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Early development of the gubernaculum and cremaster sac in estrogen receptor knockout mice

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Abstract AIM exogenous estrogen causes gubernacular atrophy and cryptorchidism in fetal rodents. Mice with an estrogen receptor-α (ERα) disrupted gene mutation (αERKO) were studied to determine whether ablation of endogenous estrogen action, through ERα, had an effect on gubernacular development. Serial sagittal sections were made of the pelvis in fetal and day 7 postnatal wild-type and αERKO mice with the estrogen receptor-α "knockout" gene mutation. Wild-type ($n = 24$), heterozygote ($n = 13$) and αERKO mice ($n = 12$) were sacrificed at 16, 17 and 18 days fetal life and at 7 days postnatally. The size of the gubernaculum, cremaster muscle, cremaster sac, and the width of the sac at both ends in day 7 mice were quantitated by computer analysis. Visually and statistically the ERKO mice could not be separated from the wild-type mice during fetal life. At day 7 postnatally, a thicker cremaster sac was noted morphologically, and also a statistically significant difference was seen in the width of the cremaster sac at the sac's tip. Sac area, cremaster muscle area and the width of the sac at the sac's end did not differ significantly. Overall there is minimal phenotypic change observed in the αERKO mouse compared to wild-type at the early developmental stages investigated. However, at postnatal day 7, there is a difference in the width of the cremasteric sac tip. This suggests that the effect of ERα, and thus signaling on the developing gubernaculum, occurs late in development. Alternatively, an action from the recently discovered ERβ may be involved. Exploration of a βERKO and the double knock-out αERKO/βERKO mouse should be informative in evaluating the effect of endogenous estrogens in gubernacular development.

Key words ERKO · Estrogen receptor α · Gubernacular development · Cremaster muscle · Cremaster sac

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Introduction

Estrogen receptors are found throughout the female reproductive tracts, including epithelial cells of Mullerian ducts [1] and mesenchymal cells of all fetal reproductive organs [2], and are suspected to have a role in normal female reproductive development [8]. Such a role can be seen in transgenic mice with an estrogen receptor mutation (αERKO) that are unresponsive to the ligand estradiol. Female αERKO mice exhibit hypoplastic uteri, hyperemic ovaries devoid of corpora lutea and infertility [5, 14, 15, 18] and reduced maternal behavior [21]. Male αERKO mice are also infertile [5, 14, 15] and show altered behavior with less aggression [22] and decreased male mating motivation [20]. Estrogen receptors, present in the 10-day-old fetus [7, 9] have been found extensively in the male reproductive tract, such as the efferent ductules and initial segments of the epididymis [8]; however, their role in male reproductive development during fetal life has not been clearly defined.

Exogenous estrogens have been shown to result in high intraabdominal testes at birth, with persisting Mullerian ducts and gubernacular atrophy when treated in utero [24], and interfere with testicular descent in the fetal mouse [12, 13]. The use of αERKO mice has allowed investigation of the effect of endogenous estrogens, showing a decrease in cremaster sac size with excessive cremaster muscle development, deficient

spermatogenesis, low testicular volume and postmortem testicular retraction in 21- to 25-week-old littermates [4].

Donaldson et al. [4] postulated that complete estrogen receptor absence in the male mouse may cause excess gubernacular development. The aim of this study was to evaluate the effect of estrogen lack on gubernacular development and to determine, if indeed there was an effect, and whether it occurred early in life or was a later phenotypic change. Thus αERKO mice were studied at days 16, 17 and 18 of fetal life and at 7 days postnatally. Emphasis has been placed on gubernacular and cremaster muscle development, processus vaginalis size and Mullerian duct regression.

Materials and methods

ERKO mice maintained at the Laboratory of Reproductive and Developmental Toxicology (National Institutes of Health, National Institute of Environmental Health Sciences, Research Triangle Park, N.C., USA) were sacrificed at 16, 17 and 18 days fetal life and 7 days postnatally under an approved NIEHS animal study protocol. These mice were littermates, resulting from the interbreeding of heterozygous αERKO mice and exhibited all three ERα genotypes. The morphological/histological analyses were carried out "blind" with genotyping being performed independently. The hemisected pelvic portions were embedded in paraffin for sectioning at 5-μm thicknesses. Gelatin was used as the slide fixative. The clean glass slides were dried at 60 °C for 20 min, then brought to water, dewaxed with xylene (2 × 2 min), 100% ethanol (3 × 1 min), and then water (1 min). The sections were stained with hematoxylin (5 min), washed in water (1 min), differentiated in 1% hydrochloric acid (two dips), washed in water (1 min), dipped in ammonia (two dips), and again washed in water (1 min). After staining with eosin (4 min), and then washing briefly in water (three dips), the sections were dehydrated in absolute ethanol (3 × 15 s) and cleared in xylene (3 × 1 min). Selected slides were stained using trichrome staining as outlined by Gomori [6] for use in photography.

This enabled the histological analysis of relative amounts of cremaster muscle within the gubernacular swelling. The size of the gubernaculum and cremaster muscle was quantitated by computer analysis (Sigmaplot digitizer; Jandel Scientific, Corte Madera, Calif., USA), by projecting all samples to the largest cross-section of gubernaculum and plotting the respective borders. At least four pelvic portions were analyzed per group. The specimens were genotyped independently and the histological analysis was carried out with no knowledge of the genotype.

Histology photographs were taken of typical trichrome stained sections representing the three groups, using TMX 100 (Kodak) film.

Statistical methods used for comparisons between gubernacular and cremaster muscle and sac sizes from different groups were mean, standard deviation, and Student's *t*-test.

Results

Forty-nine littermates were investigated. At 16 days fetal life, there were eight littermates. Of these, two were wild-type, five heterozygote αERKO and one homozygote αERKO. At 17 days fetal life there were ten wild-type, two heterozygote αERKO and two homozygote αERKO hemisected pelvic regions. At 18 days fetal life there were two wild-type, four heterozygote αERKO and one homozygote αERKO hemisected pelvic regions. Finally, at 7 days post-gestation pelvic regions of ten wild-type, two heterozygote αERKO, eight homozygote αERKO were hemisected.

There were no morphological differences between wild-type, heterozygote and αERKO mice at days 16, 17 and 18 of fetal life (Fig. 1). Similarly, there were no statistically significant differences in cremaster muscle development, gubernacular area or the ratio of cremaster

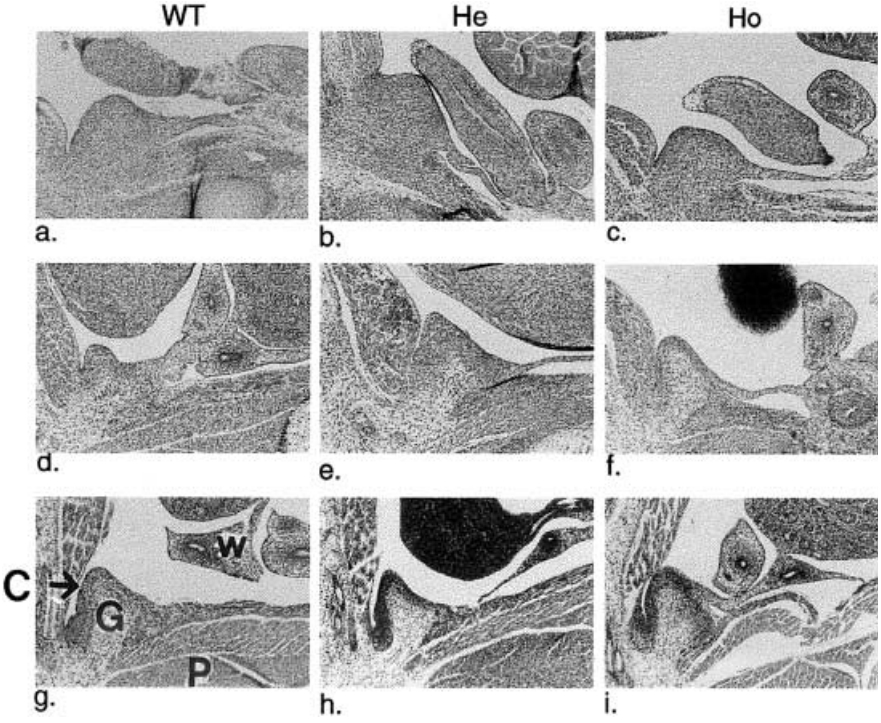

Fig. 1a–i Sagittal sections of the gubernaculum in estrogen receptor knockout (ERKO) male mice (×40, H&E; *WT* wild-type, *He* heterozygote ERKO, *Ho* homozygote ERKO). Orientation of all sections similar to **g**, which has been labeled (*P* posterior abdominal wall, *W* wolffian duct, *G* gubernaculum, *C* cremaster muscle). **a** Sixteen days fetal life wild-type, **b** 16 days fetal life heterozygote ERKO, **c** 16 days fetal life homozygote ERKO, **d** 17 days fetal life wild-type, **e** 17 days fetal life heterozygote ERKO, **f** 17 days fetal life homozygote ERKO, **g** 18 days fetal life wild-type, **h** 18 days fetal life heterozygote ERKO, **i** 18 days fetal life homozygote ERKO

Table 1 Gubernacular area (mm^2). *P*-values using two-tailed *t*-test for wild-type (*WT*) vs. estrogen receptor (*ER*) heterozygote, WT vs. estrogen receptor knockout (*ERKO*) and ER heterozygote vs. ERKO were not significant. *SD* standard deviation

	Fetal day 16 [$x \pm$ SD (*n*)]	Fetal day 17 [$x \pm$ SD (*n*)]	Fetal day 18 [$x \pm$ SD (*n*)]
Wild-type	8.52 ± 3.36 (2)	10.04 ± 4.55 (10)	8.91 ± 1.22 (2)
ER heterozygote	8.53 ± 1.93 (5)	14.27 ± 2.53 (2)	8.91 ± 1.33 (4)
ERKO	11.55 (1)	9.63 ± 3.43 (2)	10.10 (1)

Table 2 Cremaster muscle size (mm^2). *P*-values using two-tailed *t*-test for wild-type (WT) vs. estrogen receptor (*ER*) heterozygote, WT vs. estrogen receptor knockout (*ERKO*) and ER heterozygote vs. ERKO were not significant. *SD* standard deviation

	Fetal day 16 [$x \pm$ SD (*n*)]	Fetal day 17 [$x \pm$ SD (*n*)]	Fetal day 18 [$x \pm$ SD (*n*)]
Wild-type	4.29 ± 1.27 (2)	5.71 ± 4.35 (10)	5.26 ± 0.01 (2)
ER heterozygote	3.56 ± 1.42 (5)	8.70 ± 0.32 (2)	5.88 ± 0.66 (4)
ERKO	5.11 (1)	5.05 ± 1.14 (2)	6.65 (1)

Table 3 Ratio of cremaster muscle/gubernacular area. *P*-values using two-tailed *t*-test for wild-type (WT) vs. estrogen receptor (*ER*) heterozygote, WT vs. ERKO and ER heterozygote vs. estrogen receptor knockout (*ERKO*) were not significant. *SD* standard deviation

	Fetal day 16 [$x \pm$ SD (*n*)]	Fetal day 17 [$x \pm$ SD (*n*)]	Fetal day 18 [$x \pm$ SD (*n*)]
Wild-type	0.51 ± 0.05 (2)	0.48 ± 0.24 (10)	0.60 ± 0.08 (2)
ER heterozygote	0.41 ± 0.12 (5)	0.62 ± 0.13 (2)	0.66 ± 0.05 (4)
ERKO	0.44 (1)	0.54 ± 0.07 (2)	0.66 (1)

muscle area in the gubernaculum (Tables 1–3). At day 7 post-gestation, however, a trend towards a thicker cremasteric sac was noted in αERKO vs. wild-type (Fig. 2). A statistically significant difference was found in the width of the cremaster sac at the sac's tip (Table 4). In the wild-type mice, the sac tip width was 0.43 ± 0.09 mm compared to the αERKO mice which showed a mean width of 0.607 ± 0.17 mm. Using the 2-tailed *T* test, the *P*-value was < 0.01. Sac area, cremaster muscle area and the width at the sac end did not differ significantly amongst the three groups of mice (see Table 4).

Separation of the littermates morphologically, that is, based on the presence of the Mullerian duct and the appearance of the cremaster muscle, could not be performed reliably. PCR genotyping was required for identification.

Discussion

The fetal αERKO mouse showed no statistically significant differences of cremaster muscle development, gubernacular area, or the ratio of cremaster muscle area in the gubernaculum compared to wild-type. Likewise in the postnatal mouse no differences were noted except at the tip of the cremaster sac. The αERKO mice showed a thicker layer of cremaster muscle at the tip of the cremaster sac compared to the wild-type (see Table 4). Developmentally, this is consistent with the observation that older αERKO mice have uniformly thicker cremaster muscles [4].

Although αERKO mice have demonstrated variances from typical male and female behaviors [5, 20–22], they have shown normal genital morphology [5, 14, 18]. Lubahn et al. [18] even concluded that prenatal male and female reproductive tracts can still develop. This is surprising if we consider that administration of exogenous estrogens results in gubernacular atrophy [24] and cryptorchidism [19], with incomplete formation of the ductus deferens and epididymis [1, 11]. This implies that a lack of endogenous estrogens may cause a large difference in gubernacular development.

Eddy et al. [5] found no obvious differences in the seminiferous tubules of 10-day-old αERKO mice until 10 weeks, after which spermatogenesis was disrupted and the seminiferous tubules degenerated. Donaldson et al. [4] who investigated 21- to 25-week-old littermates found thicker cremaster muscles and a smaller cremaster sac with deficient spermatogenesis and testicular volume. Our study and that of Eddy et al. [5] and Donaldson et al. [4] suggest that the effect of estrogen on gubernacular development is a late effect, that is, it occurs between days 7 and week 21. However, this finding does not necessarily negate a role for estrogen in early gubernacular development, as ERβ expression may play a role. ERβ was recently detected in granulosa cells of the rat ovary [16] and implicated in prostate growth [10]. It has 97% DNA binding domain and 60% ligand binding

Table 4 Day 7 postnatally. *ER* estrogen receptor, *ERKO* estrogen receptor knockout, *SD* standard deviation

	Sac area ($x \pm$ SD; mm^2)	Sac tip width ($x \pm$ SD; mm)	Sac end width ($x \pm$ SD; mm)	Cremaster muscle size ($x \pm$ SD; mm^2)	Ratio sac/cremaster area ($x \pm$ SD)
Wild-type ($n = 10$)	10.55 ± 5.73	0.43 ± 0.09*	0.27 ± 0.14	4.94 ± 1.11	2.32 ± 1.35
ER heterozygote ($n = 2$)	4.66 ± 0.75	0.48 ± 0.03	0.15 ± 0.04	3.42 ± 1.38	1.53 ± 0.84
ERKO ($n = 8$)	13.15 ± 9.14	0.607 ± 0.17*	0.22 ± 0.05	4.45 ± 1.22	3.12 ± 1.95

* $P < 0.01$ for WT vs. ERKO. Remaining comparisons were insignificant

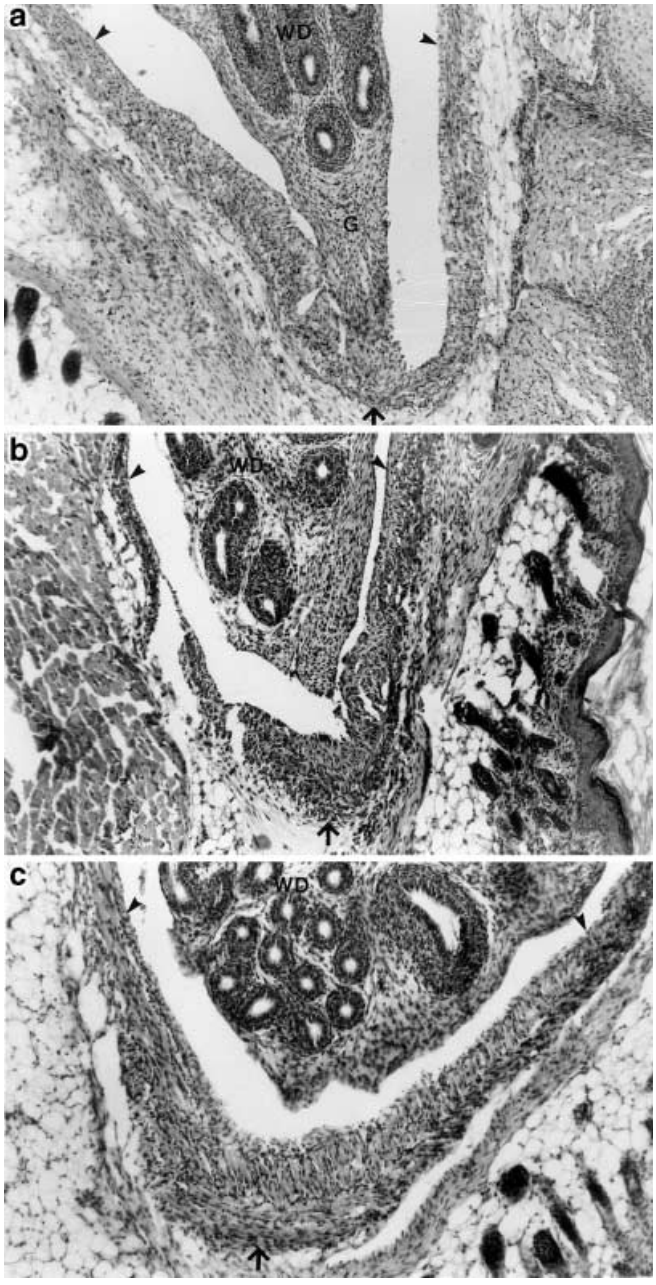

Fig. 2a–c Sagittal sections of the gubernaculum in estrogen receptor knockout (ERKO) mice on day 7 postnatally (×100, H&E). **a** Wild-type, **b** heterozygote ERKO, **c** homozygote ERKO [*WD* wolffian duct (epididymal tubules), *G* cord of gubernaculum, *solid arrow* tip of cremaster sac, *arrowhead* end of cremaster sac]

domain homology with ERα [25]. ERβ, found on the central region of chromosome 12 [25], is expressed in prostate, ovary, lung, bladder, uterus and testis (in descending order of concentration) while ERα is expressed in uterus, testis, pituitary, ovary, kidney, epididymis and adrenal [17]. Estradiol binding affinity for ERβ is similar to ERα [3], and Paech et al. [23] found that ERα and ERβ exhibit different responses to certain ligands at an AP1 element. The molecular mechanisms that regulate ERβ transcription are considered to be different from those for ERα to account for 17-β-estradiol inhibiting ERβ transcription and promoting ERα transcription [23, 25].

With both ER subtypes being located in the rat uterus, ovary, testis, epididymis and pituitary [17], there is a strong possibility that ERβ complementation, in the αERKO, could account for the surprising lack of effect of estrogen on the gubernaculum during early development. Future investigation into a βERKO and αERKO/βERKO double knock-out mouse should provide more information regarding the role of estrogen and receptor-mediated mechanisms in the first phase of testicular descent and gubernacular development.

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