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Targeted Disruption of the Estrogen Receptor Gene in Male Mice Causes Alteration of Spermatogenesis and Infertility

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ABSTRACT

The reproductive system of male mice homozygous for a mutation in the estrogen receptor (ER) gene (ER knock-out; ERKO) appears normal at the anatomical level. However, these males are infertile, indicating an essential role for ER-mediated processes in the regulation of male reproduction. Adult ERKO male mice have significantly fewer epididymal sperm than heterozygous or wild-type males. Although spermatogenesis is occurring in some seminiferous tubules of 3- to 5-month-old ERKO males, other tubules either have a dilated lumen and a disorganized seminiferous epithelium with few spermatogenic cells or lack a lumen and contain mainly Sertoli cells. There are no obvious differences in seminiferous tubules at 10 days of age between wild-type and ERKO mice, but the lumen in ERKO males is dilated in all seminiferous tubules by 20 days. However, spermatogenesis progresses and similar numbers of sperm are present in the cauda epididymis of ERKO and wild-type males until 10 weeks of age. Disruption of spermatogenesis and degeneration of the seminiferous tubules become apparent after 10 weeks in the caudal pole of the testis and progresses in a wave to the cranial pole by 6 months. However, the seminal vesicles, coagulating glands, prostate, and epididymis do not appear to be altered morphologically in ERKO mice. Serum testosterone levels are somewhat elevated, but LH and FSH levels are not significantly different from those in wild-type males. Sperm from 8- to 16-week-old mice have reduced motility and are ineffective at fertilizing eggs *in vitro*. In addition, ERKO males housed overnight with hormone-primed wild-type females produce significantly fewer copulatory plugs than do heterozygous or wild-type males. These results suggest that estrogen action is required for fertility in male mice and that the mutation of the ER in ERKO males leads to reduced mating frequency, low sperm numbers, and defective sperm function. (*Endocrinology* **137:** 4796–4805, 1996)

ALTHOUGH the importance of the estrogen receptor (ER) in the function of the female reproductive system is well established, less is known about the role of the ER in the male. Previous studies have indicated that estrogen is involved in regulating male reproductive processes, but the mechanisms have not been defined. For example, it is clear that prenatal or early postnatal treatment with estrogens has detrimental effects on the morphogenesis and function of the male reproductive system (1–3). In addition, low doses of estrogen in adult rats can lower serum LH and testosterone levels, male accessory tissue weights, and testicular content of spermatids and sperm (4). Furthermore, ER has been detected by steroid binding assays in rat Leydig cells (5), a mouse Leydig cell line, in rat and mouse Sertoli cell lines, and rat Sertoli cells in primary culture (6). ER was also found by steroid autoradiography in the duct system of the adult mouse, with the highest level in the epithelium of the efferent ductules and lower levels in the epididymis (7). In fetal mice, ER was detected by steroid autoradiography in the epithelium of the efferent ductules and epididymis (8) and by immunocytochemistry in the epididymal epithelium (9). ER was more abundant in newborn mice in the efferent ductules and initial segment than in the more distal epididymis and was not observed in the ductus deferens (8).

It was reported that the reproductive system appears anatomically normal in male mice homozygous for the ER gene knock-out (ERKO) and that these mice were fertile (10). However, reanalysis of the genotypes with an improved assay (11) determined that the males that had produced offspring were heterozygous for the mutant ER gene. The present study verifies that ERKO male mice are infertile and demonstrates that ER-mediated processes are essential for regulating male reproductive processes. Mutation of the ER gene leads to disruption of seminiferous tubule structure in juvenile mice and affects the quantity and quality of sperm produced in adults. Although it is unknown whether ER mutations affect human reproduction, the recent report of a male patient homozygous for a mutation in the ER gene indicates that this condition can occur in humans (12).

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Materials and Methods

ERKO mice

The ER gene was disrupted in embryonic stem cells by homologous recombination and used to create mice lacking the ER (10). The effects

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of this mutation on female mice were reported previously (10, 11). This study examined the effects of the ER gene mutation on the structure and function of the male reproductive system. Mice were maintained on a 12-h light, 12-h dark cycle at 22–24 C and provided water and food *ad libitum*. Food and bedding were tested and found to be free of estrogenic activity. The embryonic stem cells were from 129J mice and were injected into blastocysts from C57Bl/6 mice. The animals used in this study were produced by backcrossing the agouti offspring from chimeras onto the C57Bl/6 background. All procedures involving animals were approved previously by the NIEHS animal care and use committee and were performed in accordance with USPHS guidelines.

Matings

Continuous mating studies were carried out in an initial attempt to compare the fertility and fecundity of adult male mice that were homozygous for the mutant ER gene ($ER^{-/-}$; n = 11), heterozygous for the mutant gene ($ER^{+/-}$; n = 10), or homozygous for the wild-type gene ($ER^{+/+}$; n = 9). Mice with these three ER genotypes are referred to hereafter as ER knock-out (ERKO), heterozygous, and wild-type, respectively. Males 12–15 weeks old were housed individually, and two 6-week-old female mice (C57Bl/6) were placed with each male. After 1 month, the two females were removed and replaced with two additional 6-week-old females for another month. After each of the 1-month mating periods, females were held for an additional 3 weeks to determine the number of litters and offspring produced. The results of these mating studies are shown in Table 1. At the end of the 2-month mating period, the males were killed, and organ weights and serum hormone levels were determined. These values are shown in Tables 3 and 4.

Short term mating studies were conducted with another group of mice to determine the relative reproductive performance of the males. Groups (five mice per group) of ERKO, heterozygous, and wild-type male mice, 21–23 weeks of age, were housed individually. Two 6-week-old female mice were housed with each male for 2 weeks and then removed. One week later, 6-week-old female mice (C57Bl/6) that had received 5 IU PMSG 2 days earlier and 5 IU hCG 2 h earlier were placed in each male's cage overnight and examined the next morning for copulatory plugs. This was repeated three times at weekly intervals, with each male receiving a total of three opportunities to mate with receptive females. The results of these studies are shown in Table 2.

Sample collection

ERKO, heterozygous, and wild-type males were anesthetized with carbon dioxide and killed by cervical dislocation. Approximately 50 mice of each genotype were used. They ranged from 10 days to 10 months of age and included the mice from the mating studies. The combined seminal vesicles and coagulating glands, the right testis, and the right epididymis and vas deferens were trimmed free of fat, blotted, and weighed. Blood was collected for serum hormone analysis by heart puncture and centrifuged, and the serum was frozen at −70 C until assayed.

Tissues were fixed for histology by immersion in Bouin's solution for 12–16 h, washed in PBS, dehydrated in increasing concentrations of ethanol, and embedded in paraffin. Sections 6 μm thick were stained with hematoxylin and eosin or used for immunohistochemistry. Rabbit antiserum to a peptide sequence in a heat shock protein (HSP70-2), synthesized specifically in spermatogenic cells, was used as described previously (13).

TABLE 1. Reproductive outcome with continuous matings[a]

Estrogen receptor genotype	Litters		Offspring	
	Total	Per male	Total	Per litter
Wild-type (+/+) [N = 9][b]	19	2.1	131	6.9
Heterozygous mutant (+/−)[N = 10]	21	2.1	149	7.1
ERKO mutant (−/−) [N = 11]	0	0	0	0

[a] 12- to 15-week-old males were mated for 2 months.
[b] Two males produced no litters.

TABLE 2. Reproductive performance with short-term matings[a]

Estrogen receptor genotype	Total copulatory plugs per group	Average copulatory plugs per male per mating
Wild-type (+/+) [N = 5]	13	0.87[b]
Heterozygous mutant (+/−) [N = 5]	12	0.80[c]
ERKO mutant (−/−) [N = 5]	2	0.13[d,e]

[a] Males 21 to 23 weeks old were mated once per week for 3 weeks.
[b] Three males produced plugs in all three matings; two males produced plugs in two of three matings.
[c] Three males produced plugs in all three matings, one male produced plugs in two of three matings, and one male produced a plug in one of three matings.
[d] Two males produced one plug each and three males produced no plugs in three matings.
[e] Using a likelihood ratio test, [−/−] differs from both [+/+] and [+/−] ($P \geq 0.001$).

Serum hormones

Serum hormone levels were determined by RIA. Testosterone, FSH, and LH were analyzed, with the estimated values based on a standard with a correction factor applied to each sample for volume. Only single samples could be used because of the small volumes of serum obtained. Assays were performed by Dr. Patricia Fail (R.T.I., Research Triangle Park, NC). Results were analyzed by determining means and SEMS, and ANOVA was performed on the log scale for statistical significance.

Sperm function

Initial studies indicated that sperm function might be impaired in ERKO mice and that the effect was more pronounced in older mice. To examine these possibilities, the sperm counts, motility, and *in vitro* fertilization rates were determined. Sperm were collected from two to four wild-type and ERKO mice each at 8, 10, 12–13, and 15–16 weeks of age. Cauda epididymides were placed in 0.5 ml capacitation medium (14, 15) or commercially available M16 medium (Specialty Media, Lavallette, NJ) brought to 20 mg/ml BSA (crystalline; Life Sciences, Gaithersburg, MD). After most of the sperm had been expressed from the caudae, the tissue was minced with scissors and incubated at 37 C for 15 min to allow sperm dispersal. Clumps of tissue were avoided as aliquots were removed and diluted 1:5 and 1:10. Sperm suspensions were capacitated by incubation for 90 min at 37 C. Sperm counts, estimated percentages of motile sperm, and types of flagellar movement were determined visually by phase microscopy. For *in vitro* fertilization assays, 6- to 12-week-old CD-1 (Charles River, Raleigh, NC) or C57Bl/6 (Frederick Cancer Research and Development Center, Frederick, MD) female mice were hormone primed as before. Cumulus masses containing eggs were collected from the oviducts of these mice under paraffin oil 13–16 h after hCG injection. Cumulus masses were mixed, divided into equivalent groups, and transferred to 50- to 100-μl droplets of medium under paraffin oil. Eggs were inseminated with an equal number of motile sperm in a final droplet volume of 125–200 μl at a final concentration of $1–2 \times 10^6$ motile sperm/ml. This usually required using 5–10 times more ERKO sperm than wild-type sperm. After 8–10 h of incubation at 37 C, eggs were removed from the insemination droplet and washed through three drops of M2 medium (Specialty Media), fixed in 2.5% glutaraldehyde, transferred to slides, and stored in 3.7% formaldehyde overnight. Eggs were dehydrated in 95% ethanol, stained with acetolacmoid (16), and scored as fertilized if two pronuclei and a sperm tail were present within the vitellus. Data were analyzed for statistical significance by calculating the means, SEMS, and ANOVA. *P* values were determined by *t* test (two sample, assuming unequal variances). The results of these studies are shown in Table 5.

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Results

Matings

Continuous mating studies were performed to compare the fertility of wild-type, heterozygous, and ERKO male mice. Reproductive outcome was examined by determining the number of litters and offspring produced by males with different ER genotypes (Table 1). During the 2-month mating period, wild-type males sired an average of 2.1 litters and 6.9 offspring/litter, whereas heterozygous males sired an average of 2.1 litters and 7.1 offspring/litter. Although the reproductive outcome of the wild-type and heterozygous groups did not differ significantly for these two criteria, ERKO males sired no offspring.

A short term mating study was carried out to determine whether the absence of the ER might affect the mating performance of ERKO males compared to that of wild-type and heterozygous males (Table 2). Each male was paired 1 night/week for 3 weeks with individual females that had been hormone-primed with PMSG and hCG to induce superovulation and enhance receptivity to mating. The female mice were examined for the presence of a copulatory plug the next morning. Wild-type males produced copulatory plugs in 13 of 15 mating opportunities, heterozygous males produced plugs in 12 of 15 mating opportunities, and ERKO males produced plugs in 2 of 15 mating opportunities. All of the wild-type and heterozygous males produced plugs in at least 2 of 3 mating opportunities, whereas 2 ERKO males produced 1 plug each.

Reproductive organ weights

At the end of the 2-month mating study, the weights of the seminal vesicles and coagulating glands combined did not differ significantly among age-matched wild-type, heterozygous, and ERKO male mice (Table 3). The testis weight for ERKO mice was significantly less than that for wild-type or heterozygous mice. Although the weight of the epididymis differed statistically between ERKO and heterozygous mice, this may not be meaningful because there was no significant difference in the weight of the epididymis between ERKO and wild-type mice.

Serum hormones

Serum testosterone levels were moderately higher for ERKO males than for wild-type or heterozygous males, with the difference between wild-type and ERKO being significant at the $P = 0.02$ level (Table 4). LH and FSH levels were also somewhat higher in ERKO males than in wild-type or heterozygous males, but these differences were not statistically significant.

Histology

There were no remarkable anatomical or histological differences in appearance in the seminal vesicles or prostate among adult wild-type, heterozygous, and ERKO male mice at necropsy (data not shown). However, the epididymides of ERKO mice were often hypospermic by 20–24 weeks of age, particularly in the caput and corpus regions. The testes were small compared to those of wild-type mice (Fig. 1A) and contained many atrophic and degenerating seminiferous tubules (Fig. 1B). Some seminiferous tubules appeared relatively normal, but others either had a dilated lumen and a thin lining layer of Sertoli cells or a disorganized seminiferous epithelium with few spermatogenic cells, or lacked a lumen and appeared to contain mainly Sertoli cells.

Immunohistochemistry was used to determine whether spermatogenic cells were present within the disrupted seminiferous tubules. The antibody used recognizes a protein (HSP70-2) abundant in pachytene spermatocytes (Fig. 1C) (13, 17) and encoded by a gene containing an imperfect ER binding sequence (estrogen response element) in its promoter region. The HSP70-2 protein was readily detected in ERKO mice, even within severely disrupted seminiferous tubules (Fig. 1D), indicating that some germ cells develop to spermatocytes within these tubules and confirming that the estrogen response element-like sequence does not have a significant role in regulating the expression of this gene (18).

Initial studies noted considerable variability in the proportions of intact, degenerating, and atrophic seminiferous tubules in different ERKO males. The disruption appeared to be more extensive in older males, suggesting a progressive degenerative process. This possibility was examined by comparing the histological appearance of testes from wild-type and ERKO animals at selected ages from early postnatal to adult life. At 10 days after birth there were no obvious differences in the appearance of testes or seminiferous cords between wild-type (Fig. 2A; n = 10) and ERKO mice (Fig. 2B; n = 2). Lumen formation was beginning in an occasional cord at this age in all genotypes. By 20 days after birth, a lumen was present in all seminiferous tubules. However, the lumen was dilated considerably in all of the seminiferous tubules in ERKO mice (Fig. 2D; n = 6). Furthermore, the seminiferous

TABLE 3. Organ weights

Estrogen receptor genotype	Seminal vesicle and coagulating gland	Testis	Epididymis and vas deferens
Wild-type (+/+) [N = 9]	380 (±50)[a]	123 (±3)	59 (±3)
Heterozygous mutant (+/−) [N = 10]	360 (±30)	121 (±4)	65 (±3)
ERKO mutant (−/−) [N = 11]	460 (±30)	58 (±6)[b]	53 (±3)[c]

[a] mg (± SEM); organ weights were from mice 20 to 23 weeks old.
[b] [−/−] differed significantly from [+/+] and [+/−] ($P \geq 0.001$).
[c] [−/−] differed significantly from [+/−] ($P \geq 0.01$).

TABLE 4. Serum hormone levels

Estrogen receptor genotype	Testosterone	LH	FSH
Wild-type (+/+) [N = 9]	9.3 (±4.0)[a]	2.4 (±1.2)[b]	26 (±1.4)
Heterozygous mutant (+/−) [N = 10]	8.8 (±2.8)	1.8 (±0.9)	24 (±1.9)
ERKO mutant (−/−) [N = 11]	16.0 (±2.3)[c]	3.7 (±0.7)	30 (±1.1)

[a] ng/ml (± SEM); serum was from mice 20 to 23 weeks old.
[b] [N = 7].
[c] [−/−] *vs.* [+/+] ($P \geq 0.02$, ANOVA on log scale).

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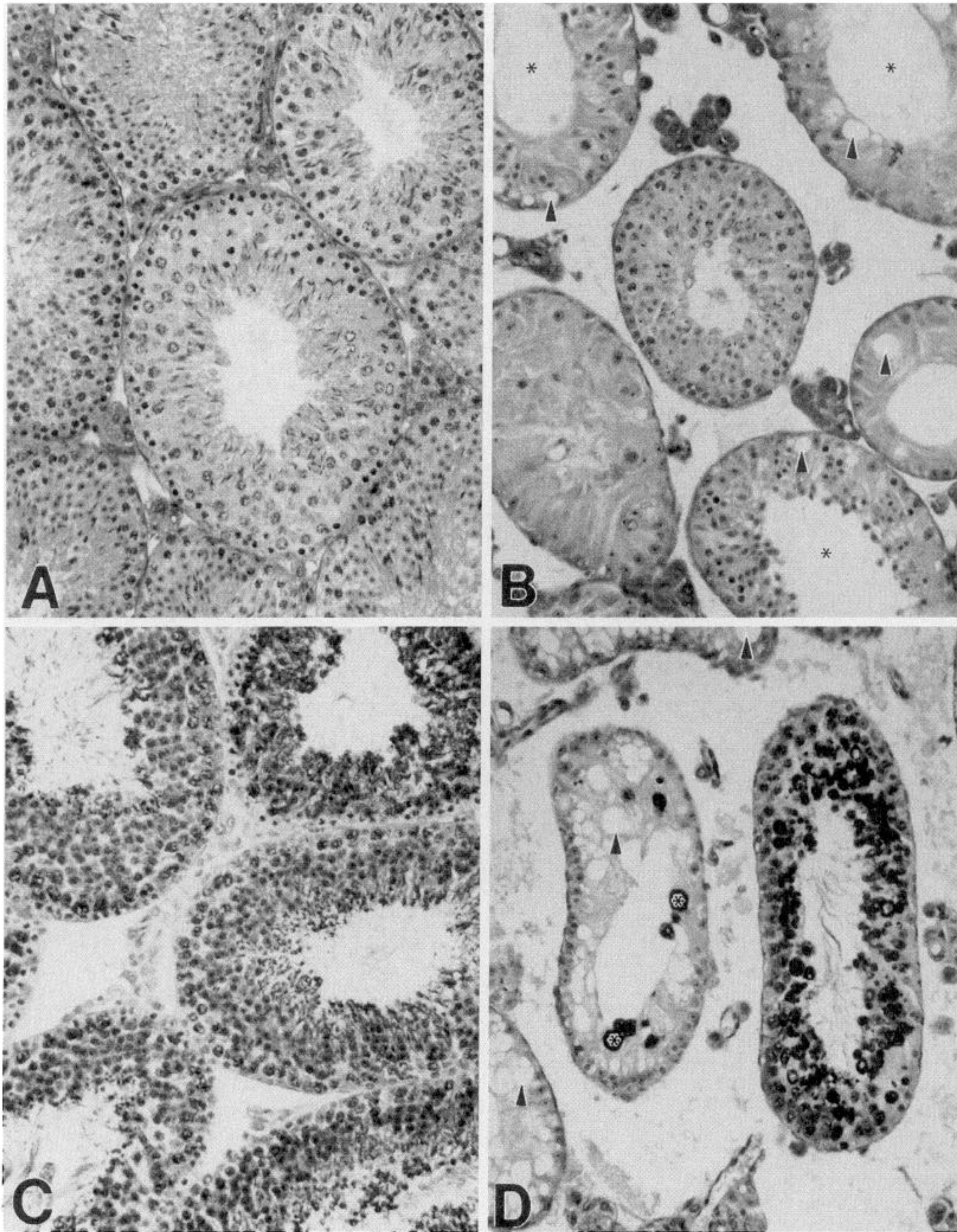

FIG. 1. Histology of testes from adult ERKO and wild-type mice. A, The testis of a wild-type adult mouse (90 days old) contains closely packed seminiferous tubules and limited interstitial space. The diameter of seminiferous tubules, the thickness of the seminiferous epithelium, and size of the lumen vary with the stage of spermatogenesis. B, The testis of an adult ERKO mouse (160 days old) contains seminiferous tubules separated by fluid-filled interstitial space. Although some seminiferous tubules appear similar to those in wild-type mice, most contain vacuoles (*arrowheads*), and the lumen is often dilated (*asterisk*) or obliterated. C, The testis of a wild-type adult mouse (143 days old) immunostained with an antiserum to the HSP70-2 protein specific to spermatogenic cells. The protein is present in pachytene spermatocytes and spermatids, but not in spermatogonia or somatic cells of the testis. D, The testis of an adult ERKO mouse (160 days old) immunostained with the antiserum to HSP70-2. The seminiferous tubules appear to consist mainly of Sertoli cells and some contain vacuoles (*arrowheads*). However, the HSP70-2 protein is present at scattered locations within these tubules (*asterisks*), indicating that some spermatocytes are present. Final magnifications, ×850.

epithelium appeared thinner in ERKO than in wild-type (Fig. 2C; n = 7) or heterozygous (data not shown; n = 8) mice. All tubules were also dilated in ERKO mice at 40 days (data not shown). These features were more pronounced in ERKO mice at 60 days (Fig. 3B; n = 3), but were not seen in wild-type (Fig. 3A; n = 4) or heterozygous (data not shown; n = 4) mice of the same age.

Although the lumen of seminiferous tubules from 20- to

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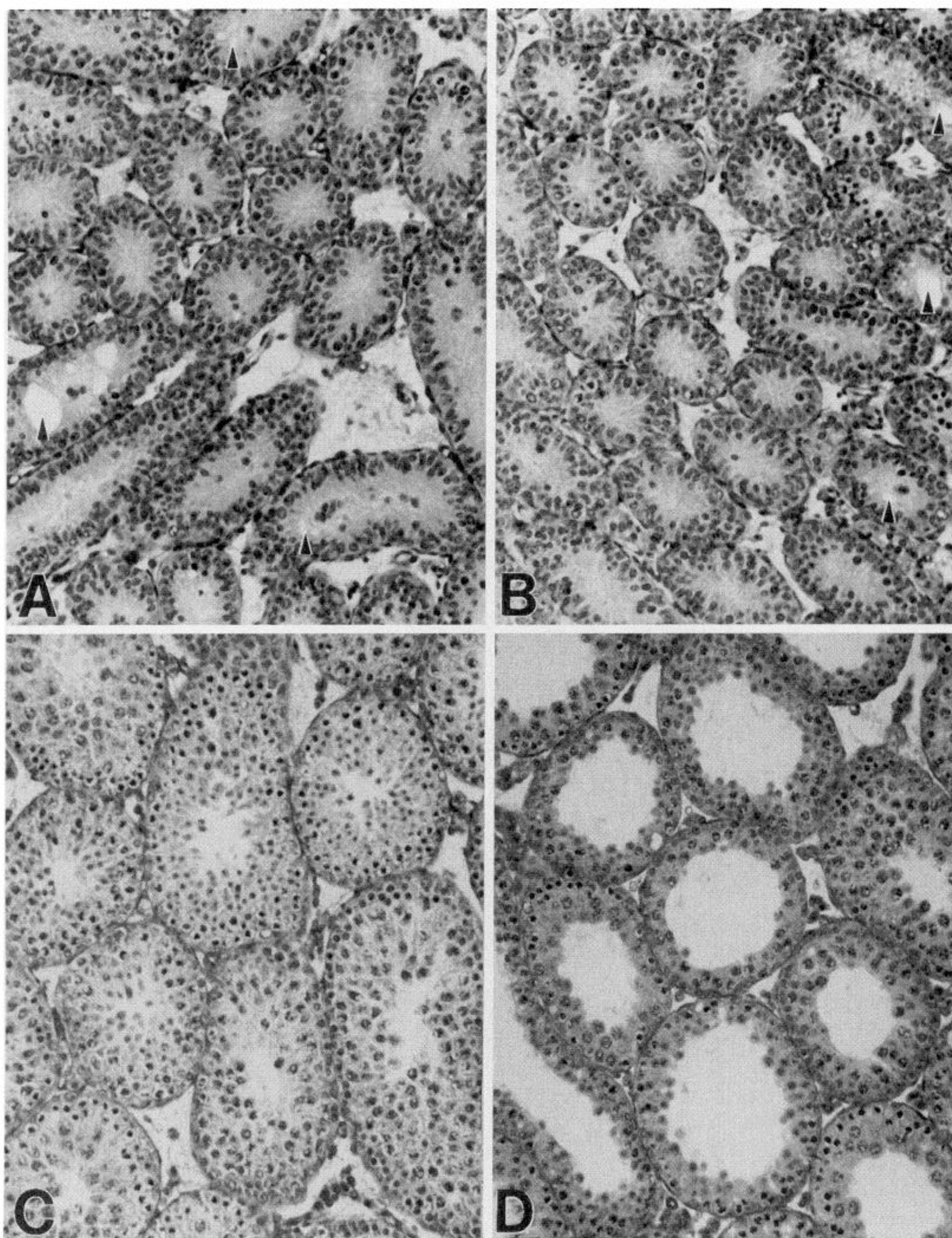

FIG. 2. Histology of testes from juvenile wild-type and ERKO mice. A, The testis of a 10-day-old wild-type mouse. The seminiferous cords are packed loosely, and interstitial spaces are present at scattered locations. Lumen formation is beginning in a few cords at this age (*arrowheads*). The seminiferous epithelium consists of Sertoli cells and spermatogonia, and their nuclei lie near the periphery of the tubule. B, The testis of a 10-day-old ERKO mouse. The association and appearance of the seminiferous cords are comparable to those in wild-type mice, and a lumen is beginning to form in some cords (*arrowheads*). C, The testis of a 20-day-old wild-type mouse. The seminiferous tubules are closely arrayed in most areas and contain a lumen and multiple layers of cells. Most tubules are in stages XII–I of the cycle because the first wave of spermatogenesis is nearly synchronous. In addition to Sertoli cells and spermatogonia, the seminiferous epithelium contains many spermatocytes and a few spermatids. D, The testis of a 20-day-old ERKO mouse. The associations between tubules and the amount of interstitial space are similar to those in wild-type mice. Spermatogenesis also appears to have progressed to about the same stages as in the 20-day-old wild-type mouse. However, the lumen is larger, the epithelium is thinner, and the number of cell layers in the seminiferous epithelium is less than in 20 day-old wild-type mice. Final magnifications, ×850.

60-day-old ERKO mice was dilated, there were no obvious indications of tubule degeneration, and spermatogenesis appeared to be progressing similarly in ERKO and wild-type mice. The lumen of the rete testis also became dilated (Fig. 4), but the efferent ductules and epididymides did not show obvious signs of dilation or other major morphological

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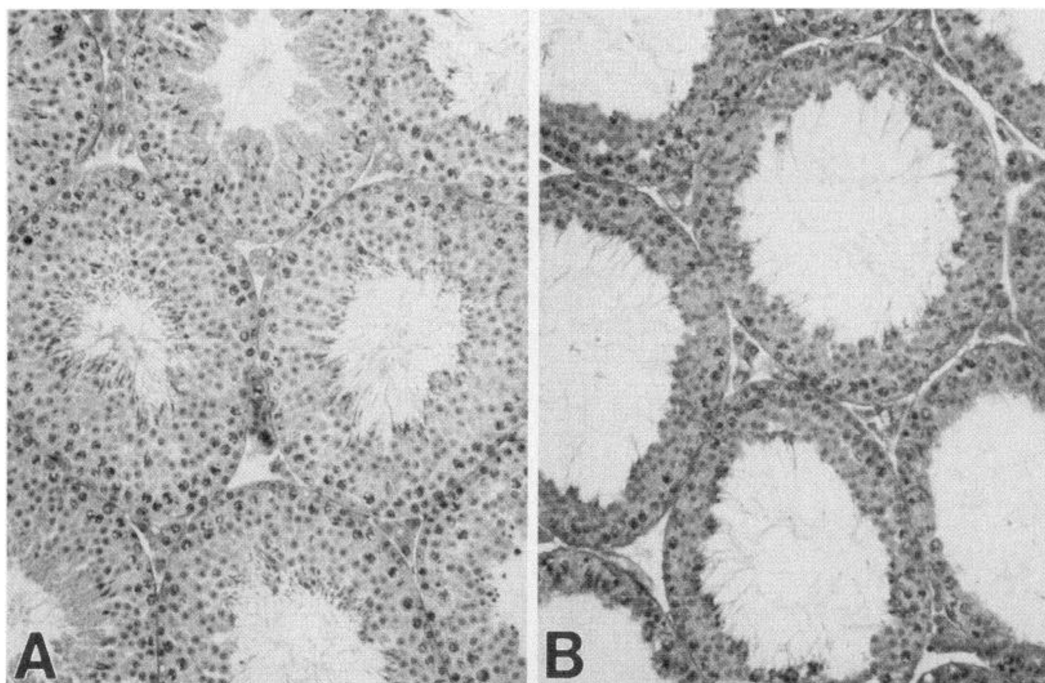

FIG. 3. Histology of young adult wild-type and ERKO males. A, The testis of a 60-day-old wild-type mouse. The seminiferous tubules are closely packed, and the interstitium is filled with Leydig cells and connective tissue. The seminiferous tubules are at different stages of spermatogenesis, and the diameter of the lumen and the thickness of the seminiferous epithelium vary with the stage of spermatogenesis. B, The testis of a 60-day-old ERKO mouse. The association between tubules is similar to that in the testis of 60-day-old wild-type mice, although the interstitium appears somewhat expanded. The tubule lumen is dilated, and the seminiferous epithelium is low in all tubules, but spermatogenesis is occurring. Final magnifications, ×850.

changes in juvenile or adult mice (data not shown). A reduction in sperm concentration was apparent by 10 weeks in the caput region of the epididymis of ERKO males and in more distal portions of the epididymis by 20 weeks when they were examined under a dissecting microscope.

Degenerating seminiferous tubules were seen in ERKO mice beginning at 10–12 weeks of age, and the region of the testis where this appeared was constant. The degeneration first appeared at the caudal pole of the testis (Fig. 4) and progressed in a wave from caudal to cranial as ERKO males became older. Only a few partially intact seminiferous tubules were present at the cranial pole of the testis of most ERKO mice at 6 months of age (data not shown; n = 15).

Sperm function

After incubation in capacitation medium for 90 min, 50–60% of sperm from cauda epididymides of 8- to 16-week-old wild-type males were motile, whereas the motility of sperm from ERKO males declined from 20% to less than 1% during this period (Table 5). The majority of the motile sperm from wild-type males had a vigorous whiplash flagellar beat that produced forward sperm progression. However, sperm from ERKO males beat less vigorously and had less forward progression. In addition, the sperm counts of wild-type mice remained relatively constant from 8–16 weeks of age, whereas those of ERKO mice declined and became significantly lower than those in wild-type mice by 12–13 weeks of age (Table 5). It was common with ERKO males to find sperm heads separated from the flagellum and sperm with retroflexed bending in the midpiece area, but there was no apparent increase in sperm with abnormally shaped heads or other major malformations.

To determine whether ERKO males were infertile only due to low sperm numbers and percent motility, *in vitro* fertilization was carried out with equivalent numbers of motile sperm from ERKO and wild-type males, 8–16 weeks of age (Table 5). Proven fertile C57Bl/6 males were included in each assay, and sperm from 6 of 7 mice fertilized 22 ± 6% of the eggs (data not shown). Previous studies have shown that *in vitro* fertilization rates are low in this strain of mouse (19). Sperm from 6 of 12 wild-type males that previously had not been test-mated fertilized 10 ± 3% of eggs when all age groups were combined, whereas sperm from 13 ERKO males fertilized 0.2 ± 0.2% of eggs (1 of 443 eggs). Although *in vitro* fertilization was ineffective for sperm from ERKO mice in each age group, these differences were not statistically significant because of the small number of animals in each age group. However, there was no obvious age trend, and when groups were combined, *in vitro* fertilization was significantly reduced ($P = 0.008$) for sperm from ERKO mice compared to those from wild-type mice.

Discussion

These studies demonstrate that adult ERKO male mice are infertile and suggest that the lack of ER and estrogen action affects at least three aspects of male reproduction. First, the number of epididymal sperm was substantially lower in ERKO mice than in wild-type mice by 12 weeks of age, indicating that spermatogenesis was compromised. Second, the functional ability of the sperm produced in ERKO males

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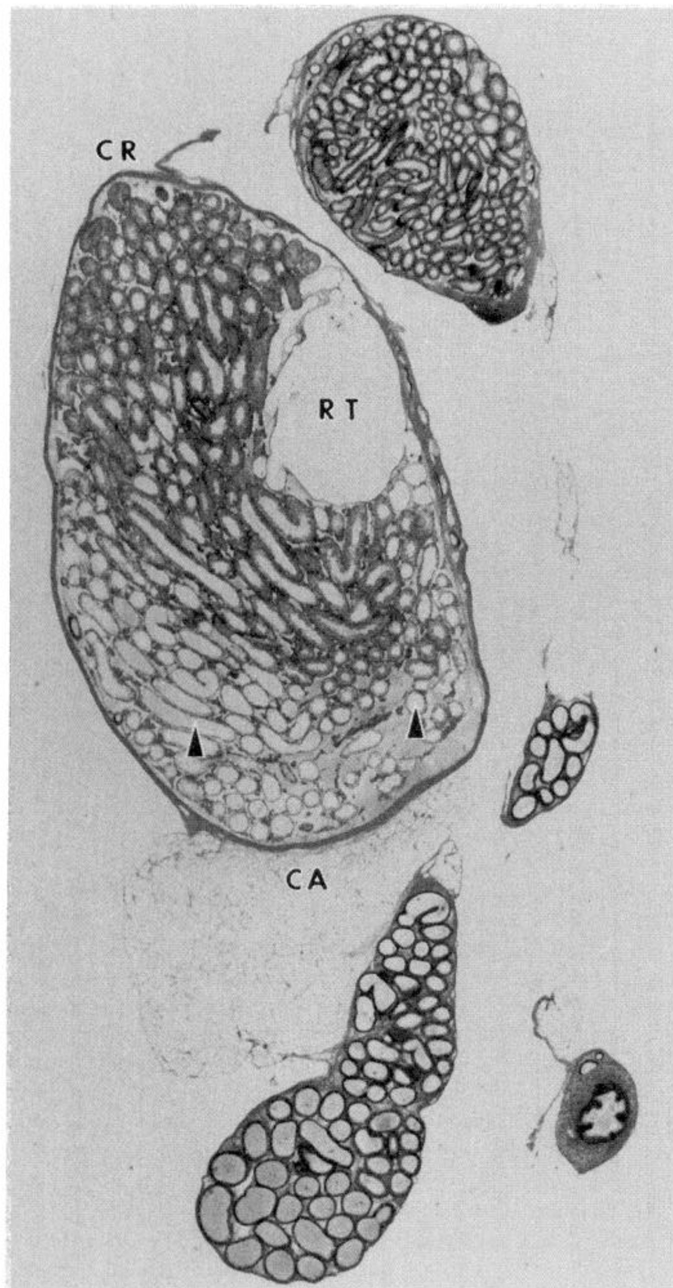

FIG. 4. Longitudinal section of a testis and portions of an epididymis from an adult ERKO mouse (79 days old). The cranial pole of the testis (CR) and part of the caput epididymis are in the *upper part* of the figure, and the caudal pole of the testis (CA) and cauda epididymis are in the *lower part*. The rete testis (RT) is located in the *upper right portion* of the testis section and is conspicuously dilated. The seminiferous tubules in the caudal pole of the testis have a thin seminiferous epithelium and a considerably dilated lumen (*arrowheads*), spermatogenesis is disrupted, and fluid is present in the interstitium surrounding these tubules. However, in the seminiferous tubules in the cranial pole of the testis, the seminiferous epithelium is thicker, the lumen is smaller, spermatogenesis is ongoing, and the tubules are closely situated. Final magnification, ×185.

was impaired. Sperm motility was less vigorous and a lower percentage of the sperm from ERKO males was motile compared to those from wild-type males, even in 8- and 10-week-old mice, with epididymal sperm concentrations being similar for ERKO and wild-type males. In addition, sperm from 8- to 16-week-old ERKO mice were ineffective at *in vitro* fertilization, even when equivalent numbers of motile sperm from ERKO and wild-type males were used. These findings indicate that disruption of the ER gene affects both the production and function of spermatozoa. Third, ERKO males produced fewer copulatory plugs than wild-type or heterozygous males mated with hormone-primed females. This suggests that behavioral aspects of reproduction also are affected in ERKO males and is consistent with preliminary observations that ERKO males mate, but intromit and ejaculate less frequently than wild-type mice (20).

Although disruption of the ER gene had severe effects on male fertility, relatively minor changes were found in the reproductive endocrine parameters measured. The seminal vesicles and coagulating gland weights were not significantly different among 5- to 6-month-old wild-type, heterozygous, and ERKO mice. The testis and epididymis weights were lower in ERKO mice at the end of the 2-month mating study, but this was probably secondary to the disruption of spermatogenesis and the presence of fewer sperm in the epididymis. The average serum testosterone levels of ERKO males were significantly higher than those of wild-type males, but the range of plasma testosterone levels in mice is substantial (21).

The serum LH and FSH levels were not significantly different among ERKO, heterozygous, and wild-type males. This result suggests that estrogen action through the ER is not essential for regulating gonadotropin levels in adult male mice. However, treatment of adult rats with low doses of the antiestrogen tamoxifen reduced serum testosterone and LH levels, caused atrophy of accessory reproductive glands, and led to disruption of spermatogenesis (22–24). Although these effects were thought to be due to suppression of LH release (24), other studies have indicated that tamoxifen treatment of rat Leydig cells *in vitro* reduces testosterone production directly (25). It is unclear if these differences in results are due to differences in species or in the levels, routes, and time span of tamoxifen treatment. However, ERKO mice provide a unique model for further examination of the role of estrogen in regulating male endocrine functions, including gonadotropin release and the initiation of spermatogenesis and testosterone production during puberty (26).

There were not substantial differences in the epididymis, seminal vesicles, coagulating glands, and prostate among ERKO, heterozygous, and wild-type males at the histological level, but the absence of ER in these tissues may have important functional effects not apparent in this analysis. Obvious morphological differences were seen in the seminiferous tubules and rete testis, with both becoming dilated in ERKO males between 10–20 days after birth. This coincides with the period of Sertoli-Sertoli tight junction formation (27–29), and the beginning of fluid production and lumen formation in the seminiferous tubule (30, 31).

Ligation of the efferent ductules causes seminiferous tubules to become dilated (32–34), suggesting that seminiferous tubule dilation in ERKO males might be caused by blockage of the duct system. However, this seems unlikely because sperm are abundant in the cauda epididymis well after the dilation occurs. Another possibility is that dilation is caused

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TABLE 5. Sperm function

Age (Weeks)	Sperm Counts (10^6/ml)		Sperm motility (%)		*In vitro* fertilization (%)	
	(+/+)	(−/−)	(+/+)	(−/−)	(+/+)	(−/−)
8	37 (±19)[a] [N = 3][b]	21 (±12) [N = 3]	58 (±8) [N = 4]	17 (±3) [N = 3]	6 (±6) [N = 2]	0 (±0) [N = 2]
			(P > 0.01)[c]			
10	26 (±2) [N = 4]	21 (±4) [N = 4]	58 (±5) [N = 4]	23 (±1) [N = 4]	13 (±8) [N = 4]	1 (±1)[d] [N = 4]
			(P = 0.03)			
12–13	35 (±4) [N = 3]	6 (±2) [N = 3]	57 (±7) [N = 3]	7 (±3) [N = 3]	14 (±9) N = 3]	0 (±0) [N = 3]
	(*P* = 0.002)		(*P* = 0.003)			
15–16	43 (±7) [N = 4]	6 (±4) [N = 4]	55 (±5) [N = 4]	0.5 (±0.3) [N = 4]	4 (±4) [N = 3]	0 (±0) N = 4]
	(*P* = 0.004)		(*P* > 0.001)			

[a] Mean (± SEM).
[b] Number of animals analyzed.
[c] *P* values are results of *t* tests for [+/+] *vs.* [−/−].
[d] One of 162 eggs apparently was fertilized.

by overproduction of luminal fluid by Sertoli cells. Although testosterone appears to be involved in regulation of fluid production by Sertoli cells (35), the role of estrogen in this process is uncertain. Estrogen is reported to regulate *N*-cadherin expression in the mouse testis (36), suggesting that the lack of ER may alter *N*-cadherin expression in ERKO males and perturb cell-cell contacts essential for maintaining appropriate fluid distributions in the testis. In addition, seminiferous tubule fluid is high in potassium (37), and estrogen has been found to regulate voltage-activated potassium channels in smooth muscle cells by a cGMP-dependent mechanism (38). Lack of ER may alter fluid production in the seminiferous tubules of ERKO mice by disrupting such a mechanism in Sertoli cells. These reports suggest that determining whether fluid transport by the seminiferous epithelium is altered in ERKO mice would be helpful for understanding the role of estrogen in testicular function.

Dilation of seminiferous tubules in ERKO mice might also be caused by reduced fluid reabsorption in the efferent ductules. It has been estimated that the efferent ductules reabsorb 83% of the fluid leaving the testis in the rat (39) and 85% in the ram (40). Ligation of the efferent ductules, but not of the distal epididymis or ductus deferens, caused seminiferous tubule dilation in 24–36 h and led to their subsequent degeneration in the rat (32–34). In addition, sodium-potassium adenosine triphosphatases involved in regulating fluid movement across epithelia are abundant in the efferent ductules (41, 42). It is thus significant that the efferent ductules have higher levels of ER than other regions of the male duct system (7, 8, 43). If mechanisms for fluid reabsorption in the efferent ductules are regulated by estrogen, functional deficiencies in these mechanisms in ERKO mice may lead to fluid accumulation within the rete testis and seminiferous tubule lumen and the subsequent disruption of spermatogenesis. However, additional studies will be needed to determine whether fluid reabsorption in the efferent ductules is regulated by estrogen and to learn whether disruption of this process is a cause of infertility in ERKO males.

An interesting finding in this study was that seminiferous tubule degeneration in ERKO mice occurs in a wave from the caudal to the cranial pole of the testis. Pathological conditions in the testis (44) or efferent duct ligation (32–34) can lead to dilation and subsequent degeneration of some or all of the seminiferous tubules. However, degeneration has not been reported to occur in a caudal to cranial progression in these conditions. One possibility is that the wave of seminiferous tubule degeneration in ERKO mice is a reflection of the pattern of distribution of the vasculature within the mouse testis. The cranial pole is well vascularized, whereas the caudal pole appears to receive distal branches and tributaries of the arterial, venous, and lymphatic systems (45). Furthermore, testicular blood flow was reduced by 24 h (34), and testicular weight was increased by 36 h after efferent duct ligation in the rat (32). We hypothesize that accumulation of fluid within the lumen of seminiferous tubules in ERKO mice leads to increased intratesticular pressure and gradually compromises testicular blood flow. This might first affect the less vascularized caudal pole and subsequently cause reduced blood flow to the cranial part of the testis, resulting in a progression of tubule degeneration from the caudal to the cranial part of the organ. In addition, it has been found that the cremaster muscle is thickened and the cremaster sac is smaller in ERKO males than those in heterozygous and wild-type males (46). These changes may also affect testicular blood flow and contribute to the promotion of seminiferous tubule degeneration in ERKO mice.

Epididymal sperm motility and concentration were reduced progressively with age in ERKO mice compared to wild-type mice, with reduced motility occurring at an earlier age than reduced concentration. Previous studies suggest that there may be more than one cause for these changes. Although androgens are the major steroids involved in regulating testicular function, ER is present in Sertoli cells (5, 6) and epithelial cells lining the male duct system (7–9, 43). In addition, mouse germ cells contain P450 aromatase and are capable of converting androgen to estrogen (47). It has been hypothesized that estrogen produced in germ cells could influence androgen production by Leydig cells, gene expression in spermatogenic cells, or epithelial cell function in the epididymis (47). This suggests that changes in epididymal sperm motility and concentration in ERKO mice could be

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directly caused by the loss of estrogen action on Sertoli cells and germ cells.

There may also be indirect causes for the reduced sperm concentration and motility in ERKO males. The lower sperm concentration in ERKO mice is probably caused by dilation of the seminiferous tubules, leading to disruption of spermatogenesis. We have suggested that this dilation is the indirect result of reduced fluid reabsorption in the efferent ductules. In addition, estrogen affects sperm transport and maturation in the epididymis (48), processes essential for sperm to gain motility and the ability to fertilize (49). If sperm transport and maturation are altered in ERKO mice, these are likely to contribute to the reduced sperm motility and may be a major cause of the failure of these sperm to fertilize.

In conclusion, this study demonstrated that the ER is required for fertility in male mice, and that the absence of the ER is detrimental to spermatogenesis, sperm function, and mating performance. It also showed that the seminiferous tubules become progressively disrupted during the postpubertal period, and that a wave of tubule degeneration progresses from the caudal to the cranial pole of the testis between approximately 3–6 months of age. These results suggest that the ER has a role in regulating multiple aspects of male reproduction, but it remains to be determined what mechanisms in ERKO males are perturbed that lead to the disruption of reproductive processes.

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