: 16,800X.



6

Figs. 8a, b: Apical Sertoli cell processes enveloping the heads of elongating spermatids near the tubular lumen of 6-month old FORKO mice. In (a), an extremely dilated space (solid stars) envelops the heads of elongating spermatids and contains no apparent ground substance or organelles of identifiable nature, aside from several membranous profiles (curved arrows). Such spaces are not territories of the tubular lumen as they are consistently delimited by a plasma membrane (short arrows). Note that the latter is closely applied to the plasma membrane delimiting the neighboring elongating germ cell cytoplasm (Gc). The ectoplasmic specialization enveloping the spermatid head shows few bundles of filaments (small arrows), while the ER cisternae overlying them (arrowheads) are at times dilated (open stars). In (b), the grossly dilated Sertoli cell process (solid stars) enveloping the elongating spermatid head contains organelles such as mitochondria (M), lysosomes (Ly) and ER cisternae, embedded in what appears to be a highly diluted ground substance. The plasma membrane of the dilated Sertoli process (short arrows) closely approximates that of the intact germ cell cytoplasm. A, acrosome. a: 10,750X; b: 8,600X.

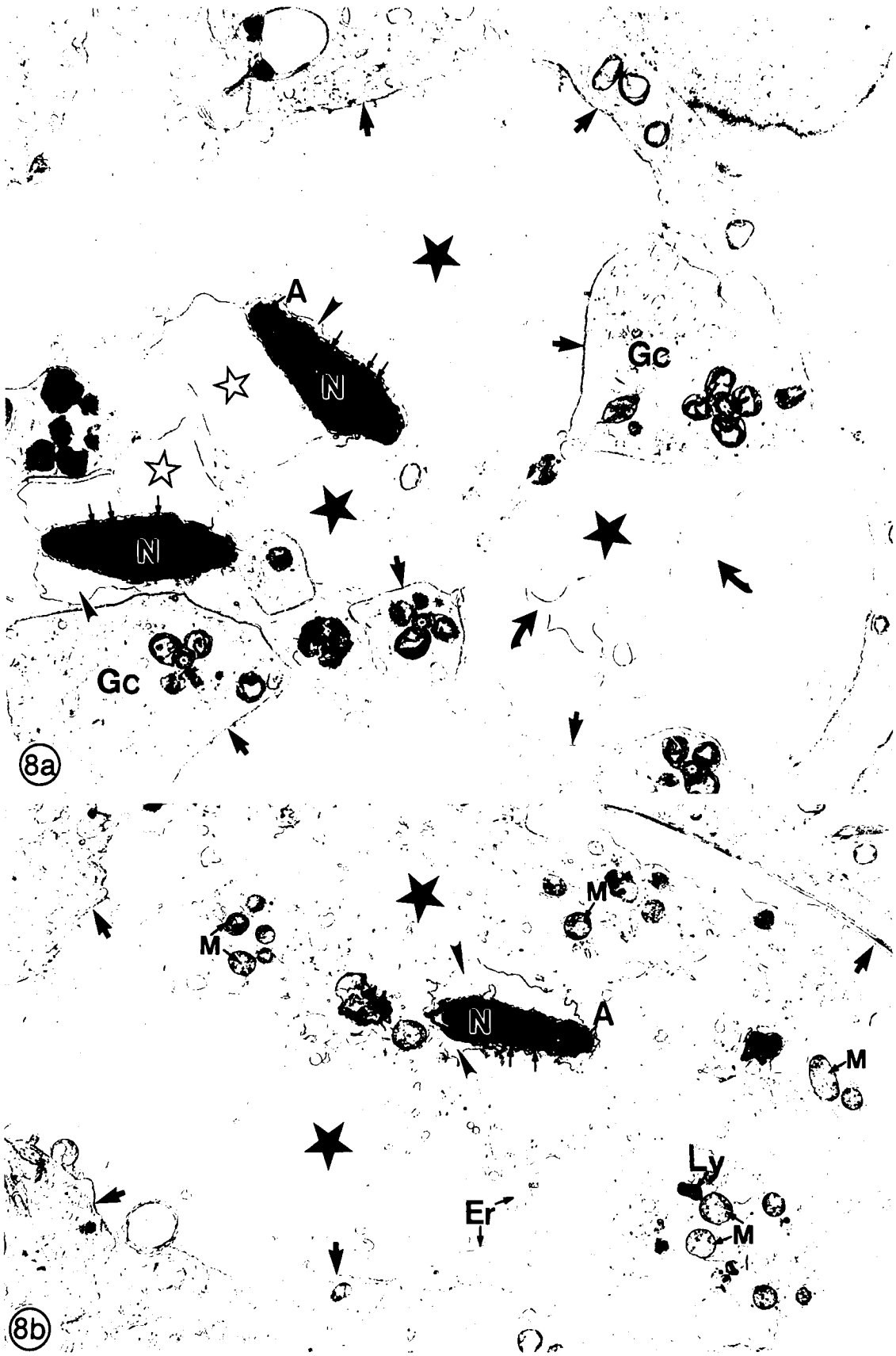

A
N
Gc
N
Gc
8a
M
M
N
A
M
Ly
Er
M
8b

Figure 9. Mean cross sectional profile area of seminiferous tubules in wild type (circles, solid line) and FORKO (squares, dashed line) mice at different ages. The mean profile area of tubules in FORKO mice is significantly less than ($p<0.05$) the mean profile area of tubules in wild type mice at all ages. The mean profile area of the tubules also increases markedly between 3 months and 12 months of age, and in the same ratio from 6 months to 12 months of age, in both groups.

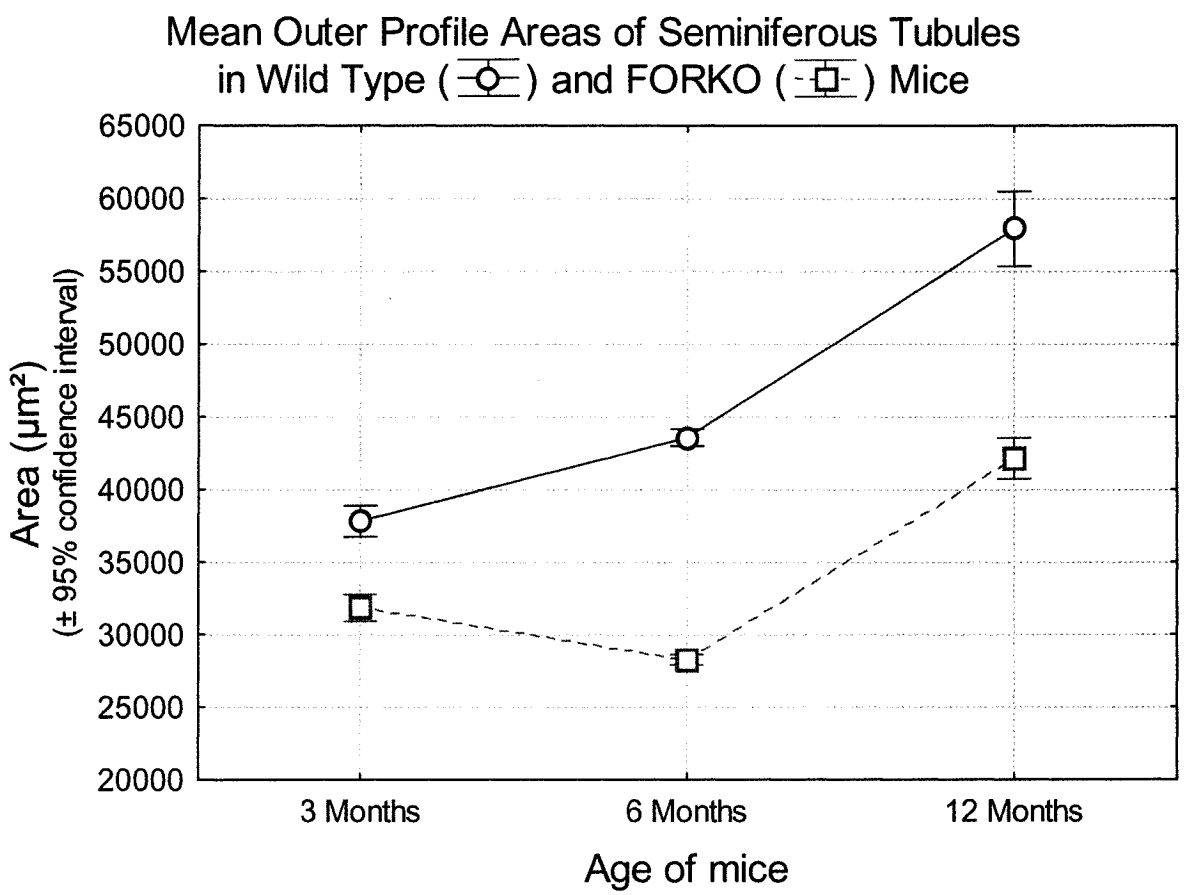

Mean Outer Profile Areas of Seminiferous Tubules
in Wild Type ( ) and FORKO ( ) Mice
65000
60000
55000
50000
45000
40000
35000
30000
25000
20000
Area (µm²)
(± 95% confidence interval)
3 Months
6 Months
12 Months
Age of mice

Figure 10: Western blot of anti-ABP peptide antibody on testicular extracts of 6-month-old wild type and FORKO mice (three lanes each from left to right) and corresponding density scan (graph at bottom). A band near 75 kDa, the molecular weight expected for ABP, is seen in all extracts. Average density scans from the three lanes for each group suggest that the amount of ABP protein present in 30 μg whole homogenate of FORKO mice is considerably less than 30 μg of whole homogenate from the wild type mice.

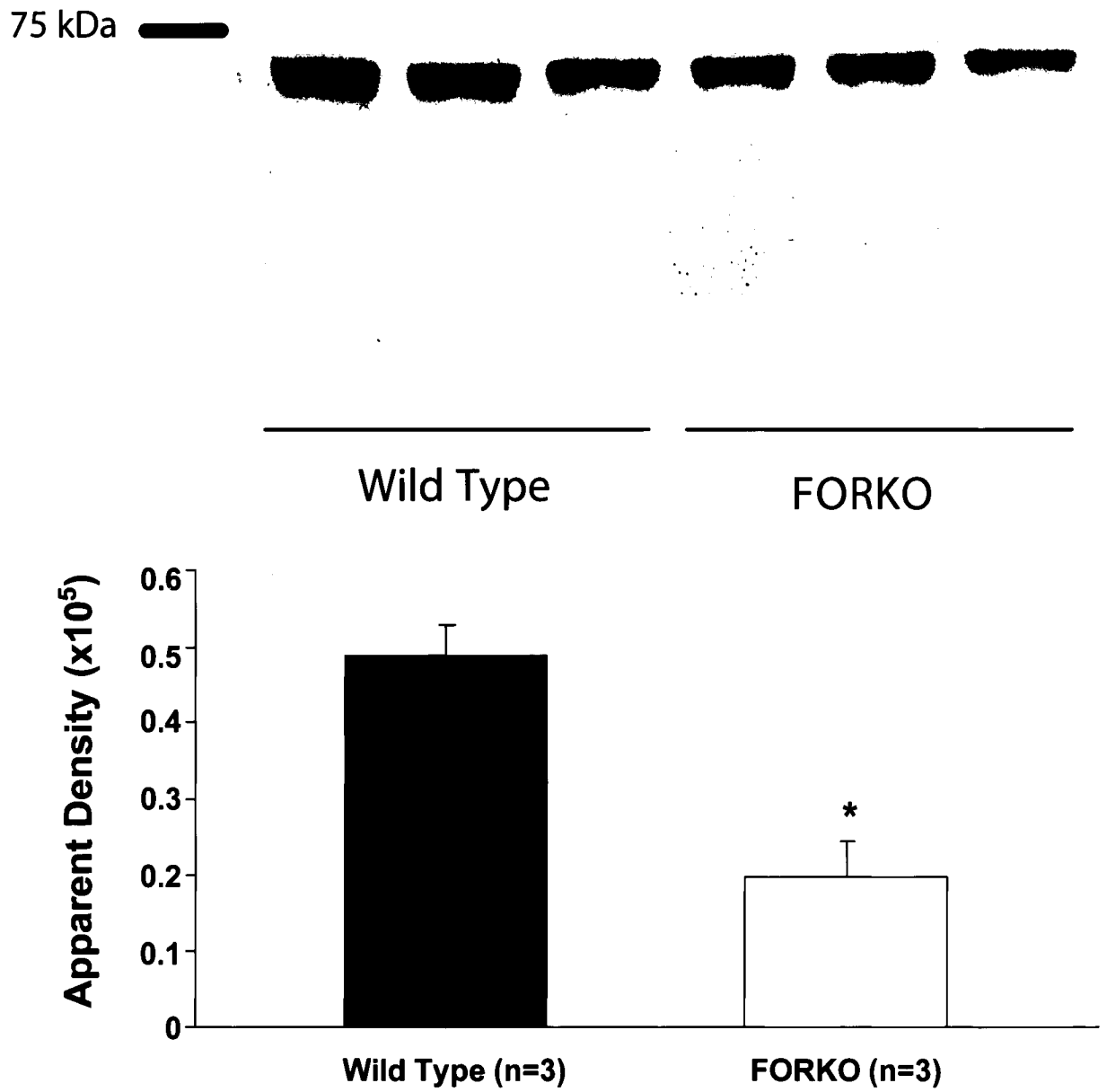
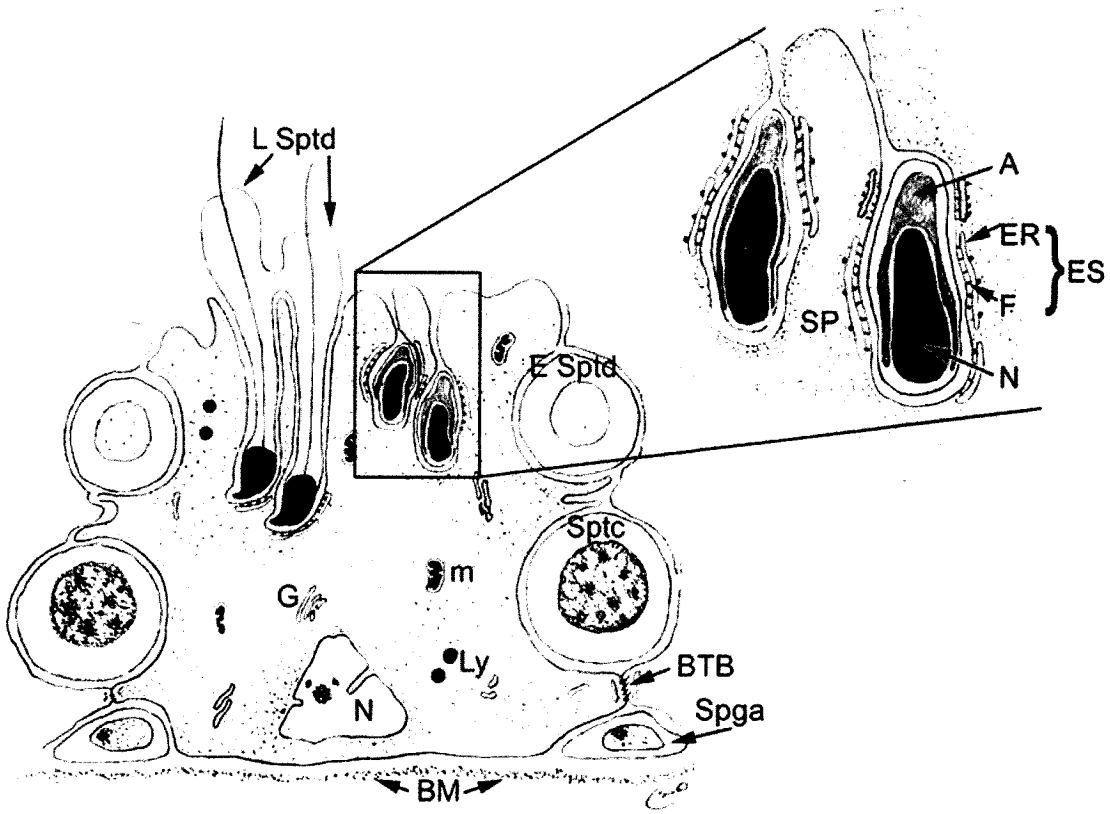

75 kDa
Wild Type
FORKO
Apparent Density (x10^5)
0.6
0.5
0.4
0.3
0.2
0.1
0
*
Wild Type (n=3)
FORKO (n=3)

Figure 11: Schematic drawing of a portion of the seminiferous epithelium of a wild type (upper) and FORKO (lower) mouse at 3 months of age as seen in the electron microscope. In wild type mice (upper panel), there is a close relationship between Sertoli cells and adjacent spherical and elongating germ cells. The cytoplasm of the Sertoli cell is compact containing numerous organelles embedded in a finely flocculent ground substance. The thin attenuated apical Sertoli cell processes (Sp) extend between the elongating spermatids seen near the lumen and contain various organelles. The ectoplasmic specializations (ES, also seen in inset) adhere to the heads of elongating spermatids and are composed of filaments (f) and flattened ER cisternae. The blood testis barrier (BTB) is intact and seen in the basal region of the epithelium. In FORKO mice (lower panel), the Sertoli cell cytoplasm of some cells is greatly distended and they show a lack of structural organization in tubules where abnormalities prevail. Also prominent are large dilated apical Sertoli cell processes, which encompass elongating spermatids. In affected Sertoli cells, there is an absence of the finely flocculent ground substance in the cytoplasm, and few organelles are evident amidst membranous whorls and profiles of varying shapes and sizes. Ectoplasmic specializations (ES, also seen in inset) while apparent continue to show filaments (f) and occasional dilated ER cisternae. In the basal region, the blood testis barrier (BTB) is present and apparently intact. G, Golgi apparatus; m, mitochondria; Ly, lysosomes; N, nucleus of Sertoli cell; Spct, spermatocyte; ESptd, early spermatid; LSptd, late or elongating spermatid; Spga, spermatogonia; BM, basement membrane.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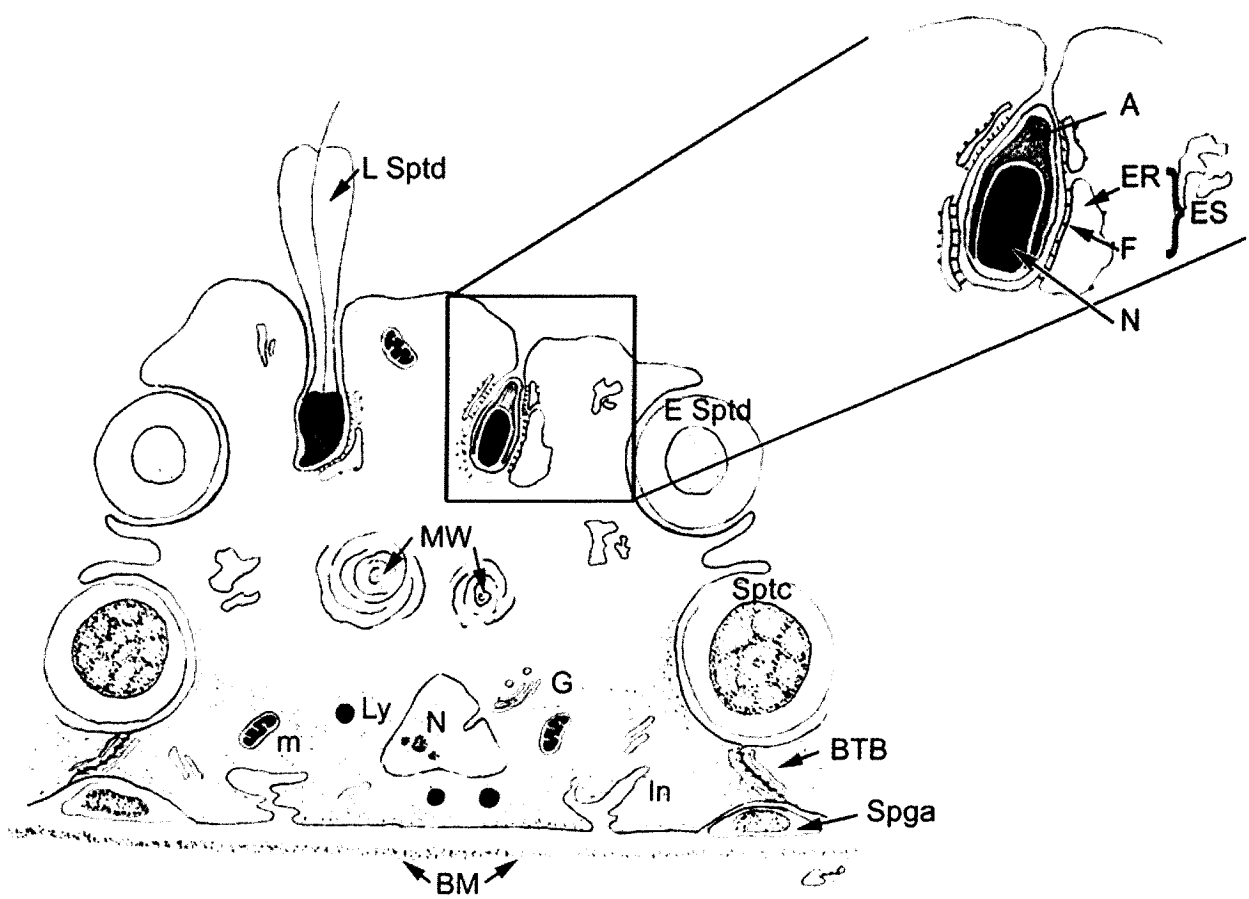
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Summary results for outer profile areas (μm^2)

Group	Age (no.)	Mean ± SD (num. obs.)
Wild type	3	37 822 ± 8815 (245)
	6	43 580 ± 4543 (235)
	12	57 918 ± 17 089 (172)
FORKO	3	31 863 ± 7244 (239)
	6	28 289 ± 2737 (220)
	12	42 179 ± 10 477 (218)

Two-factor univariate ANOVA tests of significance for outer profile areas

Effect*	SS	Degrees of freedom	MS	*F*	*P*
Treatment	5.4218	1	5.4218	652	0.0000
Age	5.3993	2	2.6996	325	0.0000
Treatment × age	0.6651	2	0.3325	40	0.0000

* Treatment (wild type of FORKO); age (3, 6, or 12 mo.).

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CHAPTER III

Effects of FSH receptor deletion on Epididymal tubules, Sperm numbers and Sperm Motility Parameters[1]

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ABSTRACT

Follicle stimulating hormone (FSH) interacts with its cognate receptor (R) on Sertoli cells within the testis and plays an important role in the maintenance of spermatogenesis. FSH-R knockout (FORKO) male mice exhibit reduced Sertoli cell numbers, serum testosterone, androgen binding protein (ABP) and reduced fertility levels. To assess the secondary response of testicular abnormalities of FORKO mice on the epididymis, we quantitatively examined differences in profile areas of epididymal tubules in the caput, corpus and cauda epididymides of 3- and 6-month old FORKO and wild type (WT) mice. Sperm counts and sperm motility parameters were also evaluated in WT and FORKO mice at 12-months of age using a Hamilton-Thorne IVOS automated semen analyzer. The quantitative data revealed that at 3- and 6-months of age, changes in epididymal tubule and lumen profile areas resulted in alterations of the lumen-to-epithelium ratio, such that the epithelium was decreased in size in FORKO mice in both the caput and corpus epididymidis compared to WT mice. Significantly lower sperm counts (47%) were detected in the cauda epididymidis of FORKO mice compared to their WT counterparts at 12-months of age. Furthermore, a total of 8 out of 14 sperm motility parameters, related primarily to velocity measurements, were significantly different in the FORKO mice. The greatest changes were observed in the percent of static sperm, which were significantly higher by 24% in FORKO mice. Principal component and factor analyses indicated there was a near perfect correlation between the increase in the percent static sperm and the decrease in the percent motile sperm. Taken together these data suggest an important role for the FSH receptor in maintaining adequate Sertoli cell numbers to sustain normal sperm numbers. In addition, effects on the epididymis observed in FORKO mice may be reflective of direct or indirect effects on the functions of these cells and their role in relation to sperm motility which is dramatically altered in FORKO mice.

INTRODUCTION

Follicle stimulating hormone (FSH), a pituitary glycoprotein hormone considered essential for mammalian fertility, acts on Sertoli cells [1-6]. The G_s-protein coupled, seven transmembrane FSH receptor (FSH-R) is localized to the basal surface of Sertoli cells, and upon stimulation activates the cAMP signaling pathway [1;7]. This pathway, as well as other signaling pathways, leads to a cascade of events that regulate various transcription factors ultimately giving rise to the numerous Sertoli cell functions [7]. Sertoli cells participate in a sustentacular role, providing nourishment, support and anchorage for the various generations of germ cells within the seminiferous epithelium [8]. The importance and requirement of Sertoli cells for spermatogenesis is unequivocal, demonstrated simply by the fact that germ cells do not differentiate properly in the absence of Sertoli cells [9].

In addition to its sustentacular role, Sertoli cells are responsible for the secretion of many proteins into the seminiferous tubule lumen, such as the glycoprotein androgen binding protein (ABP) [9-11]. ABP binds androgens with high affinity and transports them to the epididymis [11-13]. The precise role of ABP on spermatogenesis and maturation is not well known, but is associated with the regulation of steroid levels within the testis and epididymis [13]. Given its ability to bind androgens, and that endocytosis of ABP has been reported in both the germ cells of monkeys [14] and rats [15]; and through its role in the creation, or maintenance, of an epididymal specialized androgen microenvironment, ABP has a prominent role in sperm maturation [12]. The regulation of ABP by both FSH and testosterone has been demonstrated [16]; however, whether one or the other or both are required for complete function remains to be resolved [12]. It has also been demonstrated, through transgenic mice overexpressing ABP, that altered levels of ABP are associated with impaired fertility [12;17;18].

The development of various gene-knockout mouse models has opened new doors in the study of male reproduction. Mice deficient in the FSH-receptor (FORKO) provide a model to study alterations in testicular structure and function. Mutant mice at 2, 3 and 6 months have smaller testes and reduced fertility [1;19-21]. Serum FSH levels are elevated [19], while testosterone levels are greatly reduced [19;20;22] amid normal circulating levels of LH [22]. In our recent study, we noted that Sertoli cells of FORKO mice

exhibited a dilated cytoplasm suggestive of water retention, impaired ectoplasmic specializations and reduced levels of ABP production [1]. Fewer numbers of Sertoli cells in 21-day old FORKO mice [20] revealed a reduction in number of spermatozoa produced, as each Sertoli cell has a fixed capacity for supporting a given complement of germ cells.

Spermatozoa acquire full motility and fertilizing capacity during their transit through the epididymis [23-25]. The epithelial cells of the epididymis play important roles in relation to sperm maturation. In addition to the secretion of proteins and lipids into the lumen that interact with the sperm surface, these cells endocytose substances from the lumen and are involved in maintaining the correct ionic composition within the lumen [26-28]. The epididymis is well known to be an androgen dependent tissue [23;29]. Testosterone bound to ABP travels to the epididymal lumen, and in this way high epididymal luminal levels of testosterone, 10x higher than in the circulation, are maintained thereby creating an androgenic luminal microenvironment essential for sperm maturation [28]. In proximal regions, ABP bound to testosterone is endocytosed by principal cells [30], where it is converted to DHT, the prime regulator of epididymal functions, by the enzyme 5α-reductase [29].

In previous studies of FORKO mice, several key modifications, such as reduced testosterone levels, lower ABP production and reduction in fertility, suggest that the epididymis is also affected in FORKO mice [1;20;21]. The purpose of the present work was to examine morphologically and morphometrically, via quantitation of epididymal profile areas, alterations to the size of epididymal tubules of FORKO mice as compared to wild type mice of similar ages. In addition, sperm numbers as well as changes to various sperm motility parameters of cauda sperm were obtained and compared between FORKO and wild type mice. The data reveal that both the epididymides and sperm numbers are abnormal, and these changes may contribute to the reduced fertility levels found in FORKO mice.

MATERIALS AND METHODS

Animals

This investigation was approved by ethics committees of the Clinical Research Institute of Montreal and McGill University and was conducted according to accepted standards of animal experimentation. The FORKO mice were generated by homologous recombination as described by Dierich et al. [19]. This alteration eliminates the entire repertoire of FSH-R forms, producing complete loss of hormone signaling. Breeding F2 heterozygous males and females produced mice of all three genotypes in the SV129 background. The animals were maintained under controlled conditions of temperature (22°C) and light (12L: 12D), with food and water provided ad libitum. The primers and amplification conditions used for the multiplex polymerase chain reaction (PCR) to identify the phenotypes have been described in detail elsewhere [31]. In this manner, a single PCR performed on each sample allowed unambiguous identification of +/+, +/-, and -/- mice.

Routine Light Microscopic Methods

A total of 16 mice at 3-and 6-months of age (wild type, n=4; FORKO, n=4, for each age group) were used for detailed quantitative analyses of epididymal profile areas. The mice were anesthetized by an intraperitoneal injection with sodium pentobarbital (Somnitol, MTC Pharmaceuticals, Hamilton, ON, Canada). Prior to perfusion, a hemostat was placed over the testicular vessels entering the testis of each animal; both the testis and epididymides were removed and subsequently immersed in Bouin's fixative. After 10 minutes, each epididymis was cut along its long axis such that each region of the epididymis could be viewed. Thereafter, the epididymides were dehydrated in alcohol, embedded in paraffin and sectioned at 5 μm.

The remaining right epididymides of each animal was kept intact and immediately fixed by cardiac perfusion with 5% glutaraldehyde buffered in sodium cacodylate (0.1 M) containing 0.05% calcium chloride (pH 7.4). After 10 min of perfusion, the right epididymides were removed and cut into small 1-mm cubes and placed in the same fixative for an additional 2 h at 4°C. Thereafter, the tissues were thoroughly rinsed three times in 0.1 M sodium cacodylate buffer containing 0.2 M sucrose and left in this buffer overnight. The following day, the epididymides were post fixed in ferrocyanide-reduced

osmium tetroxide for 1 h at 4°C, dehydrated in a graded series of ethanol and propylene oxide, and embedded in Epon. Thick sections (0.5 μm) were cut with glass knives and stained with toluidine blue and observed by light microscopy.

Immunocytochemistry

The following affinity-purified polyclonal antibodies were used at 1: 100 dilution (v/v) for routine peroxidase immunostaining: 1) anti-prosaposin antibody (provided by Dr. C.R. Morales, McGill University, Montreal, Canada; purified and characterized as described previously [32]); 2) anti-clusterin antibody (provided by Dr. C.R. Morales, McGill University, Montreal, Canada; purified and characterized as described previously [33]); 3) anti-androgen receptor (AR) (Santa Cruz Biotechnologies); and 4) anti-aquaporin-9 (Aqp-9) (Alpha Diagnostics).

For the anti-prosaposin antibody, 5-μm-thick paraffin sections of Bouin's fixed testes were deparaffinized in Histoclear (Diamed Lab Supplies Inc., Mississauga, ON, Canada) and hydrated through a series of graded ethanol solutions. During hydration, residual picric acid was neutralized in 70% ethanol containing 1% lithium carbonate, and endogenous peroxidase activity was abolished by treating the sections with 70% ethanol containing 1% (v/v) H_2O_2. Once hydrated, the sections were washed in distilled water containing glycine to block free aldehyde groups. Non-specific binding sites were blocked using 10% goat serum for 30 min. The sections were then incubated at 37°C in a humidified chamber for 90 min with 100 μl of primary antibody diluted in Tris-buffered saline (TBS). Following washes in 0.1% Tween20 in TBS, the slides were incubated with secondary antibody (1:250; 100 μl) labeled with horseradish peroxidase for 30 min at 37°C in a humidified chamber. Reactions were revealed with diaminobenzidine tetrahydrochloride (DAB). Sections were counterstained with methylene blue, dehydrated in ethanol and Histoclear, and mounted with cover slips using Permount. TBS substitution for primary antibody was used as a negative control. No reactions were observed in these sections.

For the anti-AR, anti-AQP-9, and anti-clusterin antibody, paraffin sections were processed for immunostaining using the ImmunoCruz ABC Staining System (Santa Cruz Biotechnology Inc., Santa Cruz, CA). The sections were deparaffinized and hydrated as described above. Sections then were microwaved for antigen retrieval in citrate buffer

[34]. After boiling and cooling, the ImmunoCruz system was employed as per the suppliers' instructions. Sections were incubated with the various antibodies at a dilution of 1:100 overnight at 4°C. The sections were then washed in phosphate buffered saline (PBS) and incubated in biotinylated secondary antibody (1:250) for 30 min at room temperature. Sections were washed again in PBS and incubated in an avidin-biotin-horseradish peroxidase solution for 30 min at room temperature. Reactions were visualized by DAB. The sections were counterstained with methylene blue, dehydrated, and mounted with cover slips. For negative controls, normal blocking serum was substituted for primary antibody, no reactions were detected in the epithelium; examples of which can be seen in our previous FORKO work [1].

Quantitative analyses

Scaled digital images of 5-μm-thick paraffin sections of epididymal tubules from the caput, corpus and cauda epididymides from wild type and FORKO mice at 3- and 6-months of age were captured on a Zeiss Axioscop 2 equipped with an AxiocamMR camera. The peripheral outline of more-or-less spherical tubules was traced and the profile areas determined using measurement tools available in Version 3.1 of the Axiovision Imaging Software (Carl Zeiss Canada Ltd., Montreal, QC). Initial analyses of the data indicated that profile areas of the tubules were not distributed along a normal curve in either the wild type or the FORKO mice, and $\log_{10}$ transformations of raw data had to be done in order to obtain normal distributions. These transformations and subsequent Univariate Factorial ANOVA test and Post-hoc unequal N HSD *t*-tests were done using Version 6.1 of STATISTICA for Windows (Statsoft, Inc., Tulsa, OK); p values < 0.05 were considered significant.

Sperm Collection for Motility Analyses

Twelve mice, 6 FORKO and 6 wild type, at 12-months of age were used for this part of the study. The mice were weighed and then anesthetized using isofluorine. Both epididymides were removed, trimmed free of fat and placed in a 35mm Petri dish containing Hank's media (Gibco, Burlington, Ontario, Canada) supplemented with 0.5% bovine serum albumin (crystallized and lyophilized; Sigma-Aldrich Canada, Oakville, Ontario) pH 7.4 at 37°C. The cauda region of the left epididymidis of each mouse was dissected and placed in the freezer (-20°C) for subsequent sperm count analysis. The right

cauda epididymidis of each animal was clamped both proximally and distally, removed from the epididymis and transferred to a separate 35mm plastic Petri dish containing 3ml of fresh medium at 37°C. Using a surgical blade (Number 11; Fisher Scientific, Ottawa, Ontario), the cauda was then pierced at several sites along the tubule. The hydrostatic pressure of fluid within the lumen of the tubule caused the sperm to move rapidly into the medium. After approximately 30 seconds, once fluid turbidity was apparent, the tissue was removed and the Petri dish was placed in an incubator at 37°C in 5% CO_2 atmosphere for 5 minutes allowing the sperm to disperse. Subsequently, 100μL of the sperm suspension was aliquoted into a glass cannula and analyzed using Computer Assisted Sperm Analysis (CASA).

Assessment of Sperm Motility

Sperm were analyzed at 37°C using the Integrated Visual Optical System (IVOS; Hamilton Thorne Research Inc, Beverly, Mass). The operational settings were described by Slott et al (1993) and analyses were carried out for 30 image frames at a frequency of 60 frames/sec. CASA provided data on the percentage of motile sperm (Motile p), the percentage of static sperm (Static p), percent progressive sperm (Prog p) which reflects the number of moving sperm; and a multitude of kinetic parameters: Path Velocity (VAP) – the average velocity of the smoothed cell path (μm/s); Progressive Velocity (VSL) – the average velocity measured in a straight line from the beginning to the end of the track; Curvilinear Velocity (VCL) – the sum of the incremental distances moved in each frame along the sampled path divided by the time taken for the sperm to cover the track; Amplitude of Lateral Head Displacement (ALH) - the mean width of the head oscillation as the cell swims; Linearity (LIN) - the departure of the cell track from a straight line. Statistical analyses were done using Version 6.1 of Statistica for Windows (Statsoft Inc., Tulsa OK). Initial analyses of the data indicated there were outliers present in the dataset for several variables and these were removed using the Grubb's test. Normality tests further indicated that the spread of values for many variables did not follow a Gaussian distribution and therefore the nonparametric Mann-Whitney U test was used to define levels of significance in comparing results of wild type animals to FORKO animals. In all cases, p values < 0.05 were considered significant. In order to carry out principal component and factor analyses, raw data were converted to normal distributions using a

$\log_{10}$ transformation for ordinal variables (VAP, VSL, VCL, ALH, BCF) and an arcsin of the square root transformation for ratio variables (motile, progressive, str, lin, elong, rapid, medium, slow, static).

Sperm Counts

The left cauda epididymidis of each animal was dissected and frozen (-20°C) for subsequent analysis of sperm concentration. At the time of concentration determination the cauda was thawed and homogenized in a 50mL conical tube containing 20mL of distilled water. The "IDENT fluorescent dye" (Hamiton-Thorne Research) was resuspended in 100μL of distilled water in a small 1.5mL microcentrifuge tube. A 100μL aliquot of the homogenized sample was added to the resuspended IDENT solution and incubated at room temperature for 2 minutes. The solution was mixed and a 5μL aliquot was placed on a 20μm sperm analysis chamber (2X Cel; Hamilton-Thorne Research) slide and analyzed with the IVOS semen analyzer under ultraviolet light. As with the motility parameters, normality tests further indicated that the spread of values for the sperm count data did not follow a Gaussian distribution and therefore the nonparametric Mann-Whitney U test was used to define levels of significance in comparing results of wild type animals to FORKO animals. In all cases, p values < 0.05 were considered significant.

RESULTS

Structural and quantitative analyses of epididymal profile areas of wild type and FORKO mice

At 3- and 6-months of age, at the light microscopic level, epididymal tubules of FORKO males of the different epididymal regions revealed a normal looking epithelium and lumen containing sperm (Figs. 1, b, d, and f). The epithelium appeared to be composed of the normal complement of epithelial cells as seen in wild type mice (compare Figs. 1, a, c, e with 1, b, d, and f). Despite these features, it was apparent that the tubular diameters of various epididymal regions were smaller in size in FORKO males as compared to the wild type males. To verify these changes, quantitative analyses were performed on 3- and 6-month-old wild type and FORKO mice for the caput, corpus and cauda epididymidis. Both the outer profile area and luminal area of spherical epididymal tubules from each region were measured; the epithelial area was calculated as the difference between the two. Results of morphometric analyses revealed that at the 3-month age group the outer profile areas, luminal areas, and epithelial areas of tubules from the caput epididymides; and epithelial areas from the corpus epididymides were significantly decreased in FORKO mice as compared to the wild type (Table 1). On the other hand, the cauda epididymidis showed an unexpected increase in luminal areas in FORKO compared to the wild type mice; however the epithelial area remained unchanged (Table 1 and Fig. 2). At 6 months of age, profile, luminal and epithelial areas were significantly reduced in both the caput and corpus epididymides. In the cauda epididymidis however, only the profile and luminal areas were reduced and the epithelial area showed no significant change (Table 3 and Fig. 2). The cauda epididymidis at 3 months was the only region to show an increase in area, luminal area, and comparatively speaking was the only region that showed surprisingly different results (Fig. 2).

Immunocytochemical Analyses

Using an anti-androgen receptor antibody, it was noted that the nuclei of the epithelial cells were reactive in the different epididymal regions (Figs. 1, c, e, and f). In both wild type and FORKO mice, the nuclei showed similar staining patterns and intensities of reaction product and no observable differences were seen between the two groups of animals (compare Figs. 1, c, e with 1, d, f). In addition, some nuclei were

intensely reactive, others were only moderately, or weakly reactive; while some were unreactive. This staining pattern was reminiscent of the mosaic reaction seen for several secretory proteins and defined as a checkerboard pattern of staining [35;36]. The anti-clusterin antibody revealed no apparent change in staining patterns in the epithelial cells of both FORKO and WT mice (Figs. 3, a, and b), and as described previously [35] showed a checkerboard staining pattern.

Immunolocalizations of anti-prosaposin showed a uniform principal cell reaction throughout the epididymis of both wild type and FORKO mice comparable to that shown by others [37] (data not shown). Aquaporin-9 localization was seen apically on the microvilli of principal cells of the initial segment, caput and cauda epididymidis and within the efferent ducts of the wild type mice. The expression pattern within the FORKO epididymis was the same as in the wild type and comparable in pattern and intensity (Figs. 4, a-d). Control sections showed no staining over the epithelium in all reactions (data not shown).

Sperm Counts and Motility Analyses

The data on sperm counts obtained from the cauda epididymidis of FORKO versus wild type mice revealed a significant decrease of 47%, 11.94 M/ml versus 6.28 M/ml, between the two different groups of animals (Fig. 5).

For sperm motility parameters, 14 different measurements were calculated via CASA. When analyzed statistically a total of 8 out of 14 sperm motility parameters, related primarily to velocity measurements, were different in the FORKO mice. While not dramatic, these differences were significant. These included VSL, VAP, VCL, ALH, LIN, Prog p, Motile p, Static p. The greatest changes were observed in the percent of static sperm, which were significantly higher by close to 25% in FORKO mice. The remaining parameters all showed a significant decrease in the FORKO mice (Table 3).

Principal component and factor analyses, compares variables and determines the degree of correlation between them, was used to demonstrate the relationship between the various parameters measured with CASA (Fig. 6). There was a near perfect correlation between the increase in the percent static sperm (Static P) and the decrease in the percent sperm motility (Motile P). In addition the percentage of slow moving sperm (Slow P) was inversely correlated with decreases in the velocity parameters VSL, VCL and VAP.

DISCUSSION

The epididymis of FORKO males at 3- and 6-months of age is histologically abnormal; differing from earlier observations at 2 months of age where it appeared to be normal [21]. Upon close examination of FORKO tubules of 3-month old mice, a significant decrease in epithelial area was noted in both the caput and corpus epididymidis, while a surprising increase was revealed in the luminal area of the cauda epididymidis, but no change in epithelial area. On the other hand, in 6-month old mice, all morphometric parameters within all epididymal regions were significantly decreased in FORKO mice except for epithelial area in the cauda epididymidis which was unchanged. Earlier work by Krishnamurthy et al. [21] demonstrated that the epididymis in 3-month old FORKO males is reduced in weight by 30% compared to wild type mice, which correlates with the present data, supporting the notion that the epididymis is adversely affected in FORKO mice. It is therefore plausible to hypothesize that such changes in epididymal structure may manifest themselves in changes to sperm motility and fertility parameters.

It has previously been reported that significant changes to spermatozoa, namely acquisition of motility and fertilizing capability occur within the caput and corpus epididymidis [38-41]. Principal cells, the major cell type of the epithelium, as well as other cell types, narrow, clear and basal cells control the composition of the luminal environment through secretions and endocytosis of various proteins, which by the distal corpus region allow sperm to mature. In the present study, FORKO mice exhibit a reduced fertility and a significant reduction in motility parameters as evidenced by CASA. It is plausible, therefore, to attribute such a phenotype to decreased functionality of the epithelial compartment of the caput and corpus epididymidis which is significantly reduced in FORKO mice.

The observed reduction in outer profile, luminal and epithelial areas of tubules of most epididymal regions at 3- and 6-months of age is thought to be due to indirect effects of FSH. However, whether or not the FSH receptor is present in the epididymis is still unknown. Nevertheless, it has been shown previously that serum testosterone levels in adult FORKO males are reduced by approximately 66% [22] amidst normal circulating levels of LH. Given the synergistic relationship between Sertoli and Leydig cells and

given that the former are reduced in number and altered in both structure and function in FORKO mice [1], it is suggested that the direct effects of the lack of FSHR signaling on the Sertoli cell affects Leydig cell activity and subsequent testosterone production.

It is well recognized that the epididymis is an androgen dependent organ, and in the absence of androgens, a dramatic decrease occurs in the size of the tubules and the epithelium [42;43]. Thus our data on decreased morphometric parameters of epididymal tubules correlates well with decreased serum testosterone levels of FORKO mice. Additionally, androgen binding protein (ABP), synthesized by Sertoli cells, is decreased in FORKO males by 60% of controls [1]. It would follow that a reduction in serum testosterone levels along with reduced ABP production would result in decreased circulating and luminal concentrations of testosterone in the epididymis, as it is proposed that ABP maintains high concentrations of testosterone in the epididymal lumen [28]. Together this could account for the decreased profile, luminal and epididymal epithelial areas observed in most epididymal regions.

The cauda epidiymidis was the only region, at both 3- and 6-months of age, which reflected no change in epithelial area. It has been previously reported [28] that luminal 5α-dihydrotestosterone (DHT), the active metabolite of testosterone, contributes significantly to maintaining optimal epithelial function, and that concentrations of DHT are reduced in the cauda as compared to both caput and corpus epididymidis. The present data illustrate the difference in dependence on androgens amongst the different epididymal regions. It would follow then, that the reduced levels of testosterone and ABP observed in FORKO mice [1;22] would have its most profound effects on the caput and corpus region; leaving the cauda epithelial compartment least affected. It is interesting, however, that of all the epididymal regions examined at both 3- and 6-months of age, the cauda epididymidis was the only region where an increase in luminal area was noted at 3-months and no changes in epithelial area suggesting that regulation of functions of epithelial cells of this region differ from those of the caput and corpus regions.

In FORKO males testosterone levels, both serum and intratesticular, begin to drop significantly only after day 70 postpartum [20]. However, a significant drop in epididymal weight was observed in FORKO mice from day 28 onwards [20]. As these changes occur in a background of normal circulating testosterone levels, it is plausible

that the delay or arrest in epididymal development is the consequence of a direct effect of absence of FSH receptor signaling suggesting that the FSH-receptor may be present in the epididymis; or that FSH regulates proteins present at early time points during development that become absent at later time points, as has been shown for several proteins [44]. That the changes to the epididymis persist into adulthood, may be the result of the subsequent continued reduction in both testosterone and ABP levels.

To assess if alterations of epididymal tubular size of FORKO mice was reflective of changes in epithelial functions, several major proteins present in the epididymis were evaluated by LM immunocytochemistry. One of the major proteins synthesized and secreted by principal cells in all epididymal regions is clusterin, also known as sulfated glycoprotein (SGP-2) or Apo-lipoprotein J [35;45]. In the present study, no change was noted in clusterin expression in the different epididymal regions between both wild type and FORKO mice. In the rat, it was shown that castration also had no effect on its expression and thus it was not regulated by testosterone [46]; thus the present data suggest that FSH directly or indirectly does not regulate clusterin. Similarly, prosaposin expression within the principal cells was comparable between genotypes indicating absence of a role for FSH in the regulation of this protein in the epididymis. Androgen receptor expression was also not dramatically changed between FORKO and wild type mice. This is of interest since female FORKO mice demonstrate an increase in androgen receptor expression [47]. Thus although the epididymis is compromised in FORKO mice, several key proteins show no changes. Further studies are needed to assess functional changes that may be correlated with changes to sperm motility, a function of the epididymal epithelial cells.

In the cauda epididymidis, an increase in the size of the luminal area was noted at 3-month old FORKO mice. One possibility for this observation could be the retention of water in the lumen of this region. Aquaporin-9 is expressed in principal cells of the epididymis of the rat [48;49]. To assess if AQP-9 is compromised in FORKO mice, LM immunocytochemistry was performed; however, comparable staining was seen in FORKO compared to wild type mice. Thus FSH does not appear to regulate AQP-9 expression. The data further illustrate that other factors, yet unknown, may be involved leading to the observed phenotype in the cauda at 3-months of age. At 6-months of age,

the lumen was decreased suggesting that compensatory pathways come into play to alleviate the situation, albeit to a level lower than in wild type mice. Thus while the epididymal size is unquestionably affected in FORKO mice, the specific proteins affected by the decreased epithelial size leading to the specific phenotype observed in the present study have yet to be defined.

Previous studies have demonstrated that FSH promotes spermatogenesis by maintaining an optimum number of Sertoli cells in the adult testis. As well, it is known that each Sertoli cell nurses only a finite number of germ cells [20]. This means that the number of Sertoli cells determines the maximum output of sperm from the testis [50]. In FORKO males, where there is a lack of FSH receptor signaling, Sertoli cell numbers at 21 days are reduced by 50% and the absolute number of homogenization resistant elongated spermatids was also significantly reduced [20]. Our present data indicate a 47% reduction in sperm counts in 12-month old FORKO mice, which correlates with previous data showing decreased litter size and increased time before pups are sired in FORKO mice [20], which may in part be attributed to reduced sperm counts.

To assess the effects of altered epididymal parameters of FORKO mice, we also measured various sperm motility parameters of cauda sperm of FORKO versus wild type mice at 12-months of age. Since significant changes were seen in tubules of all regions at 3- or 6-months of age, we examined sperm motility parameters at a later time point, i.e. 12-months of age. Although reduced motility was mentioned in an early study [21], the present data revealed that 8 out of 14 sperm motility parameters were altered significantly in FORKO mice as compared to wild type mice. The greatest changes were observed in the percent of static sperm, which were significantly higher by close to 25% in FORKO mice. Principal component and factor analyses indicated there was a near perfect correlation between the increase in the percent static sperm and the decrease in the percent sperm motility. In addition, the significant reduction in the various kinetic parameters (VAP, VSL, VCL) correlates strongly with the increase in percentage of slow sperm (Slow P). The loss of major sperm motility parameters correlates well with the noted reduction in size of the epididymal epithelium at 3- and 6-months and suggests that similar alterations occur for 12-month old mice leading to impaired epididymal function. In addition, poor sperm motility could be due to altered functions of epithelial cells in

relation to secretion and/or endocytosis of proteins, lipids, ions and water. Poor sperm motility within the FORKO could also be due to effects occurred in the testis such as retention of cytoplasmic droplets, kinked (bent) tails and larger head sizes indicative of incomplete nuclear condensation [21]. Some of these alterations may be indicative of the alterations noted in Sertoli cells and as a consequence of their functions in relation to germ cell development [1]. Thus poor sperm motility could in part be due to altered epididymal epithelial functions as well as deficits occurring during spermatogenesis.

Sperm motility characterizations by CASA have also been done under different conditions. In aging Brown Norway rats, major changes in the motility of cauda sperm from regressed testes arise as a consequence of age. Parameters affected included straightness, velocity and lateral head displacements [51]. In addition, CASA has been used to examine changes in sperm motility in rats treated with various toxicants [52-54]. It is interesting that the sperm motility parameters affected under a given condition varied across different experimental conditions, suggesting that epididymal functions are multifactorial resulting in different motility phenotypes.

In summary, adult FORKO mice exhibit decreased epididymal tubule sizes (outer profile, luminal and epithelial areas) in most regions at 3- and 6-months of age. These differences appear to affect sperm motility that could in part result in the observed reduced fertility levels seen in FORKO mice. In addition, as a result of reduced Sertoli cell numbers, germ cell numbers are lower resulting in a significant reduction in sperm counts in the cauda epididymidis. Taken together, the current findings suggest an important role for FSH receptor signaling in the development and proper functioning of the epididymis.

ACKNOWLEDGMENTS

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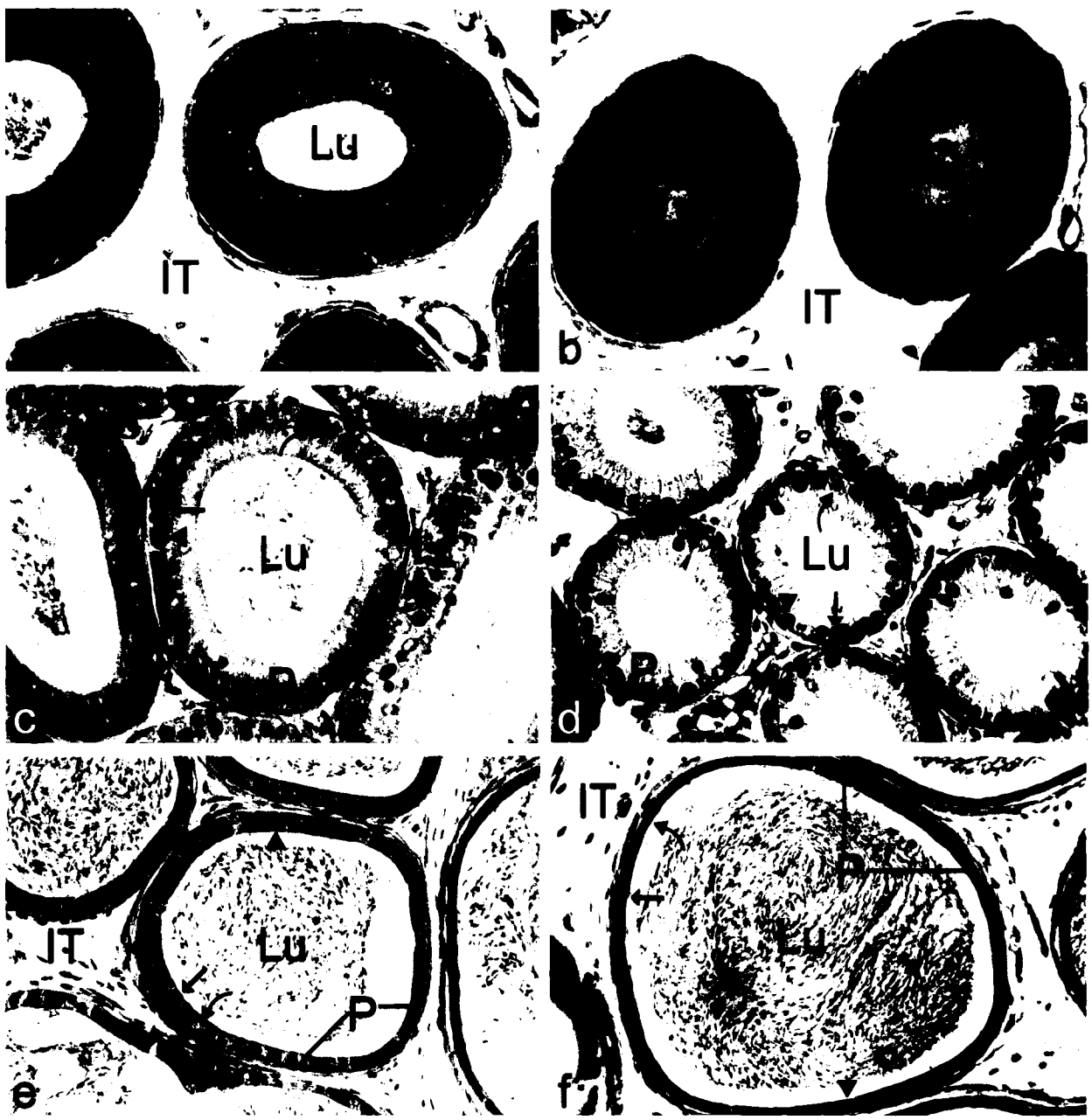
Figs. 1a-f: Light micrographs of epididymal tubules of wild type (a, c, e) and FORKO (b, d, f) mice at 3- (a-d) and 6- (e, f) months of age of the initial segment (a, b), caput (c, d) and cauda (e, f). Note the full complement of epithelial cells, composed mainly of principal cells (P), in both wild type and FORKO (compare a, c, e to b, d, f). Also note the presence of sperm within the lumen (Lu) of the tubules in both wild type and FORKO. Tissues were fixed either by glutaraldehyde (a-b) or Bouin's fixative (c-f). A reduction in tubular size is evident in knockout tubules (b, d,), as well as the epithelial compartment (b,d) compared to wild type (a, c,). Immunostaining with an anti-androgen receptor antibody (c-f) revealed a checkerboard staining pattern within the nuclei of principal cells (c-f) comparable between FORKO (d, f) and wild type (c, e). While some nuclei were intensely reactive (arrows), others were moderately reactive (arrowheads) or unreactive (curved arrows). IT, Intertubular space. (a-d) 390X; (e-f) 250X.


Lu
IT
b
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c
Lu
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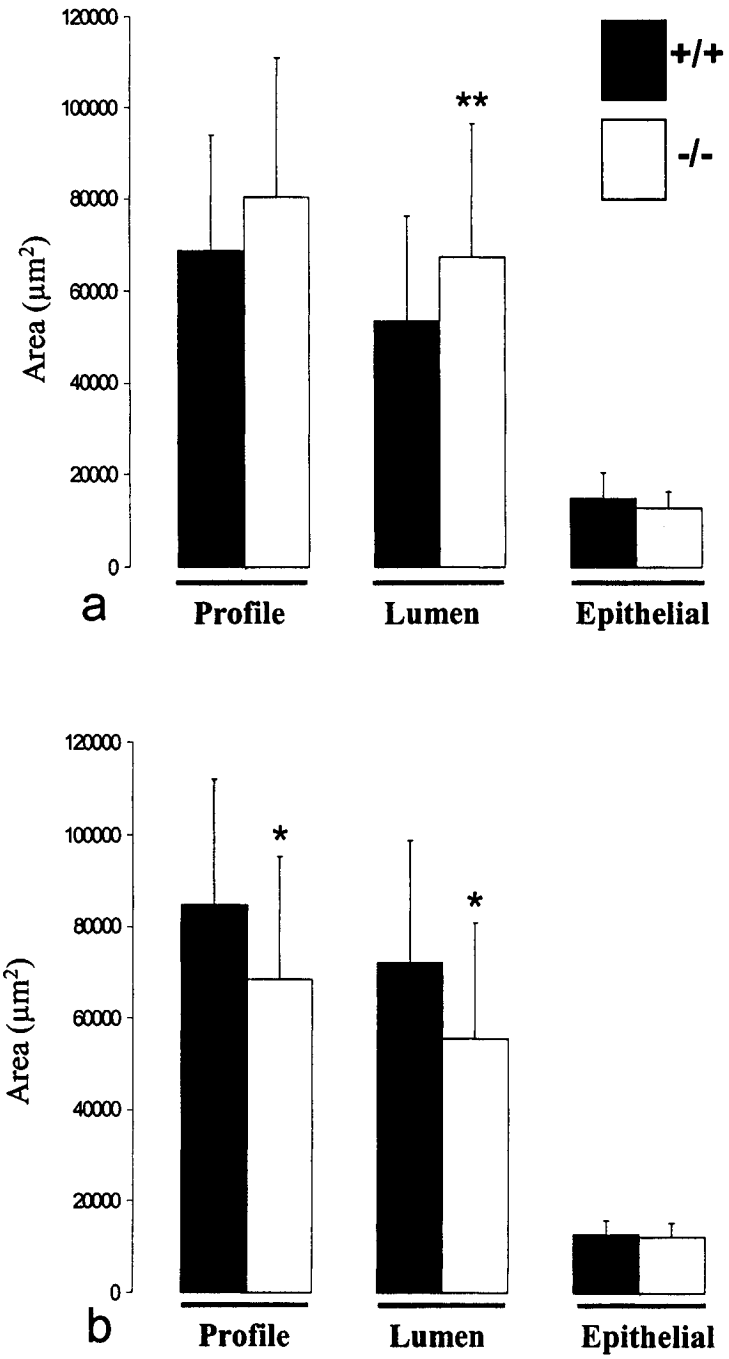
Lu
P
e
IT
Lu
P
f

Figs. 2a-b: Morphometric analysis of profile, luminal and epithelial areas of the cauda epididymidis of 3- (a) and 6- (b) month old FORKO and wild type mice. Note increase in luminal area in 3-month FORKO mice (a). Also note that epithelial area remains unchanged at both 3- and 6-months of age (a,b). The data are presented as mean ± SD, Mann-Whitney U Test, $*p<0.005$, $**p<0.05$.


120000
100000
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Area (µm²)
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120000
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Area (µm²)
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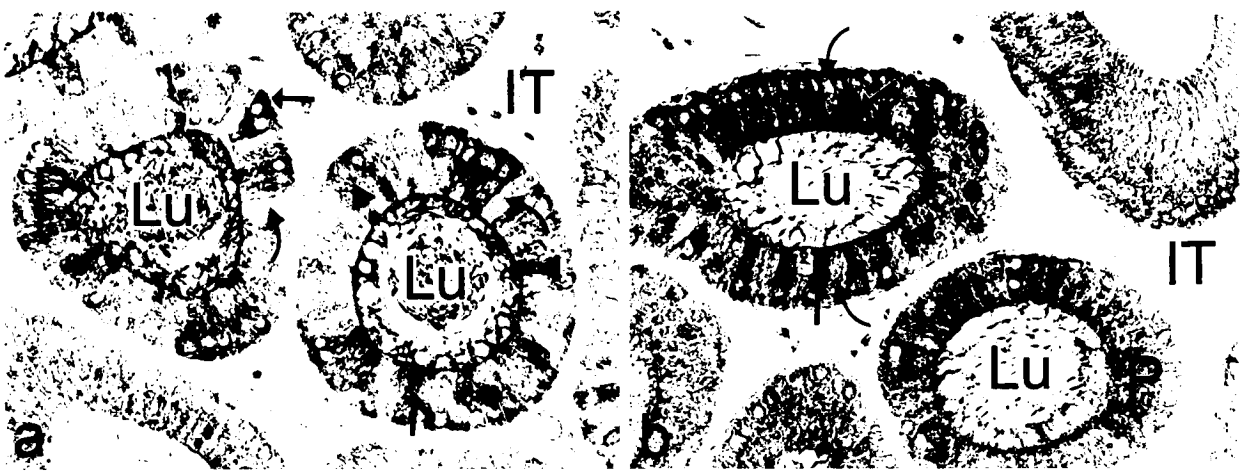
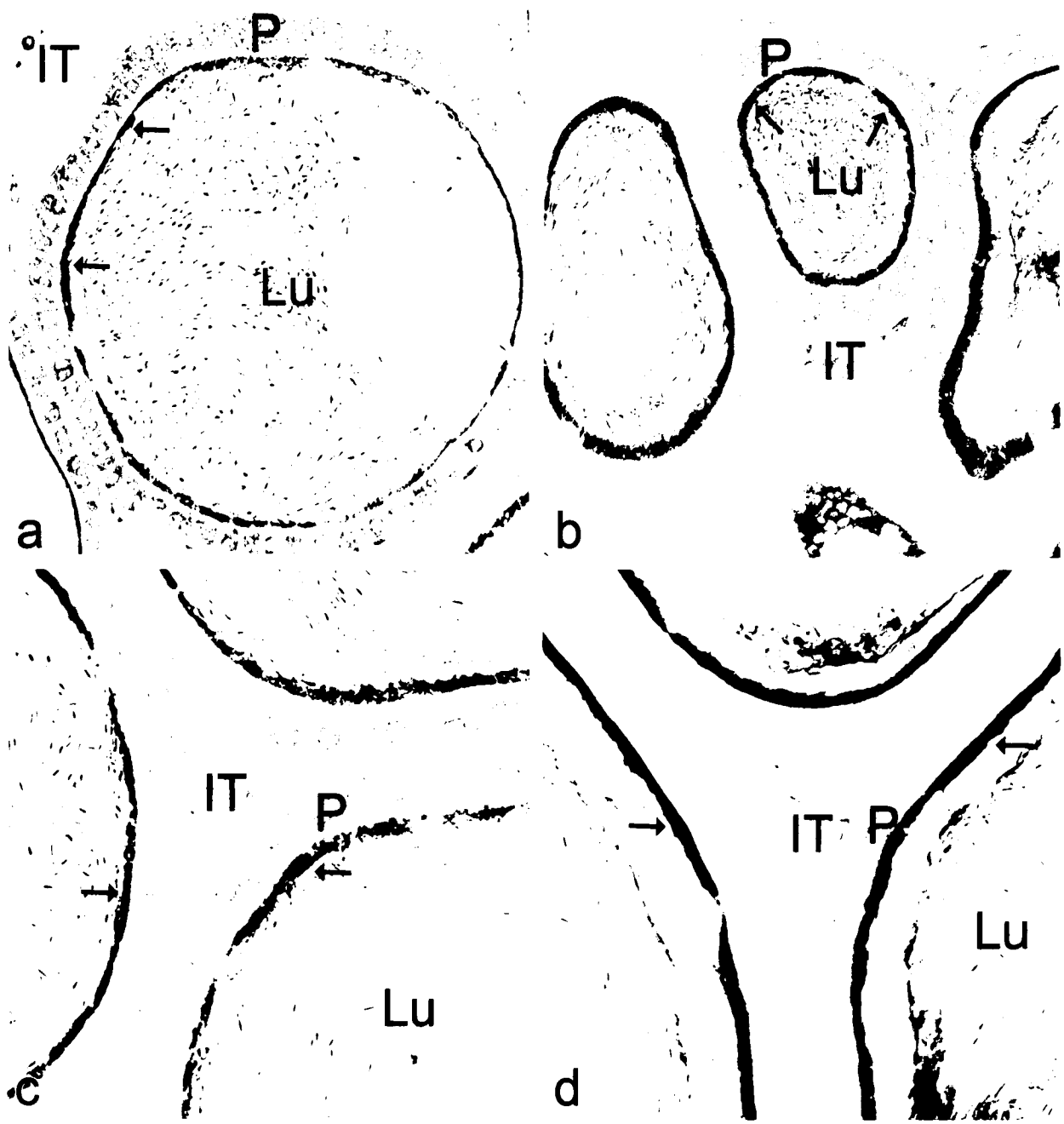
b
Profile
Lumen
Epithelial

Figs. 3a-b: Light micrographs of epididymal tubules of 6-month old wild type (a) and FORKO (b) mice immunostained with anti-clusterin antibody. Note the checkerboard pattern of staining for principal cells (P). Staining is comparable between both wild type (a) and FORKO (b). While some cells stain intensely (arrows), others are moderately reactive (arrowheads) or unreactive (curved arrows). Similar staining was observed in 3-month old FORKO and wild type tissue (data not shown). Lu, Lumen; IT, Intertubular space. (a) 250X; (b) 250X.


IT
Lu
Lu
Lu
Lu
IT
a
b

Figs. 4a-d: Light micrographs of cauda epididymidis of 6-(a-d) month old wild type (a, c) and FORKO (b, d) mice immunostained with anti-AQP-9 antibody. Note the intense reaction present over the microvilli (arrows) of principal cells (P). Staining is comparable between both FORKO (b, d) and wild type (a, c) mice. Similar staining was observed in 3-month old FORKO and wild type tissue (data not known). Lu, Lumen; IT, Intertubular space. (a, b) 250X; (c, d) 390X.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Lu
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P
Lu
d

Fig. 5: Mean sperm concentrations of 12-month old wild type and FORKO mice. The sperm concentration in the FORKO mice is significantly less, 47% ($p<0.0005$), compared to wild type. The data are presented as means ± 95% confidence intervals, Mann-Whitney U Test, $p<0.05$.

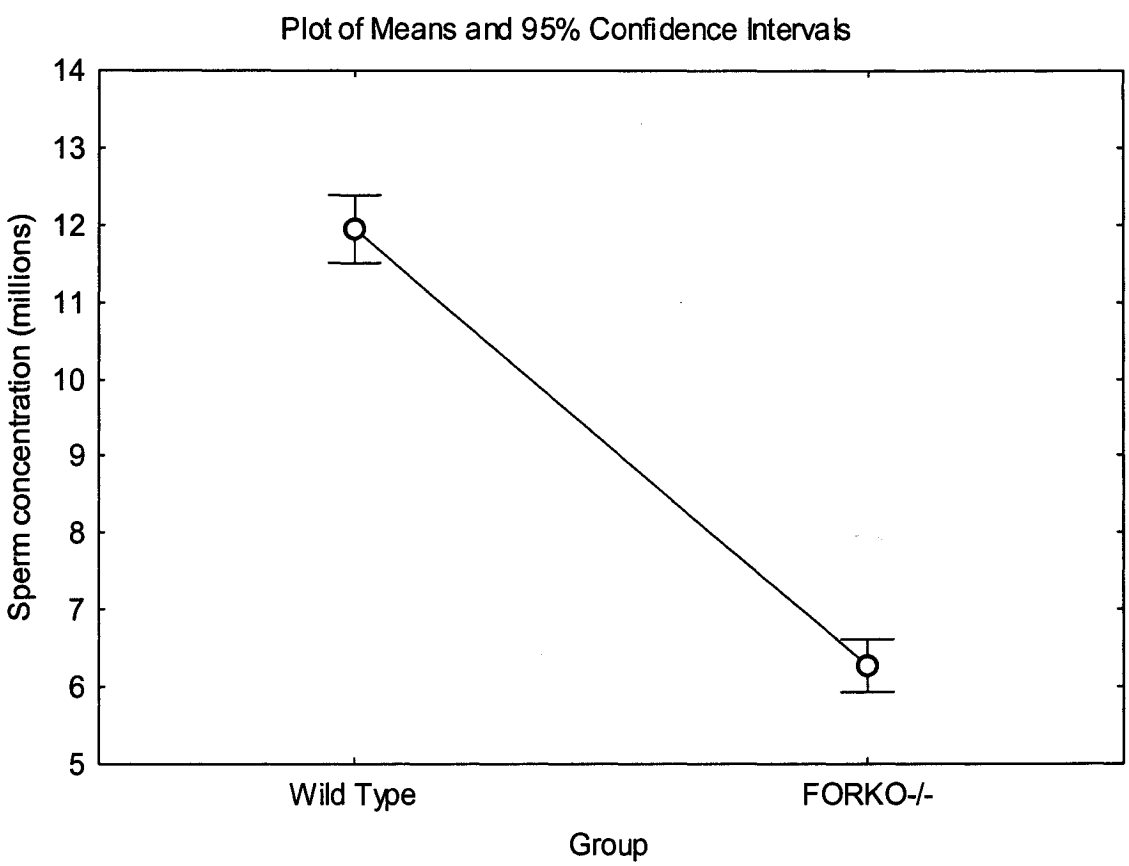
Plot of Means and 95% Confidence Intervals
Sperm concentration (millions)
14
13
12
11
10
9
8
7
6
5
Wild Type
FORKO-/-
Group

Fig. 6: Principal component and factor analysis on sperm motility parameters measured by CASA in 12-month old FORKO and wild type mice. Projected on the factor-plane, variables left of the centre point reflect decreases in those parameters, while those to the right are increases. Parameters reflecting from each other across the midline show high opposing correlations. Note the decrease in percent of motile sperm (Motile P), an active or significantly changed parameter, correlates precisely with the increase seen in percentage of static sperm (Static P). Similarly, note that the increase the percentage of slow moving sperm (Slow) correlates strongly with the significant decreases detected for the various sperm velocity parameters (VSL, VAP, and VCL). Other variables projected on the factor plane, Prog P, LIN and ALH, show reductions and associations to the northwest quadrant like Motile P but their cross correlations are unclear. Projections on the two factor planes plotted account for 73% of the total variation present in the data.

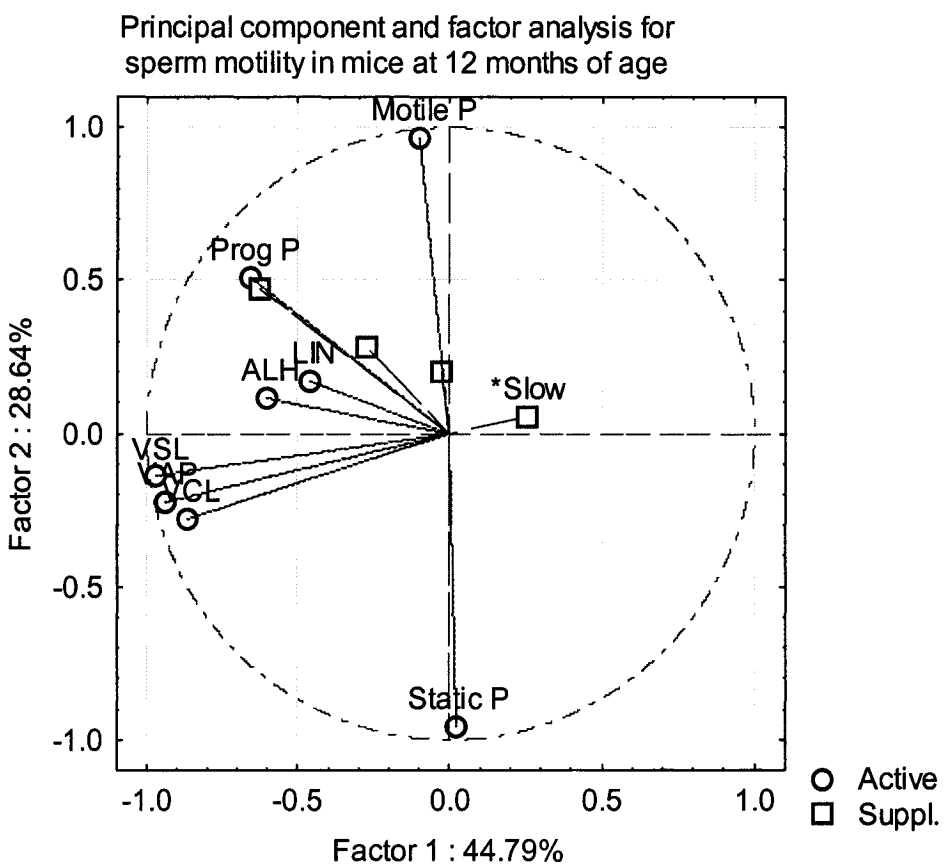
Principal component and factor analysis for
sperm motility in mice at 12 months of age
Motile P
Prog P
LIN
ALH
*Slow
VSL
VAP
VCL
Static P
1.0
0.5
0.0
-0.5
-1.0
-1.0
-0.5
0.0
0.5
1.0
Factor 2 : 28.64%
Factor 1 : 44.79%
Active
Suppl.

TABLE 1.Descriptive statistics of epididymal profile, luminal and epithelial areas of 3 month old mice.

Summary results for outer profile areas (μm^2)		
Group	Region	Mean ± SD (num. obs.)
Wild Type 3 month	Caput	13259 ± 4157 (112)
	Corpus	35966 ± 18716 (40)
	Cauda	68582 ± 25360 (42)
FORKO 3 month	Caput	10452 ± 3368 (178)[§]
	Corpus	28689 ± 12385 (92)
	Cauda	80363 ± 30502 (39)

Summary results for lumen areas (μm^2)		
Group	Region	Mean ± SD (num. obs.)
Wild Type 3 month	Caput	4674 ± 2040 (112)
	Corpus	19845 ± 14189 (40)
	Cauda	53511 ± 22729 (42)
FORKO 3 month	Caput	3404 ± 1712 (178)[§]
	Corpus	17578 ± 10806 (92)
	Cauda	67436 ± 28929 (39)[‡]

Summary results for epithelial areas (μm^2)		
Group	Region	Mean ± SD (num. obs.)
Wild Type 3 month	Caput	8585 ± 2621 (112)
	Corpus	16121 ± 6159 (40)
	Cauda	15072 ± 5579 (42)
FORKO 3 month	Caput	7048 ± 1996 (178)[§]
	Corpus	11111 ± 3030 (92)[§]
	Cauda	12927 ± 3593 (39)

Mann-Whitney U Test. [§] $p<0.0005$, [‡] $p<0.05$

TABLE 2.Descriptive statistics of epididymal profile, luminal af epithelial areas of 6 month old mice.

Summary results for outer profile areas (μm^2)		
Group	Region	Mean ± SD (num. obs.)
Wild Type 6 month	Caput	15352 ± 1782 (34)
	Corpus	34394 ± 2661 (21)
	Cauda	84826 ± 27201 (85)
FORKO 6 month	Caput	10934 ± 1228 (38)[§]
	Corpus	22361 ± 3463 (21)[§]
	Cauda	68341 ± 26734 (77)[§]

Summary results for lumen areas (μm^2)		
Group	Region	Mean ± SD (num. obs.)
Wild Type 6 month	Caput	4757 ± 1306 (34)
	Corpus	18259 ± 2635 (21)
	Cauda	72097 ± 26597 (85)
FORKO 6 month	Caput	2028 ± 550 (38)[§]
	Corpus	9831 ± 3090 (21)[§]
	Cauda	55575 ± 25096 (77)[§]

Summary results for epithelial areas (μm^2)		
Group	Region	Mean ± SD (num. obs.)
Wild Type 6 month	Caput	10595 ± 989 (34)
	Corpus	16134 ± 1467 (21)
	Cauda	12729 ± 3089 (85)
FORKO 6 month	Caput	8906 ± 951 (38)[§]
	Corpus	12530 ± 991 (21)[§]
	Cauda	12192 ± 3107 (77)

Mann-Whitney U Test. [§] $p<0.005$

Table 3. Descriptive Statistics of various motility parameters measured using CASA.

Summary Results of Sperm Motility Parameters determined by CASA			
Expressed as Mean ± SD (num. obs.)			
Parameter	Wild Type	FORKO	*p* value
Motile P	79 ± 10 (282)	75 ± 15 (352)	$p<0.05$
Static P	17 ± 10 (281)	21 ± 16 (352)	$p<0.05$
Prog P	31 ± 9 (280)	28 ± 10 (353)	$p<0.005$
VAP	116 ± 25 (282)	109 ± 25 (353)	$p<0.005$
VSL	92 ± 21 (282)	85 ± 22 (353)	$p<0.0005$
VCL	184 ± 34 (282)	177 ± 36 (353)	$p<0.05$
ALH	6 ± 1 (282)	5 ± 1 (351)	$p<0.00005$
LIN	50 ± 5 (282)	48 ± 7 (353)	$p<0.005$

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CHAPTER IV

Summary

FSH and the subsequent signaling through its cognate receptor have long been known to play an important role in reproductive development. It is through the selective knockout of the FSH-R gene, thereby eliminating the entire repertoire of all and any FSH-R isoforms that a greater understanding of the role of this receptor and its signaling pathway has been elucidated. Clinical manifestations of a similar genotype have recently surfaced and have provided even greater incentive for scientists to understand the specific role of FSH and its receptor.

While FSH-R deficiency does not appear to affect general health, male mice homozygous for the mutation are rendered sub-fertile. Female mice however, show a completely different phenotype. Namely that they are completely infertile and have also shown an increased incidence of ovarian tumogenesis, thus revealing that while this hormone may be conserved across species, its role varies extraordinarily between genders. Histological examination, at both the light (LM) and electron (EM) microscope level, of the reproductive tract revealed the presence of both structural and functional anomalies within the FORKO compared to its age matched wild type.

Within the testis, at both 3-and 6-months of age, there were significant changes to the seminiferous epithelium within knockout mice. In a non-stage specific manner, the epithelium of 60% of the seminiferous tubules appeared disrupted when compared to the wild type. Detailed examination at the level of the electron microscope revealed that the semi-lunar epithelial disruption observed in the LM was in fact Sertoli cell specific. The Sertoli cells, which solely express the FSH-R, exhibited what appeared to be a dilation of the cytoplasm. Sertoli nuclei remained intact, showed no sign of pyknosis or degeneration, the blood testis barrier appeared intact, and germ cells appeared unaffected morphologically. Organelles normally situated in compact manner within the flocculent ground substance, now appeared to be floating in a fluid filled dilated cytoplasm. Elongated spermatids, which were closely associated with thin apical Sertoli cell processes in wild type mice, appeared to be floating in thickened spaces which upon closer examination, proved to be the Sertoli processes. Based on these observations, it is evident that structurally, Sertoli cells are altered in FORKO mice compared to wild type.

Given that structure was compromised, it was plausible to want to see if function too was affected in the knockout. Examination of ABP levels, a functional indicator for Sertoli cells, revealed a significant reduction in the FORKO compared to wild type. This finding was observed by LM immunocytochemistry and confirmed by western blot analysis. In addition, morphometric studies carried out on the testis revealed a reduced tubule diameter and fewer Sertoli numbers (previous work done in our lab). Given that each Sertoli cell has a finite capacity for a given complement of germ cells, and that the seminiferous epithelium was disrupted by almost 60% would support the finding that these mice were sub-fertile. Since ABP levels were lower and considering its role in maintaining high testosterone levels in the epididymis, we therefore examined this tissue histologically and functionally.

Initial histological examination of epididymal tubules in the FORKO revealed no gross epithelial abnormalities. However, morphometry revealed that within the epididymis, the epithelium itself was reduced in the knockout compared to the wild type. The functional capacity of the epididymis resides in its epithelium. It is the epithelium which secretes the necessary proteins required for sperm maturation or carries out endocytosis of various luminal products so that the appropriate luminal microenvironment is maintained within the epididymis. With the epithelial compartment being diminished, we used Computer Assisted Sperm Analysis (CASA) to evaluate sperm itself. A total of 8 out of 14 parameters were significantly altered in the FORKO compared to wild type, all of which reflected an overall decrease in sperm quality. In addition, sperm counts were reduced significantly, close to 50%. Oddly, upon examination of functionality within the epididymis, the parameters studied, visualized immunocytochemically (clusterin and prosaposin protein expression), showed no difference between the knockout and wild type. While these are only two of the many proteins produced within the epididymis, such results provoke more questions, specifically what is causing the observed reduction in sperm motility as evidenced by CASA. Whether or not the sub-fertile nature of these mice is due to epididymal alterations, or testicular alterations, or even the lack of testicular factors which may subsequently affect epididymal function, more work remains to be done to help explain the observed phenotype.

While only one functional indicator in the testis, and two in the epididymis, were studied, it would be beneficial to examine other functional markers of these two reproductive organs. FORKO mice demonstrate significantly lower levels of testosterone, and looking at various androgen regulated proteins within the epididymis might provide more insightful data.

Additional future directions would involve in vitro work, specifically culturing FORKO Sertoli cells and wild type cells. Performing a comparative analysis would provide greater insight into what proteins, or lack thereof, are being produced and secreted by the knockout cells. By examining cells at different ages, comparisons can be made from a developmental perspective. Subsequent proteomic and genomic analysis on the cultured cells would then introduce us to specific classes of proteins and genes affected in FORKO mice.

In addition to examining Sertoli cells, an in depth analysis remains to be done on the interstitial tissue, namely the Leydig cells. Testosterone levels in the FORKO mice drop after day 70 postpartum, despite normal circulating levels of LH. The idea of an interruption in intercellular signaling between Sertoli and Leydig cells has been proposed, but not closely examined. Similarly, initial observations on sperm morphology have revealed improper nuclear condensation. It would be interesting to study the changes happening to the sperm from the time of release in the testis all the way to its storage in the cauda epididymidis.

Lastly, the exact nature of the Sertoli specific disruptions remains to be identified. One hypothesis is that water balance is altered within these cells. This hypothesis however, does not preclude other theories; such as altered cytoskeletal proteins or aberrant cell-cell communication. It can therefore be seen that there is still much to be done to determine the functional role of the FSH receptor within both the testis and epididymis.

APPENDIX I