

Expression of Aquaporins in the Efferent Ductules, Sperm Counts, and Sperm Motility in Estrogen Receptor- α Deficient Mice Fed Lab Chow Versus Casein

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ABSTRACT Estrogens play an important role in the male reproductive tract, and this is especially so for the efferent ductules, where α -estrogen receptors (ER α) have been localized. Mice deficient in ER α (α ERKO mice) are infertile, and the effect appears to be due in part to retention of water at the level of the efferent ductules. In the present study, we examined the consequences of ER α deletion on the distribution of certain aquaporins (AQPs), water protein channels, in the efferent ductules and on sperm numbers and motility. In addition, the effects of feeding mice a regular lab chow diet, which contains phytoestrogens, known to affect male reproductive tract functions, and a casein diet, which lacks phytoestrogens, were also assessed. Light microscope immunolocalizations of AQP-1 and AQP-9 revealed dramatic reduction and patchier staining in α ERKO mice with distal areas of the efferent ductules being more affected than proximal areas. No other changes in immunolocalizations were noted as a consequence of diet. Computer-assisted sperm analyses demonstrated a 62% reduction in cauda epididymal sperm/ml in α ERKO mice fed lab chow, whereas 87% fewer sperm/ml were observed in α ERKO mice fed casein, suggesting an enhanced role for sperm production and concentration in a diet containing phytoestrogens. All sperm motility parameters were altered to some degree in α ERKO mice fed lab chow. Alterations in sperm motility parameters were also detected, but were less dramatic in α ERKO mice fed casein. These data suggest that the decrease in AQP expression in the efferent ductules of α ERKO mice contributes in part to water retention in this tissue, eventually leading to backflow of water into the testis, with subsequent decreases in sperm concentration and motility. The data also suggest that phytoestrogens, which are present in regular lab chow, can influence the male reproductive tract with and without the presence of ER α , promoting efferent ductule and epididymal functions when ER α is expressed, but

inhibiting these same functions when ER α is missing. Taken together the data underscore the importance of estrogens and ER α in maintaining sperm maturation and preventing male infertility. *Mol. Reprod. Dev.* 73: 226–237, 2006. © 2005 Wiley-Liss, Inc.

Key Words: estrogen receptor; water transport; aquaporin; CASA; epididymis; efferent ductules

INTRODUCTION

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

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to urea, glycerol, and small uncharged molecules (Preston and Agre, 1991; Deen and van Os, 1998; Borgnia et al., 1999; Hatakeyama et al., 2001; Sansom and Law, 2001).

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

Water plays an important role in the male reproductive tract. In seminiferous tubules of the testis, water is secreted into the lumen by Sertoli cells in order to create a fluid environment essential for maintaining spermatogenesis and transporting sperm out of the testis. In the efferent ductules, linking the rete testis to the epididymis, up to 90% of testicular luminal fluid is reabsorbed. This process appears to concentrate sperm as an initial step to promoting fertility and motility as sperm pass along the epididymis (Hess et al., 2002). In the epididymis, water serves as a vehicle for sperm passing through this convoluted duct (Setchell et al., 1969; Setchell and Brooks, 1988).

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

Some studies on the hormonal regulation of AQPs in the male reproductive tract have been reported. In efferent ductules, expression of AQP-1 over the microvilli of nonciliated cells is not influenced by testosterone, whereas expression of AQP-9 in principal cells in some epididymal regions is dependent on both testosterone and other unidentified luminal testicular factors (Badran and Hermo, 2002) and in efferent ductule nonciliated cells are regulated by estrogen (Oliveira

et al., 2005). Recent studies have suggested an important role for estrogen in regulating water transport in the efferent ductules (Hess et al., 2001b, 2002; Hess, 2003).

In males, estrogen is present in low concentrations in blood, but it can be extraordinarily high in semen and rete testis fluids (Ganjam and Amann, 1976; Free and Jaffe, 1979). Estrogen in the rete testis fluid is derived mainly from the conversion of testosterone to estradiol by P450 aromatase present in germ cells and cytoplasmic droplets of sperm traversing the lumen of the efferent ductules, as well as in Leydig and Sertoli cells of the testis (Payne et al., 1976; Carreau et al., 1999; Hess et al., 2001a). It is well-known that male reproductive tissues express estrogen receptors (ERs) (Schleicher et al., 1984; West and Brenner, 1990; Cooke et al., 1991; Greco et al., 1993), and in particular, the efferent ductules, where ER α is abundant and where much of the fluid coming from the testis is reabsorbed (Fisher et al., 1997; Hess, 2000; Zhou et al., 2002). Use of a mouse model that lacks ER α expression, known as the α ERKO mouse, has revealed that water is retained at the level of the efferent ductules in these animals resulting in "back flooding" of seminiferous tubules. This dilutes sperm counts in the epididymis and contributes to infertility seen in these animals (Eddy et al., 1996; Hess, 2000; Hess et al., 2000, 2001b, 2002; Oliveira et al., 2001, 2002; Zhou et al., 2001; Cho et al., 2003). Although it has been shown that AQP-1 protein, but not mRNA, expression is partially regulated by ER α , AQP-1 null mice are fertile (Zhou et al., 2001) suggestive that more than one factor contributes to water retention observed in the α ERKO mouse and dependent on morphology of the microvillar border (Oliveira et al., 2001, 2002). The specific effects of absence of ER α on AQP-1, as well as on AQP-9 expression along the entire length of the efferent ductules that may contribute in part to the infertility of α ERKO mice have not been fully documented. Furthermore, while sperm counts and motility of sperm in α ERKO and antiestrogen treated mice are known to be reduced (Eddy et al., 1996; Cho et al., 2003), detailed studies on motility behavior of sperm in the α ERKO mice compared to wild type mice are presently lacking.

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

alternative diet such as purified casein, which is phytoestrogen-free.

MATERIALS AND METHODS

Animals

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

Immunocytochemistry

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

The Santa Cruz protocol was used for all immunocytochemical procedures (ImmunoCruz Staining System, Santa Cruz Biotechnology, Santa Cruz, CA). Rabbit anti-AQP-1 (Cat# AQP11-A) and anti-AQP-9 (Cat# AQP91-A) antibodies were purchased from Alpha Diagnostics International (San Antonio, TX). Sections at 5 μ m thickness were deparaffinized in Histoclear (Diamed Lab Supplies, Inc., Mississauga, ON, Canada) and hydrated in a series of graded ethanol solutions. During hydration, residual picric acid was neutralized in 70% ethanol containing 1% lithium carbonate, and endogenous peroxidase activity was abolished in 70% ethanol containing 1% (vol/vol) H_2O_2 . Once hydrated, the tissue sections were washed in distilled water containing glycine to block free aldehyde groups. Antigen retrieval was performed using a sodium citrate buffer-microwave method (Santa Cruz Biotechnology, Santa Cruz, CA).

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

lene blue. Negative controls were prepared by incubating additional sections in all solutions except primary antibody.

Sperm Motility Analyses

Mice (five per treatment type and diet type) were weighed and lightly anesthetized with isofurane and killed by cervical dislocation. The left cauda epididymides were removed and frozen (-20°) for subsequent sperm count analysis. The right cauda epididymides were clamped proximally and distally prior to excision, rinsed in prewarmed M199 medium (GIBCO) and placed in a Petri dish containing M199 + 0.5% BSA, preheated to 37°C. Each cauda was poked with the tip of a scalpel blade to permit the release of the sperm into the media. The sperm-media suspension was incubated at 37°C for 5 min, after which 100 μ l aliquots were diluted with medium and transferred into each of two compartments on a glass cannula for computer assisted sperm analysis (CASA) using the integrated visual optical system (IVOS) motility analyzer (Hamilton-Thorne Research, Inc., Beverly, MA). The operational settings of the IVOS were the standard mouse parameters as recommended by the manufacturer (IVOS, Hamilton-Thorne). For each sample, 3–5 slides, with 5–10 scans per slide were analyzed. Fourteen of 15 measurement parameters (variables) available through software were analyzed (see footnote 1 in Table 1).

Correlation and statistical analyses and power tests of motility data were done using Version 7.0 of the Statistica Data Miner for Windows (Statsoft, Inc., Tulsa, OK). Initial analyses indicated that there were some outliers present in the dataset and these were removed using the Grubb's test. Raw data for some parameters also did not follow normal distributions and these were obtained by doing \log_{10} transformations on "regular" (continuous) variables (e.g., VAP) or arcsine of the square root transformations for ratio variables (e.g., STR) as required. In subsequent Univariate Factorial ANOVA test and Post-hoc unequal N HSD *t*-tests for continuous variables and Fisher's exact tests for ratio variables, *P*-values < 0.05 were considered significant.

Sperm Counts

The frozen left cauda epididymides from each animal used for sperm motility were thawed on ice and homogenized in a 50 ml conical tube containing 20 ml of distilled water. Aliquots (100 μ l) of the resulting homogenate were diluted with 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 (2X Cel; Hamilton-Thorne Biosciences) and quantified with the IVOS semen analyzer under ultraviolet light. Data for sperm counts were analyzed as concentration (10^6 /ml). As with motility data, sperm counts in α ERKO and wild type mice were not normally distributed and \log_{10}

TABLE 1. Sperm Counts and Motility Changes Comparing α ERKO to Wild Type Mice

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

^aExplanation of parameters: Sperm counts (millions/ml); VAP, smoothed path velocity (μ m/sec); VCL, track velocity (μ m/sec); VSL, straight line velocity (μ m/sec); ALH, amplitude of lateral head displacement (μ m); BCF, beat cross frequency (hertz); Number (in millions/ml) or percent of—Motile, Progressively Motile (Prog), Rapid, Medium, Slow, and Static cells; STR, straightness (ratio of VSL/VAP); LIN, linearity (ratio of VSL/VCL); Elongation, head shape (ratio of minor to major axis of sperm head).

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

^cFor α ERKO 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 the two means are equal. The Z-test for comparing two proportions was used in power calculations for variables listed under "Ratios" and "Percentages."

transformations of raw values were done prior to carrying out *t*-tests assuming unequal variances; *P*-values < 0.05 were considered significant.

RESULTS

Immunocytochemical Localization of AQP-1 and -9 in the Efferent Ductules of Adult Wild Type and α ERKO Mice

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

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

While differences in staining patterns and intensities were noted between wild type and α ERKO mice, no major differences in the staining pattern of AQP-1 and -9 were evident by LM immunocytochemistry relative to the diet these animals consumed. Control sections in which the primary antibody was eliminated demonstrated an absence of reaction over the epithelium, luminal contents or intertubular spaces (not shown).

Sperm Counts and Motility Analyses: Lab Chow Diet

Sperm counts from the cauda epididymidis were 62% lower in α ERKO mice compared to wild type mice fed the

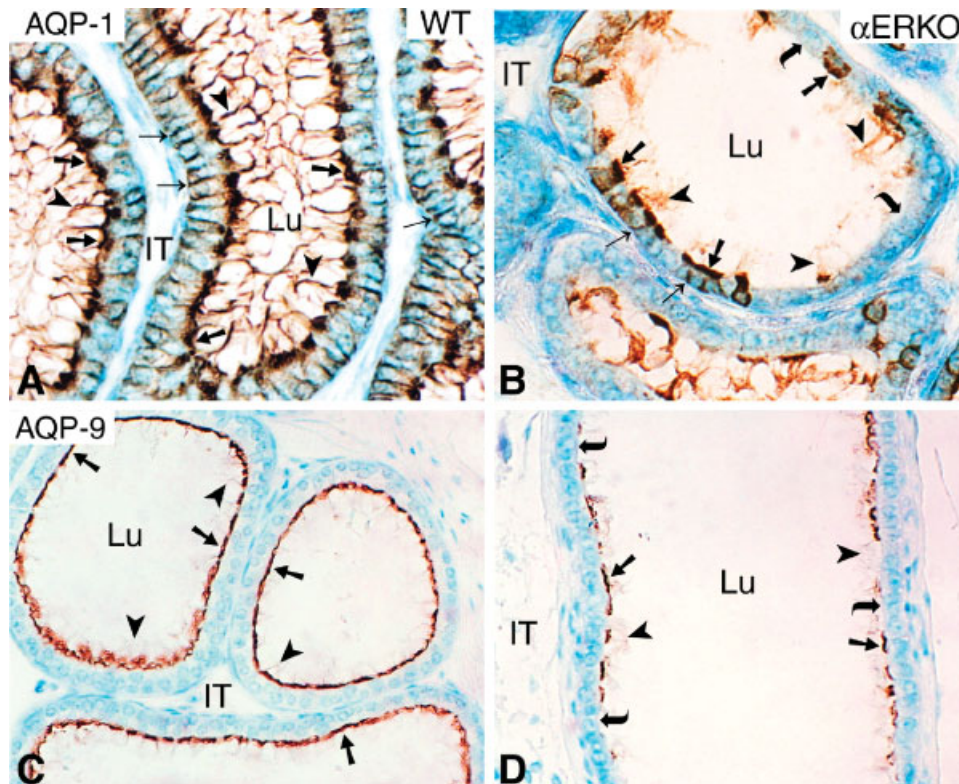


Fig. 1. A–D: Immunolocalization of AQP-1 (A, B) and AQP-9 (C, D) in the efferent ductules of wild type (A, C) and α ERKO (B, D) mice. In (A), AQP-1 expression is uniformly distributed over microvilli (thick arrows) and cilia (arrowheads) of nonciliated and ciliated cells, respectively. Basolateral staining (thin arrows) is also evident over the epithelium. In (B), there is absence of reaction over microvilli and cilia of many, but not all nonciliated and ciliated cells (curved arrows),

and basolateral staining is dramatically reduced. In (C), AQP-9 expression is uniformly distributed over the microvilli (thick arrows) of nonciliated cells, but no staining of ciliated cells (arrowheads). In (D), there is absence of reaction over the microvilli of some nonciliated cells (curved arrows). Note the lumen (Lu) of α ERKO mice (B, D) is enlarged compared to wild type mice (A, C). IT intertubular space. 420X.

lab chow diet (Table 1, lab chow, top). CASA measurements indicated that both the raw numbers and relative percentages of sperm subclassified as Motile, Progressive, Rapid, and Medium were noticeably lower, whereas sperm subclassified as Slow and especially Static were much higher, in the α ERKO mice (Table 1, lab chow, top; raw counts and percentages; Fig. 2). The data further indicated that the movement velocities of sperm (VAP, VSL, and VCL), the linearity of their motion (LIN; ratio of VSL/VCL), and the amplitudes of their lateral head displacements (ALH) were all greatly reduced in the α ERKO mice (Table 1, lab chow, top). The beat cross frequency (BCF) of sperm in α ERKO mice, in contrast, was much greater than in controls (Table 1, lab chow, top). Both the head elongation ratios of sperm (Elong) and straightness of sperm movement paths (STR; ratio of VSL/VAP) showed no significant differ-

ences in mean values between α ERKO mice and controls (Table 1, lab chow, top). There was a trend, however, for increasingly more negative correlations of the parameter Elong to occur in α ERKO mice compared to wild type mice fed the lab chow diet (Fig. 2).

Sperm Counts and Motility Analyses: Casein Diet

Similar trends for reduced motility values in α ERKO mice compared to wild type mice were observed for sperm sampled from animals fed the casein diet (Table 1, casein, bottom). As well, sperm showed increases in BCF and in the relative number of sperm that moved slowly or were static in the α ERKO mice (Table 1, casein, bottom; BCF, %Slow, %Static). Features of sperm behavior that were noticeably distinct included the findings that: (1) the degree of changes between α ERKO and control mice for the other motility parameters were generally less dramatic for animals fed casein as compared to those fed lab chow (Table 1, other parameters, top group versus bottom group; Fig. 3), and (2) raw sperm counts in the Medium, Slow, and Static categories and their expressions as percentages did not show exactly the same trends or the same proportional amount of change between α ERKO and control mice fed casein versus the lab chow diets (Table 1, Medium-%Medium, Slow-%Slow, Static-%Static, top group vs. bottom group; compare Figs. 2 and 3). In addition, sperm counts were much lower between α ERKO mice and controls in animals fed the casein diet

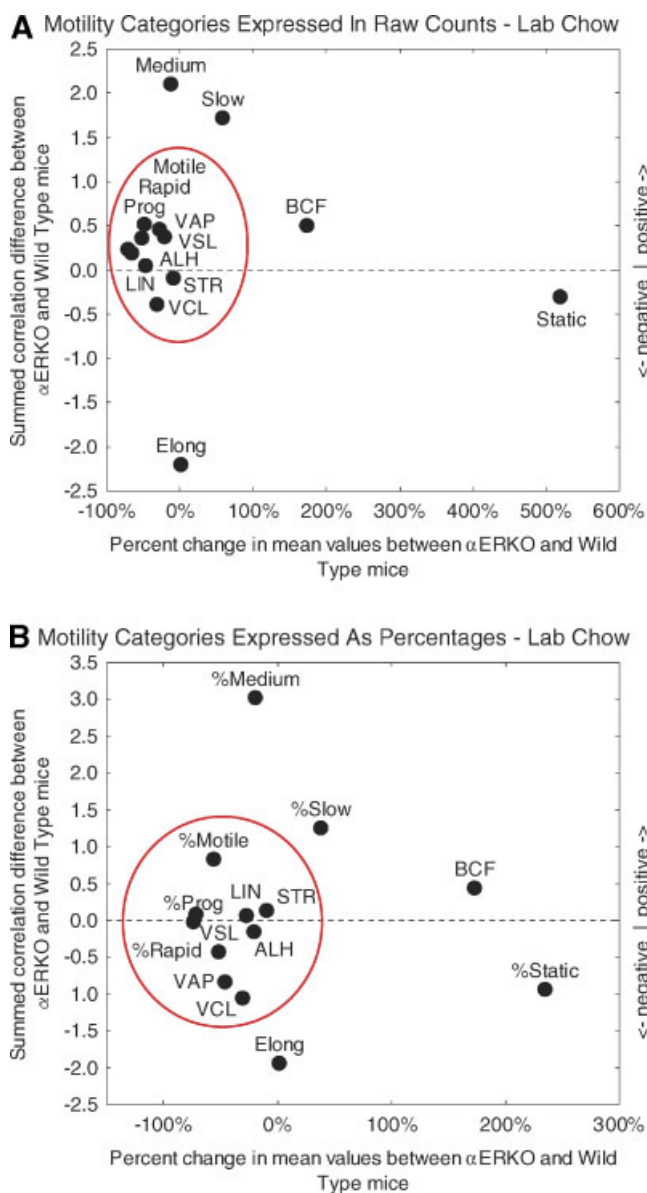


Fig. 2. Scatter plots summarizing changes in the motility behavior of sperm from α ERKO mice compared to wild type controls for animals fed lab chow. In **Panels A and B**, differences in means determined for each of the 14 motility parameters analyzed by CASA are plotted as percentages along the abscissa (from column 4 of lab chow group in Table 1; $\text{Mean}_{\alpha\text{ERKO}} - \text{Mean}_{\text{wild type}} / \text{Mean}_{\text{wild type}} \times 100\%$), 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 α ERKO mice $-\sum \text{Pearson } r$ for Parameter_A across Parameters_{A-N} in wild type mice for lab chow). Panel A shows results for correlation coefficients computed from raw sperm counts (Table 1, raw values, lab chow) whereas Panel B shows results for correlation coefficients computed from motility data expressed as percentages of total sperm cell counts (Table 1, percentages, lab chow). If there were no differences in the motility behavior of sperm from α ERKO 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 analyses based on raw counts show a single cluster of nine mildly altered parameters and five additional parameters residing at more outlying positions representing (1) slight decrease (Medium) or increase (Slow) in mean value and correlations much more strongly positive overall in α ERKO mice, (2) no change of mean (Elong) and correlations more highly negative overall in α ERKO mice, (3) twofold increase in mean value (BCF) and correlations slightly more positive overall in α ERKO mice, and (4) fivefold increase in mean value (static) and correlations slightly more negative overall in α ERKO mice. **Panel B:** motility parameters computed as percentages when plotted show clustering and outlier distribution very similar to Panel A. This indicates that changes in motility behavior in mice fed lab chow are uniform and affect sperm equally in all categories. Taken together these graphs provide a visual “fingerprint” of changes in sperm numbers and behavior that characterize the α ERKO condition in mice fed lab chow that contains phytoestrogens. [See color version online at www.interscience.wiley.com.]

compared to those fed lab chow diet (Table 1, sperm counts, top group vs. bottom group).

Sperm Counts and Motility Analyses: Wild Type and α ERKO Responses by Diet

Table 2 shows results of comparisons by diet within the wild type and α ERKO groups based on mean values given in Table 1 (results in Table 2 are “vertical” comparisons across groups as opposed to the “horizontal” comparisons made in Table 1). It is evident from Table 2 that diet affected sperm counts and sperm motility in both cases, although more dramatically in the case of α ERKO mice than in the case of wild type mice (Table 2, bottom group vs. top group; Fig. 4). In broad terms, the lab chow diet for wild type mice appeared mildly stimulatory resulting in higher sperm counts, higher velocities of sperm movement, greater

amplitude of lateral sperm head movement (ALH), as well as increases in the numbers of sperm subclassified as Motile, Prog, Rapid, and Medium without significant changes in their relative proportions (%Motile, %Prog, %Rapid, etc. same for both diets) (Table 2, top group; Fig. 4A,B). α ERKO mice fed the lab chow diet showed substantially higher sperm counts relative to α ERKO mice fed casein, but the lab chow diet otherwise appeared inhibitory both in terms of sperm movement velocities and in terms of the numbers of sperm subclassified as Medium, Slow, and especially Static (Table 2, bottom group; Fig. 4C). In relative terms, the %Motile, %Prog, and %Rapid sperm in these mice were greatly reduced, whereas the %Static sperm showed a huge increase (Table 2, bottom group; Fig. 4D).

DISCUSSION

AQP Expression in Wild Type and α ERKO Mice and Effect of Diet

In the present study, AQP-1 expression in α ERKO mice showed patches of reactive versus unreactive epithelial cells in the efferent ductules as compared to the uniform homogeneous staining seen in wild type mice (Table 3). Thus estrogen appears to regulate expression of AQP-1 protein, either directly or indirectly, on microvilli and cilia of some but not all nonciliated and ciliated cells, respectively, suggesting the dependence of these cells on ER α activation for AQP-1 expression. Although ER β is present in the efferent ductule epithelium (Zhou et al., 2002), it is

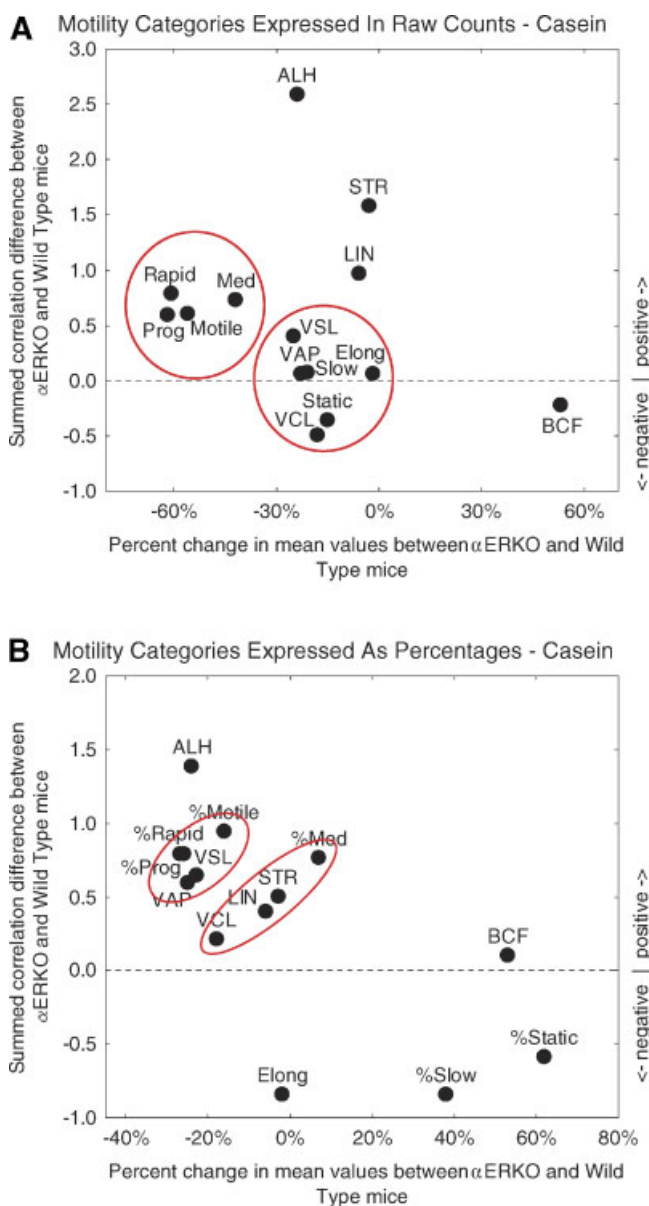


Fig. 3. Scatter plots summarizing changes in the motility behavior of sperm from α ERKO mice compared to wild type controls for animals fed casein. In **Panels A and B**, differences in means determined for each of the 14 motility parameters analyzed by CASA are plotted as percentages along the abscissa (from column 4 for casein group in Table 1), and differences between the sums of correlation coefficients computed for each parameter are plotted along the ordinate (see legend of Fig. 2 for additional details). Panel A shows results for correlation coefficients computed from raw sperm counts (Table 1, raw values, casein) whereas Panel B shows results for correlation coefficients computed from motility data expressed as percentages of total sperm cell counts (Table 1, percentages, casein). **Panel A:** motility analyses based on raw counts show a much different distribution pattern for parameters compared to the lab chow diet (compare Fig. 3 to Fig. 2). With casein, two main parameter clusters are evident. One cluster contains motility descriptors (Motile, Prog, Rapid, Medium) (means much less and correlations more positive overall in α ERKO mice), and the second cluster contains the remaining motility descriptors (Slow, Static), velocity descriptors (VAP, VSL, VCL), and a single sperm feature descriptor (Elong) (means less and correlations slightly more negative, more positive or unchanged in α ERKO mice). There are also four additional parameters residing at more outlying positions representing (1) two directional descriptors (LIN, STR) (means slightly less and correlations more positive overall in α ERKO mice) and (2) two additional sperm descriptors (ALH, BCF) altered in different ways (mean more positive and correlations more negative in α ERKO mice (BCF); mean more negative and correlations considerably more positive in α ERKO mice (ALH)). **Panel B:** motility parameters computed as percentages when plotted show clustering and outlier distribution that is similar to Panel A for six parameters (%Motile, %Prog, %Rapid, VCL, ALH, BCF) and much different for the remaining eight parameters. This indicates that changes in motility behavior in mice fed casein are not uniform and affect sperm in multiple ways. [See color version online at www.interscience.wiley.com.]

TABLE 2. Effect of Diet on Sperm Counts and Sperm Motility Comparing Mice

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

^aMeans for each parameter are explained and listed in Table 1. Comparisons in this table (Table 2) are made vertically across the diet categories of Table 1 (e.g., sperm counts in Table 1 for wild type compares 25.5 \pm 8.8–35.0 \pm 15.0 and for α ERKO compares 3.4 \pm 2.4–13.4 \pm 8.9, and so on).

^bFor mice fed regular lab chow compared to mice fed casein.

^cP-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."

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

unlikely that estrogen action through ER β would maintain AQP-1 expression, as other data have shown that ICI 182,780, which block both receptors, had little effect on AQP-1 expression (Zhou et al., 2001). Alternatively, a factor other than estrogen may regulate AQP-1 expression in the remaining reactive cells of α ERKO mice. A recent study suggests that AQP-1 is expressed constitutively in both the efferent ductules and initial segment of the epididymis (Oliveira et al., 2005), similar to its expression in the kidney (Nielsen, 1993; Borgnia et al., 1999). The sporadic loss of this water channel in the apical region of the efferent ductule epithelium in α ERKO and ICI 182,780 treated mice (Zhou et al., 2001; Oliveira et al., 2005) could be an indirect effect due to the sporadic loss of the microvillus border in these animal models (Hess et al., 1997, 2000; Zhou et al., 2001; Oliveira et al., 2002; Cho et al., 2003; Hess, 2003). In addition, targeting of AQP-1 to the basolateral membrane was dramatically reduced throughout the epithelium of α ERKO mice (Table 3), suggesting the requirement of ER α for maintaining expression on this particular membrane domain. Staining for AQP-9 on the microvilli of nonciliated cells was also reduced in α ERKO mice (Table 3), and with ICI 182,780 treatment, AQP-9 disappears from the efferent ductule epithelium, even while microvilli were still intact (Oliveira et al., 2005). Thus AQP-9 expression in some nonciliated cells appears to be closely regulated by estrogen activation of ER α ; however, this effect appears to be region specific, as the antiestrogen showed no effect on AQP-9 in the initial segment epididymal epithelium (Oliveira et al., 2005).

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

Mice fed either lab chow or the phytoestrogen-free casein diet showed no major differences in the pattern of staining for AQP-1 or -9. This suggests that the small traces of estrogenic compounds present in the lab chow diet exert minimal effects on AQP expression in the epithelium of the efferent ducts of either wild type or α ERKO mice. This is in sharp contrast to the major changes observed for sperm counts and sperm motility as a consequence of diet (discussed below).

Sperm Counts

The concentration of sperm in the cauda epididymidis was markedly lower in α ERKO mice (Table 1), which

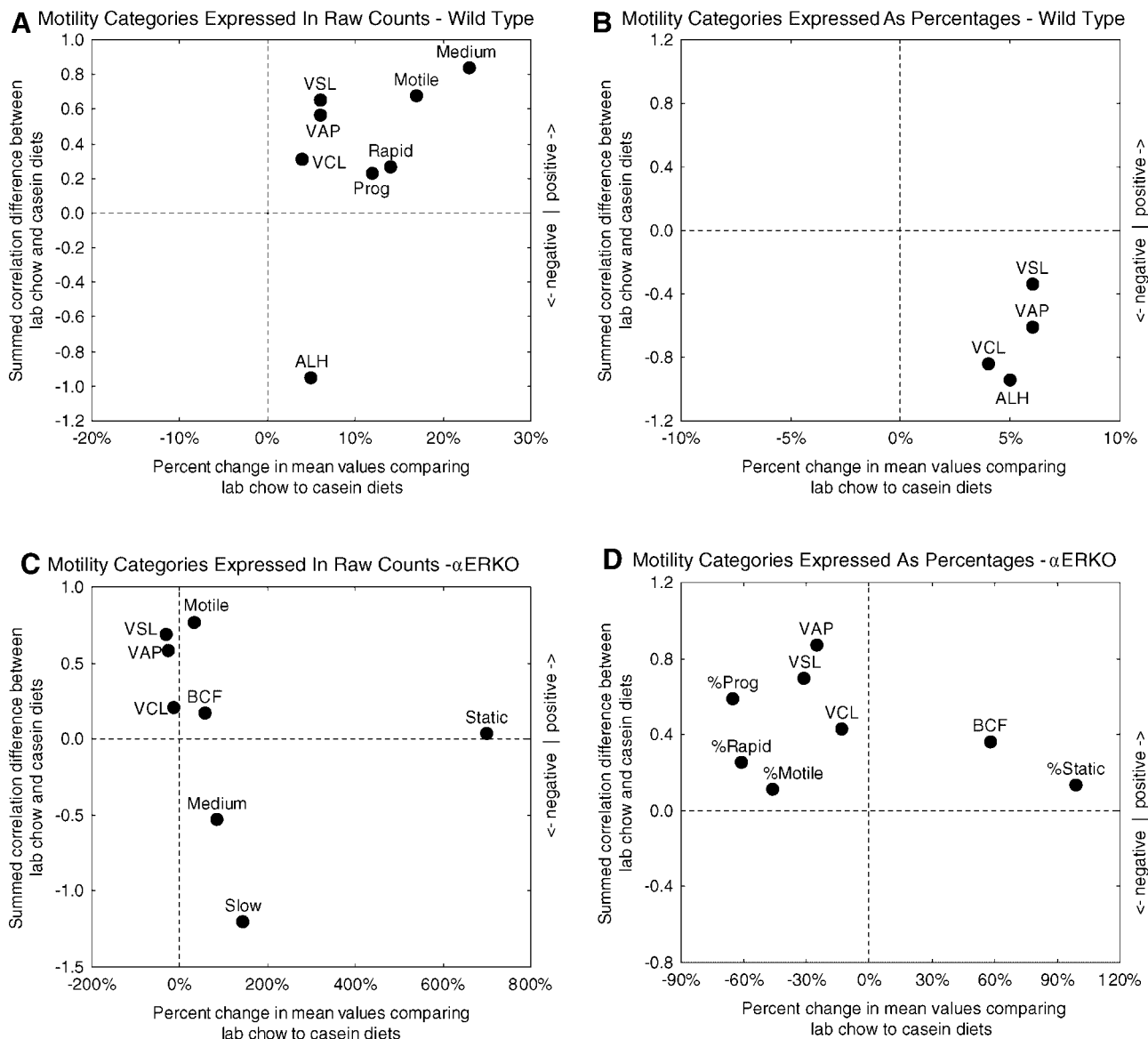


Fig. 4. Scatter plots summarizing changes in the motility behavior of sperm comparing wild type against wild type mice (**Panels A, B**) and α ERKO against α ERKO mice (**Panels C, D**) for animals fed lab chow versus casein diet. In all Panels, only those parameters which showed a significant difference between means by diet (see column 2 of Table 2) are plotted as percentages along the abscissa (from column 1 in Table 2 for wild type (Panels A, B) or for α ERKO (Panels C, D) groups), and differences between the sums of correlation coefficients computed for each of these parameters are plotted along the ordinate (see legend of Fig. 2 for additional details). Panels A and C show results for correlation coefficients computed from raw sperm cell counts (Table 2, raw values, wild type and α ERKO) whereas Panel B and D show results for correlation coefficients computed from motility data expressed as percentages of total sperm cell counts (Table 2, percentages, wild type and α ERKO). **Panels A and B:** the lab chow diet, containing phytoestrogens, is mildly stimulatory in wild type mice; sperm velocities are greater and more sperm are Motile and Progressive and

travel at a rapid or medium speed. This diet also results in more positive correlations for all parameters except ALH, which is more negatively correlated across other parameters with the lab chow diet. In terms of relative motility, velocity descriptors and ALH increase but collectively have more negative inter correlations. **Panels C and D:** the lab chow diet is mildly stimulatory to two motility descriptors (numbers of Motile and Medium) and one sperm descriptor (BCF), and strongly stimulatory to two measures of sluggish sperm (numbers of Slow and Static) in α ERKO mice. Sperm velocities are also slightly depressed in α ERKO mice fed lab chow. In relative terms, the percentage of sperm that are Motile and Progressive and moving rapidly is less and the percentage of sperm that are static is many times greater in α ERKO mice fed lab chow. Panels C and D validate that a component in the diet (phytoestrogens) is responsible for very large changes in parameters BCF and Static observed between α ERKO and wild type mice maintained on a lab chow diet (compare to Fig. 2).

confirms previous reports (Eddy et al., 1996; Cho et al., 2003). In α ERKO mice, a dramatic reduction of water reabsorption, occurring at the level of the efferent ductules, results in a highly diluted sperm. In addition, the accumulation of water leads to a backflow into the

lumen of seminiferous tubules, and disruption of the integrity of the developing germ cell population (Eddy et al., 1996; Lee et al., 2000; Hess et al., 2002). Thus, two mechanisms are involved in the reduction of sperm counts in the epididymis in the α ERKO mouse, one

TABLE 3. Expression of Aquaporins (AQPs)-1, and -9 in the Efferent Ducts of Wild Type and α ERKO Adult Mice Fed Lab Chow or Casein Diets

	Nonciliated cells (microvilli)		Nonciliated cells (basolateral)		Ciliated cells (cilia)	
	Wild type	α ERKO	Wild type	α ERKO	Wild type	α ERKO
AQP-1 ^d	+ ^a	+/- ^b	+ ^c	+/-	+	+/-
AQP-9 ^d	+	+/-	- ^c	-	-	-

^a+ indicates reactivity in all cells.

^b+/- indicates that reactivity is observed in some but not all cells.

^c- indicates absence of reaction.

^dThe effect in KO mice is more pronounced in distal than proximal regions of the efferent ducts.

which appears to be directly due to the failure of efferent ductules to reabsorb luminal water (Hess et al., 1997). From this study, it is also noteworthy that sperm counts differed in both wild type and α ERKO animals depending upon the diet (Table 1). In both animal groups, there was a marked reduction in sperm counts when fed casein rather than lab chow. This suggests that despite their small amounts, phytoestrogens in the lab chow diet enhance either germ cell production or epididymal function, or both mechanisms, in both wild type and α ERKO mice (Table 1).

A possible explanation for the dietary effects could be that phytoestrogens in the lab chow diet augment water reabsorption in the efferent ductules, possibly reducing the backflow of water. Furthermore, while AQP expression was diminished between wild type and α ERKO mice, no apparent changes in immunostaining was observed due to the different diets. Taking these points into account, it is suggested that in α ERKO mice, phytoestrogens in the lab chow diet may stimulate ER β , which is expressed constitutively in the male (Oliveira et al., 2004) or elicit a nongenomic effect that indirectly increases the concentration of sperm in the epididymis. The differences in sperm counts in wild type mice between the different diets could be due to a combined effect of the phytoestrogens on both ER α and ER β and their effects on Sertoli, germ and Leydig cells, respectively, as well as augmentation of efferent ductule functions. Hence, although we cannot rule out the possible effects of phytoestrogens on sperm production at the level of the testis, we cannot overlook the concentrating function of the efferent ductules.

Sperm Motility

While sperm counts were improved in α ERKO mice fed lab chow, the results of this study also clearly indicate that this increased concentration of sperm consists of a lower quality sperm (Table 1). The effects of diet were more dramatic in the case of α ERKO mice than in wild type mice (Table 2; Fig. 4). Taken together with results from sperm counts, these data suggest that the effects on α ERKO mice fed the different diets cannot be explained simply as the consequence of water retention at the level of efferent ducts. Rather it is suggested that altered sperm motilities in α ERKO mice are a reflection of diminished epididymal functions, as the epithelial

cells of this tissue play a major role in producing motile and fertile sperm (Robaire and Hermo, 1988; Orgebin-Crist, 1996; Cornwall et al., 2002). Furthermore, because sperm motility parameters in α ERKO mice that were fed lab chow diet were diminished compared to those fed the casein diet, the data suggest that phytoestrogens in the absence of an ER α may be inhibitory through ER β activity and adversely influence epididymal function and sperm maturation. However, wild type mice fed lab chow showed significantly improved sperm motility compared to those fed casein, suggesting that phytoestrogens in the presence of ER α may be stimulatory through ER β activity, enhancing epididymal function and hence sperm motility. In the uterus, ER β appears to play a role in modulation of ER α functions (Weihua et al., 2000), but whether ER α and ER β modulate each other's function in the male reproductive tract is not known, despite the fact that both ERs are expressed along the epididymis in mice (Fisher et al., 1997; Couse et al., 2001; Zhou et al., 2002).

The epididymis is regarded as a tissue that plays a role in sperm maturation, whereby sperm acquire their proper motility characteristics (Robaire and Hermo, 1988; Cooper, 1995; Cornwall et al., 2002). The coordinated activities of the epithelial cells of the epididymis monitor the luminal environment by secretion and endocytosis of various substances including proteins, water and ions that leads to sperm maturation (Robaire and Hermo, 1988; Cooper, 1995; Turner, 2002). In the present study, multiple motility parameters were altered in α ERKO mice as compared to wild type mice and alterations were noted in both cases depending on the diet fed to the animals. This would suggest that different functions of one or more than one epithelial cell type must play a role in the ultimate production of sperm with diverse motility characteristics, and that the composition of the diet can also affect their functions related to motility. However, despite these findings, we cannot at this time ascribe which specific function(s) of the different epithelial cells are altered that lead to the varying sperm motility parameters noted in α ERKO mice and wild type mice fed on different diets. The data would suggest, however, that the diverse motility features gained by sperm as they traverse the epididymis are governed by a host of varying epithelial cell functions. In summary, the present data reveal a role for

ER α on expression of AQP-1 and -9 in the efferent ductules. In addition, α ERKO mice show reduced sperm counts and motility as compared to wild type mice. A role for the presence of phytoestrogens in the diet is also established for sperm counts and motility in the presence or absence of ER α .

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