

Morphometric Study of the Gubernaculum in Male Estrogen Receptor Mutant Mice

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ABSTRACT: To determine role of estrogen receptors in testicular descent, a morphometric study of the testis and structures derived from the gubernaculum was made in sexually mature male mice having an estrogen receptor disrupted gene mutation (ERKO). Macroscopic dissections and sagittal serial sections were made of the pelvis of four wild-type mice, four mice heterozygous for the ERKO mutation, and four homozygous ERKO males. By external morphological examination the testes appeared to be descended in all three genotypes. All mice had development of a cremaster sac, which is derived from the gubernaculum, but this was twice as large in wild-type mice than in both the heterozygote or homozygote ERKO groups.

The cause for the smaller cremaster sac appeared to be excessive development of the cremaster muscle in ERKO mice. The thickened muscle was associated with postmortem retraction of the testes into the inguinal canal or abdomen. Spermatogenesis and testicular volume were deficient in homozygous ERKO mice at this age. This study demonstrates that estrogen has a previously unknown role in masculine sexual development of the gubernaculum and the structures derived from it, such as the cremaster muscle.

Key words: ERKO, estrogen, estrogen receptor, cremaster, processus vaginalis, testis, gubernaculum.

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Testicular descent is a complex mechanical process controlled by hormones from the testis itself (Hutson and Donahoe, 1986; Hutson and Beasley, 1992). In experimental models such as the fetal mouse, exogenous estrogens have been shown to interfere with descent (Hutson, 1987; Hutson et al, 1990). At birth, animals treated *in utero* with estrogens show high intraabdominal testes near the kidneys, persistence of the Mullerian ducts, and atrophy of the gubernaculum (Raynaud, 1958). These models have proved useful for dissecting out the hormonal regulation of testicular descent, but they give little insight into any possible effects of endogenous estrogens from the maternal circulation.

The advent of a mouse model exhibiting a functional knockout of the estrogen receptor (ER) has allowed the role of endogenous estrogens in the male to be examined. The ER knockout (ERKO) mouse is homozygous for a mutant disrupted ER gene, in which a neomycin sequence containing premature stop codons and polyadenylation sites has been inserted into exon 2 of the ER gene by homologous recombination (Lubahn et al, 1993; Korach, 1994). Both male and female mice survive to adulthood

with normal gross external phenotypes. Females have hypoplastic uteri with no detectable corpora lutea, and males are grossly normal except for small testes and low sperm counts.

The aim of this study was to examine the anatomy of the male urogenital tract in the ERKO mouse, with particular emphasis on development of the gubernaculum, the anlagen of the processus vaginalis, and the cremaster muscle.

Materials and Methods

The specimens used in the study were from 21- to 25-week-old male littermates of interbreeding of heterozygous ERKO mice, exhibiting all three ER genotypes. Animals were sacrificed by cervical dislocation under a NIEHS-approved animal protocol. Tissues were dissected immediately and fixed in 10% formalin. Caudal portions of 12 mice were examined. The specimens were in three groups: four wild-type, four ER heterozygous, and four ER homozygous mutants. Although the genotypes of the specimens were determined, the morphological studies of the tissues were carried out 'blind.'

Genotyping of offspring from heterozygote interbreeding was performed by polymerase chain reaction (PCR) analysis, as previously described (Lubahn et al, 1993). Primer pairs for the endogenous and disrupted ER gene were used to detect a PCR product of 239 base pairs for the endogenous gene and 649 base

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FIG. 1. Macroscopic dissections of the inguino-scrotal region of formalin-fixed tissue after removal of skin. (Left), Wild-type mouse (56-41), showing projection of the cremaster sac (C) caudal to the penis (P). The testes are fully descended in this specimen. (Center), Heterozygote ERKO mouse (56-61) with an appearance similar to that of the wild-type mouse. In this specimen the testes are in the processus vaginalis, although in the other three specimens the testes were retracted. (Right), Homozygote ERKO mouse (56-67), showing very small cremaster sacs and testes (T) retracted inside the abdominal cavity (anterior abdominal muscles have been excised).

pairs for the disrupted ER gene. Offspring possessing all three genotypes were detected by this procedure.

All dissections were done in an alcohol bath (80% ethanol) using a Leica dissecting microscope. The specimens were skinned and the subcutaneous fat removed. Macroscopic observations were made, determining the position of the testes and general appearance of all structures. The specimens were photographed using Kodak Ektachrome 100 ASA film (film code: EPN 404; Kodak, Rochester, New York).

The urogenital system of each specimen was excised and divided into left and right halves along the sagittal plane. Each half system was processed histologically (Tissue-Tek III, Sakura Fine Technical Co., Tokyo, Japan), and serial sections were cut from lateral to medial. The sections were stained alternately with hematoxylin-eosin (H&E) and trichrome. Histological observations were made, again noting the general appearance of structures in each group. Photographs were taken of typical sections representing the three groups, using Ektachrome 160-T (Kodak) and Ilford PAN-F 50 (Ilford, Mobberley, Cheshire, UK) film.

Quantitative measurements of the cremaster muscle and the testis were made. The section of each block where the remnant of the gubernacular cord attached to the caudal epididymis was selected. This slide was projected and the outline of the cremaster muscle traced. Using, in addition, a Leica (Deerfield, Illinois) calibrated slide and a Sigmaplot digitizer (Jandel Scientific, Corte Madera, California), the surface area of muscle was calculated. The length of the processus vaginalis (PV) within the cremaster sac was measured from the projections. The largest cross section of the testis for each block was also selected. Again by a calibrated

projection of the image, measurements of diameters were made. The volume of the testis was approximated to the volume of an ellipsoid and thus calculated using integral calculus.

Statistical methods used to compare data from different groups were mean, standard deviation (SD), and Student's *t*-test.

Results

Although no gross external morphological differences had been evident, macroscopic dissection did reveal some differences between the three groups (Fig. 1). In the wild-type group the testes were all descended (Fig. 1, left) except for one, which was atrophic and intraabdominal. The heterozygote ERKO mice had three of eight fully descended testes (Fig. 1, center), and five of eight testes were retracted, whereas in the homozygote ERKO group only one testis was in the PV, because six of eight were retracted up to near the bladder neck (Fig. 1, right). The cremaster sac was bigger in both length and width in the wild-type animals compared with the ERKO animals; the descended testes were lower in the cremaster sac in the wild-type than in the ERKO groups (Fig. 1). Only one of eight wild-type testes had retracted out of the scrotum with fixation.

Sagittal sections of the cremaster sac traced onto paper confirmed that the wild-type mice had much larger sacs than the ERKO groups. The normal sac was capacious,

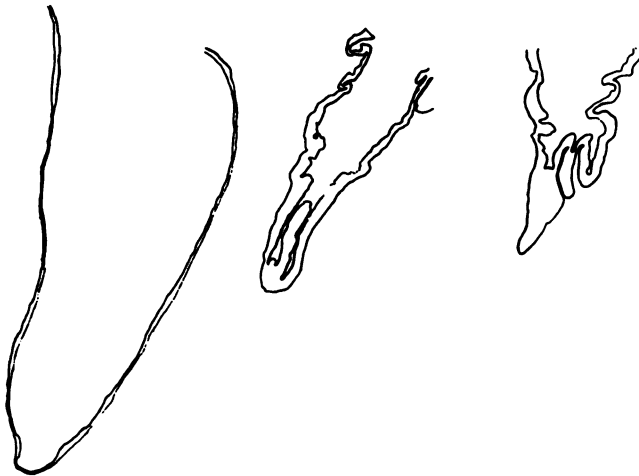


FIG. 2. Tracings of cremaster sacs as used to calculate the area of the cremaster muscle. The wild-type sac (left) is capacious and very thin-walled. The heterozygote (center) sac is small with thick muscle (the testes were retracted out of the sac in this specimen). The homozygote (right) sac is also small and thick-walled; it contained no testes.

with a very thin rim of cremasteric muscle (Fig. 2, left). ERKO mice had small, irregular sacs, with a much thicker layer of cremaster muscle (Fig. 2, center and right). The end of the sac often showed deep folds (Fig. 2, right).

Histology of the cremaster sacs showed normally appearing cremaster muscle in the three groups, apart from the different thicknesses. The microscopic appearance of the testis in the heterozygote ERKO mice was similar to that of the wild-type mice, whereas the homozygous ERKO mice had degenerated, empty seminiferous tubules. In the wild-type and heterozygote epididymis, sperm storage was normal, but in the homozygote the epididymal tubules were filled with proteinaceous debris.

Morphometric assessment of the histological sections confirmed the qualitative differences seen both macro- and microscopically (Table 1). The length of the cremaster sac (PV length), from the abdominal wall to its tip, was nearly twice as long in wild-type males compared with the ERKO groups. The cross-sectional width of the cremaster muscle near its abdominal wall attachment (proximal cremaster width) was about half that in wild-type mice compared with heterozygote ERKO ($P < 0.02$) and homozygote ERKO mice ($P < 0.05$).

Furthermore, the muscle thickness near the tip of the

sac was also less than half that in wild-type animals compared to both experimental groups ($P < 0.05$). The cross-sectional area of cremaster muscle, in the sagittal section showing the tip of the sac, using Sigmaplot, was significantly greater in the two ERKO groups compared with the wild-type ($P < 0.01$).

The testicular volume, as calculated from the section with the largest cross-sectional area, was similar in controls and heterozygous ERKO animals, but it was decreased in homozygote ERKO animals (Table 1).

Discussion

The ERKO mouse has a small cremaster sac but with a greatly increased muscle bulk compared with the wild-type animal. In addition, there is a defect in spermatogenesis, as noted previously (Korach, 1994; Washburn et al, unpublished data). Most testes were not located within the cremaster sac after fixation, despite the absence of any gross anomaly of testicular descent noticed by external examination. This suggests that the thickened cremaster muscle may retract the testis of the ERKO mice more easily into the abdomen than in wild-type mice. Whether the associated defect in spermatogenesis seen in the homozygous ERKO is secondary to abnormal retraction or an unrelated anomaly cannot be determined completely from this study, although normal sperm production and fertility are seen in the heterozygous ERKO mouse despite a similar cremaster muscle. This suggests that the testicular defect is unrelated.

These studies of the genital tract of male ERKO mice suggest that estrogen receptors (ER) have a role in male sexual development that was not recognized previously. Currently it is widely held that estrogens are not involved in early sexual development, because this would affect both males and females (Jost, 1979). ER have not been identified in human male external genitalia, but they are present in female structures, such as the stroma of the labia minora (Kalloo et al, 1993). The gonad and Wolffian duct of the fetal mouse are reported to contain ER on day 13 (the onset of sexual differentiation), but they mostly disappear by birth (Greco et al, 1992). The epididymis began expressing ER on day 19 of gestation (Cook et al, 1991).

Table 1. Dimensions of processus vaginalis (PV), cremaster muscle, and testis in ERKO mice

Genotype (n = 4)	PV length (mm)	Proximal cremaster width (mm)	Distal cremaster width (mm)	Cross-sectional area of cremaster (mm ²)	Testis volume (mm ³)	Retracted testes
Wild-type	11.3 ± 1.4	0.11 ± 0.04	0.10 ± 0.03	2.70 ± 0.69	48.1 ± 14.8	1/8
Heterozygote	5.1 ± 1.9	0.26 ± 0.04	0.28 ± 0.18	4.56 ± 0.82	57.5 ± 17.7	5/8
Homozygote	6.6 ± 4.0	0.22 ± 0.05	0.23 ± 0.17	4.37 ± 1.48	25.2 ± 13.4	6/8

ER have not been reported in the gubernaculum, but this may be because this structure has not been examined directly. The gubernaculum is the genito-inguinal ligament in the embryo, linking the gonad and genital ducts in the urogenital ridge to the developing anterior abdominal wall, the muscles of which form around the column of embryonic mesenchyme to define the site of the future inguinal canal (Backhouse and Butler, 1960). Certainly there is considerable indirect evidence for a role for estrogen in the fetal gubernaculum (Hutson, 1987). Exogenous estrogens in the fetal mouse cause atrophy of the normal swelling reaction (Raynaud and Raynaud, 1958), retention of the Mullerian ducts, and complete failure of testicular descent (Hadziselimovic, 1983; Hutson, 1987). The mechanisms postulated to explain these phenomena include inhibition by estrogen of the hypothalamic-pituitary-testicular axis (Hadziselimovic, 1983) and inhibition of putative Mullerian-inhibiting substance (MIS) action on the gubernaculum (Hutson, 1987). Undescended testes in association with retained Mullerian ducts may be secondary to mechanical inhibition of descent by the uterus (Josso et al, 1983) or may represent a dual action of MIS on both the Mullerian ducts and the gubernaculum (Luthra and Hutson, 1990). Regardless of whether estrogens act via suppression of testosterone or MIS action, there is a definite effect on the developing gubernaculum.

During the first phase of testicular descent relative to the position of the ovary, the gubernaculum undergoes a "swelling reaction" by an increase in extracellular matrix and water (Backhouse and Butler, 1960; Wensing, 1968). This enlargement of the caudal end of the male gubernaculum holds the testis near the inguinal region while the embryo enlarges. By contrast, the female gubernaculum remains thin and elongates, in proportion to embryonic growth, to become the round ligament and ligament of the ovary (Attah and Hutson, 1991). This is associated with ascent of the ovary away from the future internal inguinal ring (van der Schoot, 1993; Shono et al, 1994).

In the second, or inguino-scrotal phase of testicular descent, the gelatinous gubernaculum migrates from the inguinal region across the pubis and into the scrotum under the action of androgens (Hutson, 1985). There is now a considerable body of evidence that this migratory phase requires the genitofemoral nerve and the neuropeptide calcitonin gene-related peptide (Hutson et al, 1996). During the second phase the PV develops inside the gubernaculum as a diverticulum of the peritoneum. The cremaster muscle also develops within the outer rim of the gubernaculum outside the PV.

As exogenous estrogens cause atrophy of normal gubernacular development, complete lack of estrogens might cause excessive gubernacular development, if endogenous estrogens inhibit gubernacular growth. Excessive gubernacular development cannot be observed directly in this

study of sexually mature males, but the excessive growth of the cremaster muscle seen in ERKO mice may indicate excessive prenatal enlargement with a subsequent increase in the amount of differentiation into cremaster muscle. Further studies of fetal and neonatal ERKO mice will be needed to evaluate this possibility.

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