

Effects of FSH Receptor Deletion on Epididymal Tubules and Sperm Morphology, Numbers, and Motility

AMIT GROVER,^{1,2} CHARLES E. SMITH,³ MARY GREGORY,⁴ DANIEL G. CYR,⁴
M. RAM SAIRAM,^{1,2*} AND LOUIS HERMO⁵

¹Molecular Reproduction Research Laboratory, Clinical Research Institute of Montreal, Montreal, Quebec, Canada

²Department of Physiology, McGill University, Montreal, Quebec, Canada

³Département de Stomatologie, Université de Montréal, Montreal, Canada

⁴INRS-Institut Armand Frappier, Université du Québec, Pointe-Claire, Canada

⁵Department of Anatomy and Cell Biology, McGill University, Montreal, Quebec, Canada

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. Male FSH-R knockout (FORKO) mice show fewer Sertoli cells and many that are structurally abnormal and as a consequence fewer germ cells. Lower levels of serum testosterone (T) and androgen binding protein (ABP) also occur, along with reduced fertility. To assess the effects of FSH-R depletion as an outcome of testicular abnormalities, sperm from the cauda epididymidis were counted and examined ultrastructurally. As reduced fertility may also reflect changes to the epididymis, the secondary responses of the epididymis to lower T and ABP levels were also examined by comparing differences in sizes of epididymal tubules in various regions of FORKO and wild type (WT) mice. Sperm motility was evaluated in FORKO mice and compared to that of WT mice by computer assisted sperm analysis (CASA). Quantitatively, the data revealed that epithelial areas of the caput and corpus epididymidis were significantly smaller in FORKO mice compared to WT mice. Cauda epididymal sperm counts in FORKO mice were also much lower than in WT mice. This resulted in changes to 9 out of 14 sperm motility parameters, related mostly to velocity measures, which were significantly lower in the FORKO mice. The greatest change was observed relative to the percent static sperm, which was elevated by 20% in FORKO mice compared to controls. EM analyses revealed major changes to the structure of the heads and tails of cauda luminal sperm in FORKO mice. Taken together these data suggest a key role for the FSH receptor in maintaining Sertoli cells to sustain normal sperm numbers and proper shapes of their heads and tails. In addition, the shrinkage in epididymal epithelial areas observed in FORKO mice likely reflect direct and/or indirect changes in the functions of these cells and their role in promoting sperm motility, which is noticeably altered in FORKO mice. *Mol. Reprod. Dev.* 72: 135–144, 2005.

© 2005 Wiley-Liss, Inc.

Key Words: gene knockout; FORKO mouse; epididymis; sperm counts and motility

INTRODUCTION

Follicle stimulating hormone (FSH), a pituitary glycoprotein hormone considered essential for mammalian fertility, acts on Sertoli cells (Means and Huckins, 1974; Fritz, 1978; Kliesch et al., 1992; Bockers et al., 1994; Rannikko et al., 1996; Grover et al., 2004). 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 (Simoni et al., 1997; Grover et al., 2004). This pathway, as well as other signaling mechanisms, cause a cascade of events that regulate various transcription factors ultimately giving rise to numerous Sertoli cell functions (Simoni et al., 1997). Sertoli cells create an effective blood testis barrier, and provide support, nourishment, and anchorage for the various generations of germ cells within the seminiferous epithelium (Clermont, 1993). Sertoli cells secrete many substances both apically and basally and are involved in endocytosis and the phagocytosis of residual bodies of elongating spermatids (Clermont et al., 1987). The importance and requirement of Sertoli cells for normal spermatogenesis is unequivocal, demonstrated simply by the fact that germ cells do not differentiate properly in the absence of Sertoli cells (Griswold, 1993).

Grant sponsor: CIHR (to MRS, DGC, and LH); Grant sponsor: NIH (to CES); Grant number: DE013237; Grant sponsor: NSERC (to DGC and LH).

*Correspondence to: M. Ram Sairam, Molecular Reproduction Research Laboratory, Clinical Research Institute of Montreal, 110 Avenue des Pins West, Montréal, Québec, Canada H2W 1R7.
E-mail: m.ram.sairam@irem.qc.ca

Received 26 January 2005; Accepted 16 March 2005
Published online 22 June 2005 in Wiley InterScience
(www.interscience.wiley.com).

DOI 10.1002/mrd.20303

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 one approach to study alterations in testicular structure and function. Mutant mice at 2, 3, and 6 months have smaller testes and reduced fertility (Dierich et al., 1998; Krishnamurthy et al., 2000, 2001a; Grover et al., 2004). Serum FSH levels are elevated (Dierich et al., 1998), while testosterone levels are greatly reduced (Dierich et al., 1998; Krishnamurthy et al., 2001a,b) amid normal circulating levels of LH (Krishnamurthy et al., 2001b). In our recent study, we noted that in given cross sections of seminiferous tubules, while some Sertoli cells of FORKO mice were normal in appearance; others in the same tubule were abnormal ultrastructurally exhibiting a dilated cytoplasm suggestive of water retention and impaired ectoplasmic specializations. In addition, reduced levels of androgen binding protein (ABP) production were noted in FORKO mice (Grover et al., 2004). ABP is secreted by Sertoli cells into the lumen (French and Ritzen, 1973; Bardin et al., 1988; Griswold, 1993) and binds androgens with high affinity, transporting them to the epididymis (French and Ritzen, 1973; Danzo et al., 1974; Munell et al., 2002). In the epididymal lumen, ABP is associated with maintaining high levels of T for the epididymal epithelial cells, where T is converted to DHT by 5α -reductase, its active metabolite (Robaire and Viger, 1995). Because the epididymis is dependent on high levels of T for maintaining its structure and functions, in relation to sperm motility and fertility (Turner, 2002), it may be hypothesized that FORKO mice will have abnormalities within the epididymis.

In addition, earlier studies on FORKO mice revealed fewer numbers of Sertoli cells in 21-day-old FORKO mice (Krishnamurthy et al., 2001a), with a concomitant reduction in number of sperm produced, as each Sertoli cell has a fixed capacity for supporting a given complement of germ cells. Light microscope evidence also suggested abnormal sperm nuclear condensation, the presence of bent twisted tails and larger head sizes, suggesting that the sperm of FORKO mice were abnormal and could in part be responsible for the reduction in fertility noted in FORKO mice (Krishnamurthy et al., 2000).

To assess the respective role of FSH-R signaling loss as a cause of testicular abnormalities (fewer Sertoli cells resulting in fewer germ cells, production of morphologically abnormal sperm) versus that of epididymal epithelial alteration in FORKO mice, we investigated several key parameters. First, we examined alterations to the appearance and size of epididymal tubules of FORKO mice as compared to wild type mice of similar ages, by morphological and morphometric parameters. Secondly, caudal epididymal sperm were assessed for their morphological appearance by electron microscopy, as well as changes to their numbers and various motility parameters comparing FORKO mice to their wild type counterparts. The data revealed that epididymal profile

areas as well as sperm morphology, numbers, and motility were all altered in FORKO mice.

MATERIALS AND METHODS

Animals

This investigation was approved by the Animal Care 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. (1998). 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 constant temperature (22°C) and lighting (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 (Danilovich et al., 2000). In this manner, a single PCR was performed on each sample to identify +/+, +/-, 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 quantitative analyses of epididymal profile areas and ultrastructural analyses of their epithelial cells and sperm. The mice were anesthetized by intraperitoneal injections of sodium pentobarbital (Somnitol, MTC Pharmaceuticals, Hamilton, ON, Canada). Prior to vascular perfusion, a hemostat was clamped over the left testicular vessels entering the testis of each animal; both the testis and epididymis of this side were removed and immersed immediately in Bouin's fixative. After 10 min, the epididymides were cut longitudinally such that all regions (initial segment, caput, corpus and cauda) could be viewed in a single section. Thereafter, the epididymides were dehydrated in alcohol, embedded in paraffin and sectioned at 5 μm .

The untouched right epididymides in each animal were fixed by whole body perfusion through the heart with 5% glutaraldehyde buffered in sodium cacodylate (0.1M) containing 0.05% calcium chloride (pH 7.4). After 10 min of perfusion, the epididymides were removed and various regions were cut into small 1 mm 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. The following day, the samples 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. Semi-thin sections (0.5 μm) were cut with glass knives and stained with toluidine blue and observed by light microscopy. Thin sections of selected regions of each block were cut with a diamond knife, placed on copper grids, and counterstained with uranyl

acetate (5 min) and lead citrate (2 min). Both the epithelial cells and sperm in the lumen were assessed for differences between wild type and FORKO mice. Sections were examined with a Philips 401 electron microscope.

Four additional mice at 12-months of age (wild type, $n=2$ and FORKO, $n=-2$) were fixed by whole body perfusion through the heart with 5% glutaraldehyde buffered in sodium cacodylate buffer and processed as described above for the 3- and 6-month old mice. Both the epithelial cells and sperm in the lumen of these mice were assessed for ultrastructural differences between wild type and FORKO mice.

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 (Morales et al., 2000)); (2) anti-clusterin antibody (provided by Dr. M.D. Griswold, Washington State University, Pullman, Washington; purified and characterized as described previously (Griswold et al., 1986)); and (3) anti-androgen receptor (AR) (Santa Cruz Biotechnologies, Santa Cruz, CA).

For the anti-prosaposin antibody, 5 μ m thick paraffin sections of Bouin-fixed epididymides 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 μ l 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 μ l) 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 Permout. For the anti-AR and anti-clusterin antibodies, paraffin sections were processed for immunostaining using the ImmunoCruz ABC Staining System (Santa Cruz Biotechnology). The sections were deparaffinized and hydrated as described above. Sections then were microwaved for antigen retrieval in citrate buffer (Danilovich et al., 2001). 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. TBS substitution for primary antibody was used as a negative control, in addition to the use of normal rabbit serum.

Quantitative Histological Analyses

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

Sperm Collection for Motility Analyses

Six FORKO and six wild type mice at 12-months of age were used for this part of the study. The mice were weighed and then anesthetized with isoflurane. The left cauda epididymidis of each animal was clamped both proximally and distally, then cut out and rinsed in a 35 mm plastic Petri dish containing pre-warmed Hank's medium M199 (Invitrogen Canada, Inc., Burlington, ON) supplemented with 0.5% bovine serum albumin at 37°C. It was then transferred to a fresh Petri dish, unclamped and using a surgical blade (Number 11; Fisher Scientific, Nepean, ON), the cauda was pierced allowing sperm to disperse into the medium. The cauda tissue was removed and the Petri dish was placed in an incubator at 37°C in 5% CO_2 atmosphere for 5 min. Subsequently, an aliquot of the sperm suspension was placed into an 80 μ m glass chamber and analyzed using a Hamilton Thorne IVOS automated semen analyzer (Hamilton-Thorne Biosciences, Beverly, MA). Analyses were carried out for 30 image frames at a frequency of 60 frames/sec. Fourteen of 15 measurement parameters (variables) available through software were analyzed (see Table 2). The cauda region of the right epididymidis of each mouse was dissected and placed in a freezer at -20°C for subsequent sperm count analyses.

Correlation and statistical analyses and power tests of motility data were done using Version 7.0 of the Statistica Data Miner for Windows (Statsoft, Inc., Tulsa,

OK). Initial analyses indicated that there were some outliers present in the dataset and these were removed using the Grubb's test. Raw data for some parameters also did not follow normal distributions and these were obtained by doing \log_{10} transformations on "regular" (continuous) variables (e.g., VAP) or arcsine of the square root transformations for ratio variables (e.g., STR) as required. In subsequent Univariate Factorial ANOVA test and Post-hoc unequal N HSD *t*-tests for continuous variables and Fisher's exact tests for ratio variables, *P* values < 0.05 were considered significant.

Sperm Counts (Indicated as Experiment 1 in Table 2)

The frozen right cauda epididymides of each animal were thawed and homogenized in a 50 ml conical tube containing 20 ml of distilled water. 100 μ l aliquot of the resulting homogenate was suspended in 100 μ l of distilled water in 1.5 ml microcentrifuge tubes coated with "IDENT fluorescent dye" (Hamilton-Thorne Biosciences) and incubated at room temperature for 2 min. The solution was mixed and a 5 μ l aliquot was placed on a 20 μ m sperm analysis chamber ($2 \times$ Cel; Hamilton-Thorne Biosciences) and quantified with the IVOS semen analyzer under ultraviolet light. As with profile areas and motility data, sperm counts in FORKO and wild type mice were not normally distributed and \log_{10} transformations of raw values were done prior to carrying out *t*-tests assuming unequal variances; *P* values < 0.05 were considered significant.

RESULTS

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 (Fig. 1A,C,E) of the different epididymal regions revealed an epithelium composed of a normal complement of epithelial cells as seen in wild type mice (Fig. 1B,D,F) and a lumen containing sperm. Despite apparent structural similarities, the tubular diameters of some epididymal regions appeared smaller in size in FORKO males as compared to 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 and corpus epididymidis. Both the outer profile area and luminal area of those epididymal tubules showing a more-or-less spherical appearance from each region were measured; the epithelial area was calculated as the difference between the two. Results of morphometric analyses revealed that, in the 3-month age group, the outer profile areas, luminal areas, and epithelial areas of tubules from the caput epididymidis were significantly decreased in FORKO as compared to wild type mice (Table 1). In the corpus epididymidis, only the epithelial areas of FORKO mice were significantly decreased (Table 1). At 6 months of age, profile, luminal, and epithelial areas

were all significantly reduced in both the caput and corpus epididymidis (Table 1).

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. In both wild type (Fig. 1C) and FORKO (Fig. 1D) mice, the nuclei showed similar staining patterns and intensities of reaction product, and no observable differences were seen between the two groups of animals. In addition in both groups of mice, while some nuclei were intensely reactive, others were only moderately or weakly reactive; several were unreactive. The size and shape of the nuclei of principal cells appeared to be unaltered in FORKO mice and were comparable to those of wild type mice. The anti-clusterin antibody also revealed no apparent change in staining patterns in epithelial principal cells of both FORKO and wild type mice (Fig. 1E,F) and as described previously showed a checkerboard staining pattern (Hermo et al., 1991). Immunolocalization of anti-prosaposin antibody revealed a uniform punctate staining in principal cells throughout the epididymis of both wild type and FORKO mice comparable to that shown by others (Hermo et al., 1992) (data not shown). While not quantified, the light microscope immunocytochemical results (4 runs/antibody) were reproducible from animal to animal over the different ages and over the numerous sections stained for each run (8 slides/run). In all cases, control sections, where the primary antibody was eliminated and where normal rabbit serum was used, showed no staining over the epithelium or contents of the lumen or intertubular space in all epididymal regions; examples of which can be seen in our previous FORKO work (Grover et al., 2004).

Sperm Counts and Motility Analyses

The total numbers of sperm present in the cauda epididymidis were 26%–45% lower in FORKO mice compared to controls (Table 2, top). CASA measurements indicated that the numbers of sperm subclassified into Motile, Progressive, Rapid, Medium, or Slow categories were also reduced by 27%–35% in FORKO mice (Table 2, middle). The numbers of sperm classified as Static, however, were no different than controls (Table 2, middle). When expressed in relative terms as percentages (Table 2, bottom), sperm subclassified into these categories in FORKO mice showed similar percentages as sperm from control mice except in the case % Static sperm which appeared elevated by 20% in FORKO mice (Table 2; difference is not significant due to high variations in the counts). The data further indicated that the movement velocities of sperm (VAP, VSL, and VCL) and the amplitude of their lateral head displacements (ALH) were slightly, but nevertheless significantly, reduced in FORKO mice (Table 2). However, the beat cross frequency (BCF), head elongation ratio of sperm (Elong), and various relative ratios computed

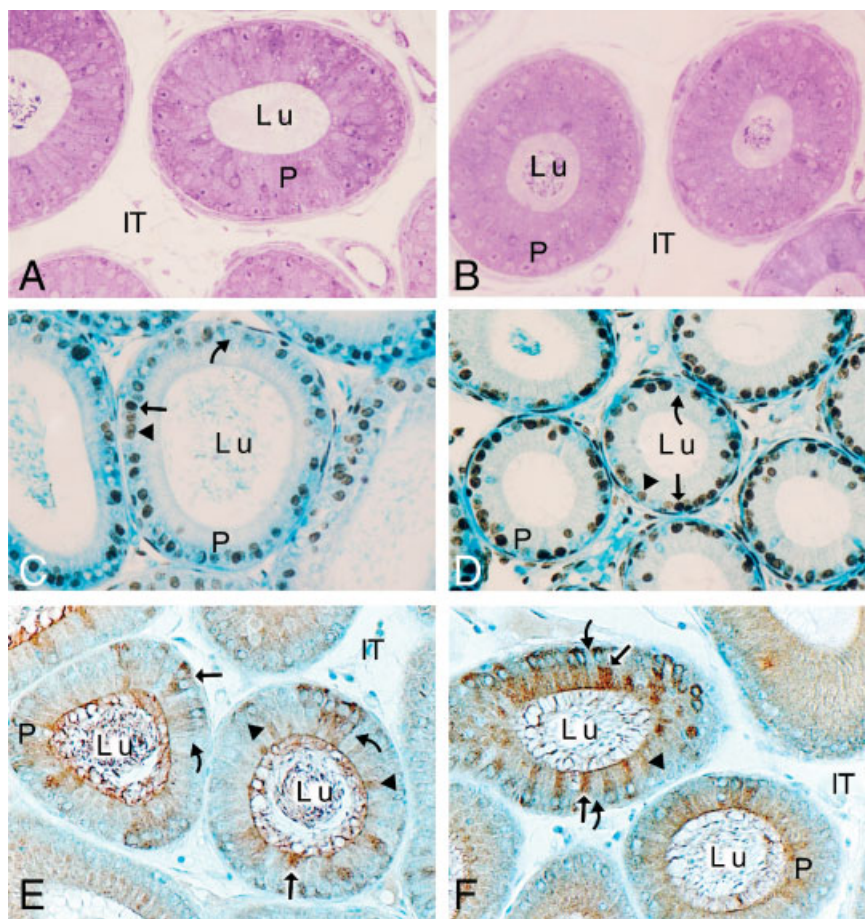


Fig. 1. A–F: Light micrographs of epididymal tubules of wild type (A, C, E) and FORKO (B, D, F) mice at 3- (A–D) and 6- (E, F) months of age of the initial segment (A, B, E, F) and caput (C, D). Note the full complement of epithelial cells, composed mainly of principal cells (P), in both wild type and FORKO (compare A, C, E–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, F), as well as the epithelial compartment (B, D, F) compared to wild type (A, C, E). 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). Immunostaining with anti-clusterin antibody revealed a checkerboard staining of principal cells (P) comparable between both wild type (E) and FORKO (F). 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). IT, Intertubular space. Original magnification; A–D, 390 \times ; (E–F) 250 \times .

from raw velocity data (STR and LIN) showed no significant differences between FORKO and wild type mice (Table 2).

EM Analysis of the Epididymal Epithelium and Sperm From the Cauda Epididymidis of FORKO Versus Wild Type Mice

Electron microscope analyses of epithelial cells in the epididymis from FORKO mice at 3-, 6-, and 12-month old mice showed no major ultrastructural differences in the various organelles compared to wild type mice (data not shown).

In the lumen, major changes were noted to sperm from FORKO mice at each of the three ages examined. In wild type mice, the varying planes of section through the heads of sperm revealed smooth tubular or elongated profiles, with a densely packed nuclear chromatin and

an acrosome closely applied to the nucleus. The cytoplasm constituted a thin investment around the nucleus and contained no large organelles (Fig. 2 inset). Various oblique, longitudinal, and cross sectional profiles of the middle, principal, and end pieces of the tail revealed that the cytoplasm around each tail contained a single axoneme and associated structures (Fig. 2 inset). In dramatic contrast were the shape and appearance of the heads and tails of many sperm of FORKO mice. The nuclei of some sperm were highly irregular in appearance taking on twisted and contorted shapes (Fig. 2A,B). In some cases, more than one nucleus occupied a common cytoplasm (Fig. 2B,E). In some cases, the cytoplasm was surrounded by vesicular profiles and a large amorphous moderately dense material (Fig. 2E). In addition, the acrosomes at times appeared to be peeling off the nucleus (Fig. 2B,C). The heads of some sperm

TABLE 1. Summary Statistics for Profile Area Measurements in Epididymis

Age	Region	Wild type mice mean \pm SD (num. obs.) ^a	FORKO mice mean \pm SD (num. obs.) ^a	Change ^b	P values ^c	Power ^d
Outer profile areas (μm^2)						
3 month	Caput	13,259 \pm 4,157 (112)	10,452 \pm 3,368 (178)	-21%	0.0000	1.0000
	Corpus	35,966 \pm 18,716 (40)	28,689 \pm 12,385 (92)	-20%	0.3038 NS	0.5057
6 month	Caput	15,352 \pm 1,782 (34)	10,934 \pm 1,228 (38)	-29%	0.0009	1.0000
	Corpus	34,394 \pm 2,661 (21)	22,361 \pm 3,463 (21)	-35%	0.0007	1.0000
Luminal areas (μm^2)						
3 month	Caput	4,674 \pm 2,040	3,404 \pm 1,712	-27%	0.0000	1.0000
	Corpus	19,845 \pm 14,189	17,578 \pm 10,806	-11%	0.9568 NS	0.1497
6 month	Caput	4,757 \pm 1,306	2,028 \pm 550	-57%	0.0000	1.0000
	Corpus	18,259 \pm 2,635	9,831 \pm 3,090	-46%	0.0013	1.0000
Epithelial areas (μm^2)						
3 month	Caput	8,585 \pm 2,621	7,048 \pm 1,996	-18%	0.0014	0.9997
	Corpus	16,121 \pm 6,159	11,111 \pm 3,030	-31%	0.0000	0.9999
6 month	Caput	10,595 \pm 989	8,906 \pm 951	-16%	0.0038	1.0000
	Corpus	16,134 \pm 1,467	12,530 \pm 991	-22%	0.0001	1.0000

^aNumber of observations = number of tubular profiles measured in sections from four rats per age per group.

^bFor FORKO mice compared to wild type mice.

^cP values < 0.05 are considered significantly different (NS, not significant).

^dThis is the power associated with rejecting the null hypothesis that the two means are equal.

TABLE 2. Sperm Counts and Motility Changes Comparing FORKO to Wild Type Mice

Parameter ^a	Wild type mice mean \pm SD (num. obs.) ^b	FORKO Mice mean \pm SD (num. obs.) ^b	Change ^c	P values ^d	Power ^e
Sperm counts					
Expt 1	11.0 \pm 4.8 (267)	6.0 \pm 3.5 (230)	-45%	0.0000	1.0000
Expt 2 ^f	51.6 \pm 18.1 (281)	38.4 \pm 18.1 (351)	-26%	0.0000	1.0000
Raw values					
VAP	116.2 \pm 24.7	108.9 \pm 25.5	-6%	0.0004	0.9472
VSL	91.9 \pm 21.2	85.2 \pm 22.0	-7%	0.0001	0.9734
VCL	183.7 \pm 34.3	176.6 \pm 36.4	-4%	0.0125	0.7065
ALH	5.8 \pm 1.0	5.5 \pm 1.3	-5%	0.0003	0.9409
BCF	2.2 \pm 2.0	2.1 \pm 2.5	-5%	0.6955 NS	0.0790
Motile	40.2 \pm 13.3	28.2 \pm 13.4	-30%	0.0000	1.0000
Prog(ressive)	15.6 \pm 5.8	11.1 \pm 6.9	-29%	0.0000	1.0000
Rapid	26.3 \pm 9.0	19.1 \pm 10.7	-27%	0.0000	1.0000
Medium	13.9 \pm 7.3	9.1 \pm 5.0	-35%	0.0000	1.0000
Slow	2.0 \pm 1.7	1.4 \pm 1.4	-31%	0.0000	0.9927
Static	9.4 \pm 7.6	8.8 \pm 9.9	-6%	0.4338 NS	0.1176
Ratios					
STR	76.9 \pm 4.1	75.8 \pm 5.9	-1%	0.7467 NS	0.0616
LIN	50.0 \pm 4.7	48.3 \pm 7.0	-3%	0.6711 NS	0.0709
Elong(ation)	49.2 \pm 4.9	48.9 \pm 5.4	-1%	0.9403 NS	0.0506
Percentages					
%Motile	79.4 \pm 9.9	75.1 \pm 15.5	-5%	0.2022 NS	0.2459
%Prog(ressive)	31.4 \pm 9.2	28.5 \pm 10.4	-9%	0.4286 NS	0.1251
%Rapid	52.5 \pm 11.3	49.6 \pm 14.0	-6%	0.4689 NS	0.1118
%Medium	26.9 \pm 10.5	25.5 \pm 11.8	-5%	0.6907 NS	0.0688
%Slow	4.0 \pm 3.6	4.0 \pm 4.3	0%	1.0000 NS	0.0501
%Static	16.7 \pm 10.3	20.9 \pm 16.1	20%	0.1818 NS	0.2647

^aExplanation of parameters: sperm counts (millions); VAP, smoothed path velocity ($\mu\text{m}/\text{sec}$); VCL, track velocity ($\mu\text{m}/\text{sec}$); VSL, straight line velocity ($\mu\text{m}/\text{sec}$); ALH, amplitude of lateral head displacement (μm); BCF, beat cross frequency (hertz); number (in millions) or percent of motile, progressively motile (Prog), rapid, medium, slow, and static cells; STR, straightness (ratio of VSL/VAP); LIN, linearity (ratio of VSL/VCL); elongation: head shape (ratio of minor to major axis of sperm head).

^bTotal number of observations (measurements) made from a pool of six mice per group.

^cFor FORKO mice compared to wild type mice.

^dP values < 0.05 are considered significantly different (NS, not significant). A Fisher's exact test was used to compare differences between means for "Ratios" and "Percentages."

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

^fThis experiment at roughly five times higher sperm concentrations was used to obtain all data listed below under "Raw Counts", "Ratios", and "Percentages."

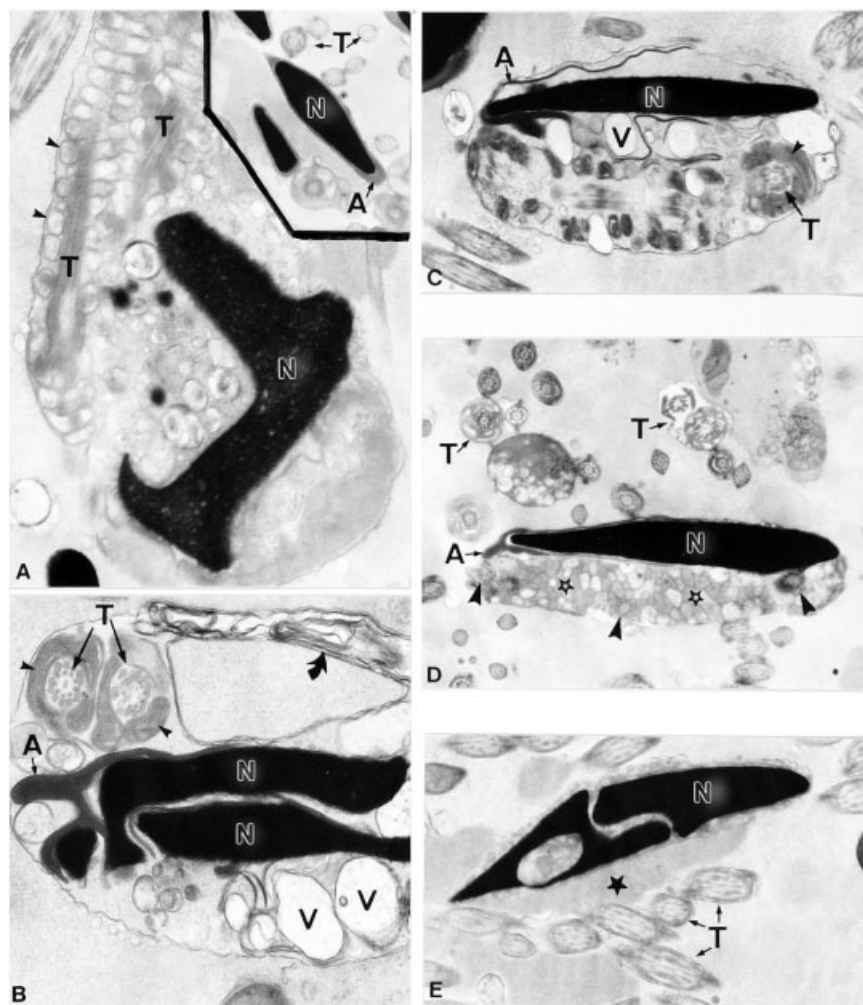


Fig. 2. Electron micrographs of sperm within the cauda epididymidis of 12-month-old wild type (inset) and FORKO (A–E) mice. In wild type mice (A inset), the sperm head appears smooth and tubular or elongated in profile and cytoplasm enveloping the head is attenuated with no prominent organelles; the chromatin is condensed within the nucleus (N) and the acrosome (A) is closely applied to it (inset). Cytoplasm surrounding sperm tails (T) reveals the presence of a single axoneme and associated structures. In FORKO mice, the nucleus (N) is often distorted or lobulated (A, B, E). The acrosome (A) shows a peeling off configuration (B, C). In FORKO mice (B–E), the cytoplasm surrounding the head is

prominent and contains vacuoles (V), membranous profiles (curved arrow), mitochondria (open stars), and several cross sectional profiles of tails (T), none of which are encountered in wild type mice. Tails of sperm show a central axoneme, outer dense fibers and enveloping mitochondria (small arrowheads). Some sperm heads (E) are associated with a moderately dense amorphous material (dense stars). In the lumen of FORKO mice (D), some cross sections of tails (T) show a double axoneme and associated structures enveloped by a common cytoplasm. Original magnification: Inset: 10,465 \times ; (A) 28,175 \times ; (B) 35,525 \times ; (C) 14,950; (D) 9,315 \times ; (E) 15,180 \times .

encompassed large masses of cytoplasm, which contained large vacuoles (Fig. 2A–C), membranous whorls (Fig. 2A) and aggregations of mitochondria (Fig. 2D). On some occasions, the cytoplasm enveloping the sperm heads contained several cross sectional profiles of tails, each presenting an axoneme and associated structures (Fig. 2B–D). Although a quantitative analysis was not preformed on sperm and tail abnormalities, it was roughly estimated, per unit area of epididymal lumen, that approximately 20%–30% of the heads and tails of sperm in FORKO mice were abnormal as compared to wild type mice.

DISCUSSION

In the present study, we noted dramatic changes to the size of the epididymal epithelium of FORKO mice,

as well as changes to the morphology, numbers, and velocity characteristics of cauda epididymal sperm. Collectively these changes would have an impact on reducing fertility in FORKO mice as will be discussed below. In the epididymis, FORKO tubules showed a significant decrease in epithelial area in both the caput and corpus epididymidis of 3- and 6-month old mice, supporting earlier data (Krishnamurthy et al., 2000) which demonstrated that the epididymis in 3-month-old FORKO males is reduced in weight by 30% compared to wild type mice. An attempt was also made to quantify changes to the cauda epididymidis. However, the data were highly variable and erratic, and this appears to be due to the diversity of size of the epithelium and lumen of its many subdivisions, a point that we will need to take into account in future studies.

The observed reduction in the various morphometric parameters of the caput and corpus epididymidis appears to be due to the reduction in serum testosterone levels occurring in adult FORKO males (Krishnamurthy et al., 2001b). It is well established 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 (Danzo et al., 1975; Orgebin-Crist, 1996). Thus our data on decreases in the size of epididymal tubules correlates well with decreased serum testosterone levels of FORKO mice. Additionally, androgen binding protein (ABP), secreted by Sertoli cells into the lumen, is decreased in FORKO males by 60% of controls (Grover et al., 2004). As ABP transports and maintains high concentrations of testosterone in the epididymal lumen (Turner, 2002), it would follow that a reduction in serum testosterone levels along with reduced ABP production would result in de-

creased circulating and luminal concentrations of testosterone in the epididymis. Together this could account for the decreased epididymal profile, luminal and epithelial areas observed in several epididymal regions. In addition, it is well established that a synergistic relationship exists between Sertoli and Leydig cells, with regard to various functions (Sharpe, 1993). Given that Sertoli cells are reduced in number and altered in both structure and function in FORKO mice (Grover et al., 2004), it may be suggested that the direct effects of the lack of FSH-R signaling on the Sertoli cell has an effect on Leydig cell functions and subsequent testosterone production (Krishnamurthy et al., 2001b).

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 Apolipoprotein J (Sylvester et al., 1984; Hermo et al., 1991). In the present study, no apparent change was noted in clusterin staining between both wild type and FORKO mice in all regions and at various ages. As for the rat, the immunostaining was seen as a checkerboard pattern, with principal cells displaying varying degrees of reaction product (Hermo et al., 1991). Since clusterin has been implicated in having a protective effect against apoptosis, the absence of significant changes in clusterin staining suggests that the epididymal epithelium is not undergoing cell death in FORKO mice (Bailey et al., 2002). In the rat, it has been demonstrated that

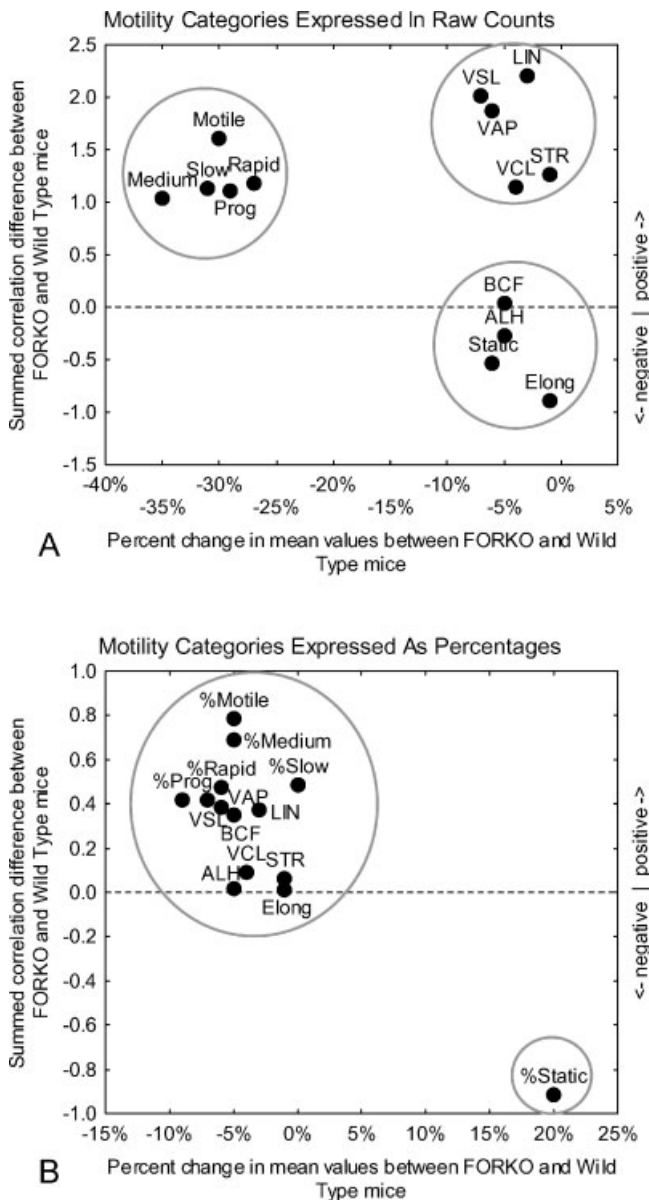


Fig. 3. Scatter plots summarizing changes in the motility behavior of sperm from FORKO mice compared to wild type controls. In **panels A and B**, differences in means determined for each of the 14 motility parameters analyzed by CASA are plotted as percentages along the abscissa (from column 4 of Table 2; $\text{Mean}_{\text{FORKO}} - \text{Mean}_{\text{WILD TYPE}} / \text{Mean}_{\text{WILD TYPE}} \times 100\%$), and differences between the sums of correlation coefficients computed for each parameter are plotted along the ordinate ($\sum \text{Pearson } r$ for Parameter_A across Parameters_{A-N} in FORKO mice - $\sum \text{Pearson } r$ for Parameter_A across Parameters_{A-N} in wild type mice). Panel A shows results for correlation coefficients computed from raw sperm cell counts (Table 2, top) whereas panel B shows results for correlation coefficients computed from motility data expressed as percentages of total sperm cell counts (Table 2, bottom). If there were no differences in the motility behavior of sperm from FORKO mice and wild type mice then all points should plot near the “0” x-axis and “0” y-axis position (which they do not). Panel A: Motility parameters based on raw counts are grouped into three main clusters, one representing sperm feature descriptors (BCF, ALH, Elong) and a single motility descriptor (Static) (means slightly less and correlations more negative overall in FORKO mice), a second cluster representing velocity descriptors (VAP, VSL, VCL) and directional descriptors derived from them (LIN, STR) (means slightly less and correlations more positive overall in FORKO mice), and a third cluster representing motility descriptors (Motile, Prog, Rapid, Medium, Slow) (means much less and correlations more positive overall in FORKO mice). Panel B: Motility parameters based on relative percentages show one major grouping (means slightly less and correlations more positive overall in FORKO mice) and a single parameter offset from the rest (%Static) (mean greater and correlations more negative overall in FORKO mice). Taken together these graphs provide a visual “fingerprint” of changes in sperm number and behavior that characterize the FORKO condition.

castration had no effect on expression of clusterin and thus it was not regulated by testosterone (Heramo et al., 2000); the present data also suggest that FSH directly or indirectly does not regulate clusterin. Similarly, prosaposin (sulfated glycoprotein-1) staining within principal cells was comparable between genotypes indicating absence of a role for FSH in the regulation of this protein in the epididymis. In the rat, as in the present study, prosaposin is localized to the lysosomes of epithelial cells (Heramo et al., 1992) and absence of changes in staining intensities suggests that these cells are not becoming more active in endocytosis in FORKO mice. Androgen receptor expression was also unaltered between FORKO and wild type mice, suggesting an absence of a role for FSH in its expression. This is of interest since female FORKO mice demonstrated a significant increase in androgen receptor expression (Balla et al., 2003). Interestingly, a checkerboard pattern of staining in principal cells was noted for the androgen receptor, not unlike that for clusterin. It would be of interest in future experiments to correlate the degree of staining of clusterin and the androgen receptor in a given principal cell and determine if they match one another. Taken together, while the epididymis is compromised in FORKO mice, several key proteins show no apparent changes in expression. Future studies using a proteomic or genomic approach would help resolve which epididymal function might correlate to reduced epithelial size in FORKO mice.

In an earlier light microscope study on FORKO mice, bent tails and larger head sizes indicative of incomplete nuclear condensation were noted for sperm in the epididymal lumen (Krishnamurthy et al., 2000). The present study confirms and extends these observations, by including detailed electron microscope analyses performed at different ages. The data reveal abnormal shapes of their heads and nuclei, detached acrosomes, multinucleated sperm and abnormal retention of cytoplasm enveloping their heads. Also evident is the occurrence of sperm tails with two axonemes and associated structures surrounded by a common cytoplasm. All of these features suggest abnormal development of spermatids in the testis. This is not surprising since in FORKO mice, Sertoli cells of several tubules are abnormal in appearance, often taking on dilated and irregular shapes (Grover et al., 2004). In addition, the ectoplasmic specializations between spermatids and Sertoli cells appeared to be diminished in size (Grover et al., 2004). As Sertoli cells are supportive cells for germ cells (Krishnamurthy et al., 2000; Grover et al., 2004) and produce many substances required for their development (Griswold, 1993), it is not surprising to find abnormal sperm in the epididymal lumen.

In the present study, almost all motility parameters estimated for cauda sperm in 12-month-old FORKO mice were reduced compared to wild type mice. Since reduced motility was reported for FORKO mice at 3-months of age (Krishnamurthy et al., 2000), the present data both confirm and extended these initial observations to a much older age. Four basic changes appear to

characterize the FORKO condition. First, the most profound alteration is in the total numbers of sperm, which are much reduced in FORKO mice (Table 2). Second, this results in significantly fewer total numbers of sperm cells being detected in all motility categories (summarized in Fig. 3A), but their distributions in terms of percentages are similar to controls except in the case of %Static sperm, which are relatively more numerous in FORKO mice (summarized in Fig. 3B). Third, FORKO sperm move somewhat slower than control sperm but expressions of the relative straightness and linearity of their migratory paths are no different than controls (Table 2; Fig. 3). Lastly, the amplitudes of side-to-side movements of sperm are slightly, but nevertheless significantly, lower in FORKO mice compared to control mice (Table 2). 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 (Syntin and Robaire, 2001). In addition, CASA has been used to examine changes in sperm motility in rats treated with various toxicants (Toth et al., 1991a,b; Slott et al., 1993; Aravindakshan et al., 2004). It is interesting that the sperm motility parameters affected under a given condition varied across different experimental conditions, suggesting that epididymal functions are likely multifactorial resulting in different motility phenotypes.

The changes in motility of sperm in FORKO mice appear to be the result of several different phenomena. On one hand, altered sperm motility may be attributed to the presence of abnormal shapes of the heads and tails of sperm seen in the cauda epididymidis, which is an indirect result of abnormal Sertoli cell functions in the testis. On the other hand, reduced motility may also be the result of impaired epididymal functions that arise as a consequence of the reduced size of the epithelium in FORKO mice and as a result reduced functions. In addition, our present data also reveal a reduction by as much as 45% in sperm counts in the cauda epididymidis. These findings clearly suggest a very important role for FSH-R signaling in the development and proper functioning of the testis and epididymis.

ACKNOWLEDGMENTS

We thank Ms. Jeanni Mui for her excellent EM technical assistance, Dr. C.R. Morales from McGill University for providing the anti-prosaposin and Dr. M.D. Griswold from Washington State University for providing the anti-clusterin antibody. The assistance of Heather Smith and Sarah Torabi in obtaining quantitative data is also gratefully acknowledged.

REFERENCES

- Aravindakshan J, Gregory M, Marcogliese DJ, Fournier M, Cyr DG. 2004. Consumption of xenoestrogen-contaminated fish during lactation alters adult male reproductive function. *Toxicol Sci* 81:179–189.

- Bailey RW, Aronow B, Harmony JA, Griswold MD. 2002. Heat shock-initiated apoptosis is accelerated and removal of damaged cells is delayed in the testis of clusterin/ApoJ knock-out mice. *Biol Reprod* 66:1042–1053.
- Balla A, Danilovich N, Yang Y, Sairam MR. 2003. Dynamics of ovarian development in the FORKO immature mouse: Structural and functional implications for ovarian reserve. *Biol Reprod* 69:1281–1293.
- Bardin CW, Gonsalus GL, Musto NA, Cheng CY, Reventos J, Smith C, Underhill DA, Hammond G. 1988. Corticosteroid binding globulin, testosterone-estradiol binding globulin, and androgen binding protein belong to protein families distinct from steroid receptors. *J Steroid Biochem* 30:131–139.
- Bockers TM, Nieschlag E, Kreutz MR, Bergmann M. 1994. Localization of follicle-stimulating hormone (FSH) immunoreactivity and hormone receptor mRNA in testicular tissue of infertile men. *Cell Tissue Res* 278:595–600.
- Clermont Y. 1993. Introduction to the Sertoli cell. In: Lonnie D Russell, Michael D Griswold, editors. *The Sertoli cell*. Clearwater, Florida: Cache River Press. pp xi–xxv.
- Clermont Y, Morales C, Hermo L. 1987. Endocytic activities of Sertoli cells in the rat. *Ann NY Acad Sci* 513:1–15.
- Danilovich N, Babu PS, Xing W, Gerdes M, Krishnamurthy H, Sairam MR. 2000. Estrogen deficiency, obesity, and skeletal abnormalities in follicle-stimulating hormone receptor knockout (FORKO) female mice. *Endocrinology* 141:4295–4308.
- Danilovich N, Roy I, Sairam MR. 2001. Ovarian pathology and high incidence of sex cord tumors in follitropin receptor knockout (FORKO) mice. *Endocrinology* 142:3673–3684.
- Danzo BJ, Eller BC, Orgebin-Crist MC. 1974. Studies on the site of origin of the androgen binding protein present in epididymal cytosol from mature intact rabbits. *Steroids* 24:107–122.
- Danzo BJ, Orgebin-Crist MC, Eller BC. 1975. Changes in 5 α -dihydrotestosterone binding to epididymal cytosol during sexual maturation in rabbits: Correlation with morphological changes in the testis and epididymis. *Mol Cell Endocrinol* 3:203–220.
- Dierich A, Sairam MR, Monaco L, Fimia GM, Gansmuller A, LeMeur M, Sassone-Corsi P. 1998. Impairing follicle-stimulating hormone (FSH) signaling in vivo: Targeted disruption of the FSH receptor leads to aberrant gametogenesis and hormonal imbalance. *PNAS* 95:13612–13617.
- French FS, Ritzen EM. 1973. A high-affinity androgen-binding protein (ABP) in rat testis: Evidence for secretion into efferent duct fluid and absorption by epididymis. *Endocrinol* 93:88–95.
- Fritz IB. 1978. Sites of action of androgens and follicle-stimulating hormone on cells of the seminiferous tubule. In: Litwack G, editor. *Biochemical actions of hormones*. New York: Academic Press. pp 249–281.
- Griswold MD. 1993. Protein secretion by Sertoli cells: General considerations. In: Lonnie D Russell, Michael D Griswold, editors. *The Sertoli cell*. Clearwater, Florida: Cache River Press. pp 195–200.
- Griswold MD, Roberts K, Bishop P. 1986. Purification and characterization of a sulfated glycoprotein secreted by Sertoli cells. *Biochemistry* 25:7265–7270.
- Grover A, Sairam MR, Smith CE, Hermo L. 2004. Structural and functional modifications of Sertoli cells in the testis of adult follicle-stimulating hormone receptor knockout mice. *Biol Reprod* 71:117–129.
- Hermo L, Wright J, Oko R, Morales CR. 1991. Role of epithelial cells of the male excurrent duct system of the rat in the endocytosis or secretion of sulfated glycoprotein-2 (clusterin). *Biol Reprod* 44:1113–1131.
- Hermo L, Morales C, Oko R. 1992. Immunocytochemical localization of sulfated glycoprotein-1 (SGP-1) and identification of its transcripts in epithelial cells of the extratesticular duct system of the rat. *Anat Rec* 232:401–422.
- Hermo L, Xiaohong S, Morales CR. 2000. Circulating and luminal testicular factors affect LRP-2 and Apo J expression in the epididymis following efferent duct ligation. *J Androl* 21:122–144.
- Kliesch S, Penttila TL, Gromoll J, Saunders PT, Nieschlag E, Parvinen M. 1992. FSH receptor mRNA is expressed stage-dependently during rat spermatogenesis. *Mol Cell Endocrinol* 84:R45–R49.
- Krishnamurthy H, Danilovich N, Morales CR, Sairam MR. 2000. Qualitative and quantitative decline in spermatogenesis of the follicle-stimulating hormone receptor knockout (FORKO) mouse. *Biol Reprod* 62:1146–1159.
- Krishnamurthy H, Babu PS, Morales CR, Sairam MR. 2001a. Delay in sexual maturity of the follicle-stimulating hormone receptor knockout male mouse. *Biol Reprod* 65:522–531.
- Krishnamurthy H, Kats R, Danilovich N, Javeshghani D, Sairam MR. 2001b. Intercellular communication between Sertoli cells and Leydig cells in the absence of follicle-stimulating hormone-receptor signaling. *Biol Reprod* 65:1201–1207.
- Means AR, Huckins C. 1974. Coupled events in the early biochemical actions of FSH on the Sertoli cells of the testis. In: Dufau M, Means A, editors. *Hormone binding and target cell activation in the testis*. New York: Plenum Press. pp 145–165.
- Morales CR, Zhao Q, El-Alfy M, Suzuki K. 2000. Targeted disruption of the mouse prosaposin gene affects the development of the prostate gland and other male reproductive organs. *J Androl* 21:765–775.
- Munell F, Suarez-Quian CA, Selva DM, Tirado OM, Reventos J. 2002. Androgen-binding protein and reproduction: Where do we stand? *J Androl* 23:598–609.
- Orgebin-Crist MC. 1996. Androgens and epididymal functions. In: Bhasin S, Swerdloff RS, editors. *Pharmacology, biology and clinical applications of androgens*. New York: Wiley-Liss. pp 27–38.
- Rannikko A, Penttila TL, Zhang FP, Toppari J, Parvinen M, Huhtaniemi I. 1996. Stage-specific expression of the FSH receptor gene in the prepubertal and adult rat seminiferous epithelium. *J Endocrinol* 151:29–35.
- Robaire B, Viger RS. 1995. Regulation of epididymal epithelial cell functions. *Biol Reprod* 52:226–236.
- Sharpe RM. 1993. Experimental evidence for Sertoli-germ cell and Sertoli–Leydig cell interactions. In: Lonnie D Russell, Michael D Griswold, editors. *The Sertoli cell*. Clearwater, Florida: Cache River Press. pp 391–418.
- Simoni M, Gromoll J, Nieschlag E. 1997. The follicle-stimulating hormone receptor: Biochemistry, molecular biology, physiology, and pathophysiology. *Endocr Rev* 18:739–773.
- Sylvester SR, Skinner MK, Griswold MD. 1984. A sulfated glycoprotein synthesized by Sertoli cells and by epididymal cells is a component of the sperm membrane. *Biol Reprod* 31:1087–1101.
- Syntin P, Robaire B. 2001. Sperm structural and motility changes during aging in the Brown Norway rat. *J Androl* 22:235–244.
- Toth GP, Stober JA, George EL, Read EJ, Smith MK. 1991a. Sources of variation in the computer-assisted motion analysis of rat epididymal sperm. *Reprod Toxicol* 5:487–495.
- Toth GP, Stober JA, Zenick H, Read EJ, Christ SA, Smith MK. 1991b. Correlation of sperm motion parameters with fertility in rats treated subchronically with epichlorohydrin. *J Androl* 12:54–61.
- Turner TT. 2002. Necessity's potion: Inorganic ions and small organic molecules in the epididymal lumen. In: Robaire B, Hinton BT, editors. *The epididymis: From molecules to clinical practice*. New York: Kluwer Academic/Plenum Publishers. pp 131–150.