Ex3 α ERKO male infertility phenotype recapitulates the α ERKO male phenotype

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Abstract

Disruption of the *Esr1* gene encoding estrogen receptor α (ER α) by insertion of a neomycin resistance gene (neo) into exon 2 (α ERKO mice) was shown previously to cause infertility in male mice. While full-length ER α protein was not expressed in α ERKO mice, alternative splicing resulted in the low-level expression of a truncated form lacking the N-terminus A/B domain and containing the DNA- and ligand-binding domains. Thus, it was unclear whether the reproductive phenotype in α ERKO males was only due to the lack of full-length ER α or was affected by the presence of the variant ER α isoform. The present study examined male mice with deletion of exon 3 of *Esr1* gene, lacking the DNA-binding domain, and null for ER α (Ex3 α ERKO). Dilution of some seminiferous tubules was apparent in male Ex3 α ERKO mice as early as postnatal day 10 and was pronounced in all tubules from day 20 onward. At 6 weeks of age, sperm numbers and sperm motility were lower in

Ex3 α ERKO mice than in wild-type (WT) mice, and the rete testis and efferent ductules were dilated. Mating studies determined that adult Ex3 α ERKO males were infertile and failed to produce copulatory plugs. Serum testosterone levels and *Hsd17b3* and *Cyp17a1* transcript levels were significantly higher, but serum estradiol, progesterone, LH, and FSH levels and *Cyp19a1* transcript levels were not significantly different from those in WT mice. These results confirm and extend those seen in other studies on male mice with deletion of exon 3 of *Esr1* gene. In addition, the reproductive phenotype of male Ex3 α ERKO mice recapitulated the phenotype of α ERKO mice, strongly suggesting that the α ERKO male infertility was not due to the presence of the DNA-binding domain in the truncated form of ER α and that full-length ER α is essential for maintenance of male fertility.

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Introduction

Previous studies showed that male α ERKO mice homozygous for a targeted disruption of the *Esr1* gene encoding estrogen receptor α (ER α) were infertile due to compromised fluid reabsorption by the efferent ductules and the initial segment of the epididymis (Eddy *et al.* 1996, Hess *et al.* 1997). This resulted in progressive dilation of the seminiferous tubule lumen, reduction in motility and loss of fertilizing ability by sperm, and disruption of the seminiferous epithelium leading to collapse of spermatogenesis. In addition, α ERKO males produced significantly fewer copulatory plugs when mated with wild-type (WT) female mice, contributing to the infertility phenotype (Eddy *et al.* 1996, Ogawa *et al.* 1997). When germ cells from α ERKO males were transplanted into the testes of WT mice depleted of germ cells, the recipients became fertile and sired offspring with the same genotype as the transplanted cells. This demonstrated that ER α is not required by male germ cells, but is required by somatic cells of the testis and/or male reproductive tract that provide the environment in which male gametes develop and mature

(Mahato *et al.* 2000, 2001). However, it is of interest that estrogen was reported to act through a G-protein-coupled receptor (GPR-30) on GC-1 spermatogonial cells to activate the epidermal growth factor receptor transduction pathway and stimulate their proliferation (Sirianni *et al.* 2008).

Several studies have provided evidence that estrogen is involved in regulating steroidogenesis in Leydig cells. The cytochrome P450, family 17, subfamily a, polypeptide 1 (CYP17A1) enzyme has an essential role in the steroidogenesis pathway in the synthesis of androstenedione, and the hydroxysteroid (17- β) dehydrogenase 3 (HSD17B3) enzyme is responsible for reducing androstenedione to testosterone. Diethylstilbestrol (DES) treatment inhibited CYP17A1 activity in testes of adult mice (Samuels *et al.* 1964) and in Leydig cells from fetal rats (Majdic *et al.* 1996). The *Cyp17a1* mRNA and CYP17A1 protein levels also were reduced in mice lacking estrogen sulfotransferase (SULT1E1), the enzyme responsible for sulfoconjugation and inactivation of estrogens (Tong *et al.* 2004). Transgenic mice expressing human aromatase (AROM⁺ mice; *CYP19A1*) had reduced testicular levels of *Cyp17a1* and *Hsd17b3* mRNA and low

testosterone and high estrogen levels (Strauss *et al.* 2009). In contrast, the levels of *Cyp17a1* and *Hsd17b3* mRNA and CYP17A1 and HSD17B3 enzymatic activity were elevated significantly in the testis of α ERKO mice (Akingbemi *et al.* 2003). Other studies with the AROM⁺ mice indicated that estrogen regulates Leydig cell steroidogenesis through ER α and not ER β (Strauss *et al.* 2009).

The original α ERKO mice were generated using the technology available at the time, which involved insertion of a neomycin resistance gene (neo) into exon 2 of the *Esr1* gene (Lubahn *et al.* 1993). Although this eliminated expression of full-length ER α , an alternative splicing event resulted in low-level expression of a truncated form of ER α lacking the N-terminal A/B domain, but containing the DNA- and ligand-binding domains (Couse *et al.* 1995, Pendaries *et al.* 2002). The present study examines mice generated with exon 3 of the *Esr1* gene flanked by loxP sites and crossed with Sox2-cre transgenic mice to produce mice with a deletion in the *Esr1* gene of the region encoding the DNA-binding domain (Ex3 α ERKO) and lacking a functional ER α (Hewitt *et al.* 2010). Other investigators have generated mice with deletion of exon 3 of the *Esr1* gene, referred to as ER α KO (Dupont *et al.* 2000) and ACTB-Cre/ER α ^{-/-} mice (Chen *et al.* 2009a), or with a knock-in of mutant forms of the *Esr1* gene, referred to as NERKI (McDevitt *et al.* 2007, Weiss *et al.* 2008) and ENERKI mice (Sinkevicius *et al.* 2009). The *Esr1* allele in NERKI mice has point mutations in the first zinc finger of the DNA-binding domain of ER α , which replace the glutamic acid at position 207 and the glycine at position 208 with alanines and disrupts the ability of ER α to bind DNA (Jakacka *et al.* 2002). The *Esr1* allele in ENERKI mice has a mutation in the ligand-binding pocket of ER α , which replaces the glycine at position 525 with lysine and disrupts the ability of ER α to bind estradiol (Sinkevicius *et al.* 2008). Male α ERKO (Eddy *et al.* 1996), ER α KO (Dupont *et al.* 2000), and ACTB-Cre/ER α ^{-/-} mice (Chen *et al.* 2009a) were found to be infertile in continuous mating studies. While ENERKI males did not undergo the disruption of the testis and excurrent ducts seen in α ERKO, ER α KO, and ACTB-Cre/ER α ^{-/-} males, they were subfertile (Sinkevicius *et al.* 2009). Disruption of the morphology of the testis and excurrent ducts in NERKI males was delayed compared to α ERKO males, but they were infertile primarily due to defects in male mating behavior (McDevitt *et al.* 2007). The present study analyzes the reproductive phenotype of male Ex3 α ERKO mice and relates the findings to the reproductive phenotypes of α ERKO, ER α KO, ACTB-Cre/ER α ^{-/-}, NERKI, and ENERKI males.

Materials and Methods

Animals

All experiments involving animals were carried out according to US Public Health Service (USPHS) guidelines, and the studies were approved by the National Institute of Environmental Health Sciences (NIEHS) Institutional

Animal Care and Use Committee. The generation of mice lacking exon 3 of *Esr1* was described previously (Hewitt *et al.* 2010).

Fertility studies

The fertility and fecundity of 7- to 14-week-old C57BL/6 male Ex3 α ERKO ($n=9$) and their WT littermates ($n=6$) were determined in a continuous mating study in which each male was mated with two C57BL/6 females (6 weeks of age) for 1 month and with two different females the following month. The females were monitored for pregnancy during and after the mating periods, and the number of litters and offspring was recorded. At the end of the second month, the males were euthanized and organs were weighed, sperm numbers and sperm motility were determined, and serum hormone levels were assayed. A separate study was carried out with Ex3 α ERKO males ($n=4$) to determine whether they exhibited typical mating characteristics. Each male was mated with two C57BL/6 females (6 weeks of age) for 5 days, and the females were checked daily for copulatory plugs and this was repeated 2 weeks later.

Sample collection

Ex3 α ERKO and WT males 6–7 and 18–33 weeks of age were anesthetized with carbon dioxide and euthanized by cervical dislocation. Blood for measuring serum hormone levels was collected by heart puncture or orbital bleeding on anesthetized mice prior to euthanasia. Body weights were determined, and the reproductive organs were excised and cleaned of fat and blotted before the testis, epididymis, and combined seminal vesicle, and coagulating gland weights were determined. Testes and epididymides were fixed in Bouin's solution for 12–16 h, washed in PBS, dehydrated in increasing concentrations of ethanol, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for histological examination. In addition, 10- and 20-day-old and 6-week-old Ex3 α ERKO and WT mice were euthanized, weights (body, testis, and epididymis) were recorded, and testes and epididymides were prepared for histological examination as described. Images were recorded using an Axioplan microscope (Carl Zeiss, Thornwood, NJ, USA), and QImaging camera and software (QImaging, Tucson, AZ, USA).

Sperm motility assays and counts

The caudae epididymides of 6- to 7-week-old and 14- to 35-week-old Ex3 α ERKO and WT mice were collected in PBS (Ca²⁺/Mg²⁺-free) at room temperature, cleaned, and transferred to 500 μ l of M2 medium (Chemicon, Phillipsburg, NJ, USA). Cuts were made with iridectomy scissors, sperm were allowed to swim out into the medium for 10 min at room temperature, and motility was assayed using computer-assisted sperm analysis (CASA). Sperm tracks (1.5 s, 30 frames) were captured at 60 Hz and analyzed

using HTM-IVOS Sperm Analyzer software (Hamilton Thorne Biosciences, version 12.2L, Beverly, MA, USA). Sperm collected in the same manner from 6-week-old and adult Ex3αERKO and WT mice were diluted 1:10 or 1:2 in water, and sperm counts were determined on duplicate samples using a hemocytometer.

Serum hormone levels

The serum hormone levels were assayed for individual 6- to 7-week-old and adult male mice in the NIEHS Clinical Pathology Support Laboratory using an APEX Automatic Gamma Counter (ICN Micromedex Systems, Inc., Huntsville, AL, USA). RIA kits were used to measure testosterone, estradiol, progesterone (Coat-A-Count, Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA), FSH, and LH serum levels (ALPCO Diagnostics, Salem, NH, USA).

Quantitative real-time RT-PCR assay

Male mice (three WT and six Ex3αERKO) were euthanized at 2–3 months of age, and testes were snap frozen in liquid nitrogen. Ovaries were collected from adult female WT mice. Testes and ovaries were pulverized individually and homogenized in Trizol, and RNA was prepared as recommended by the manufacturer (Invitrogen, Carlsbad, CA, USA). Analysis was performed in duplicate for each testis (6 WT and 12 Ex3αERKO mice) and ovary (2 WT mice) by quantitative real-time RT-PCR (qPCR) with an ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) as described previously (Hewitt *et al.* 2010). Primer sequences included *Cyp17a1*: F-GATCGGTTTATGCCTGAGCG, R-TCCGAAGGGCAAATAACTGG; *Hsd17b3*: F-ATGGA-GTCAAGGAGGAAAGGC, R-GGCTGTAAAGAGGCC-AGGG; *Cyp19a1*: F-TGATCATGGGCCTCCTTCTC, R-CCCAGACAGTAGCCAGGACCT; *Rpl7*: F-AGCTGG-CCTTTGTCATCAGAA, R-GACGAAGGAGCTGCAG-AACCT. Expression ratios were calculated according to the method of Pfaffl (2001).

Statistical analysis

Statistical analyses were performed using Student's *t*-test (two samples, assuming unequal variances) to calculate the mean and the S.E.M.

Table 1 The total number of litters and offspring produced by adult Ex3αERKO and wild-type (WT) male mice during a 2-month mating study

	Litters	Offspring
Ex3αERKO (n=8)	0	0
WT (n=6)	29	175

Figure 1 Organ weights of wild-type (WT) and Ex3αERKO mice. (A) Combined testis and epididymis (T+E) weights were determined for 10- and 20-day-old WT and Ex3αERKO mice. Testis weights were determined for 6-week-old and adult WT and Ex3αERKO mice. Data are expressed as mean $\pm$ S.E.M.; (**P* < 0.001). (B) Combined seminal vesicle and coagulating gland weights were determined for 6-week-old WT and Ex3αERKO mice. Data are expressed as the mean $\pm$ S.E.M.; (***P* < 0.005).

Results

Fertility studies

Two-month continuous breeding studies were performed to compare the fertility and fecundity of WT and Ex3αERKO male mice. The 6 WT males sired 29 litters and 175 offspring during this period, while the 8 Ex3αERKO males sired no offspring (Table 1). In addition, 4 Ex3αERKO males were housed twice for 5 days each with 2 WT females, and the females were examined each morning for copulatory plugs. No copulatory plugs were detected, suggesting that the males failed to breed.

Reproductive organ weights

The combined testis and epididymis weights of 10- and 20-day-old WT and Ex3αERKO mice were not different. However, the testis weights of 6-week-old Ex3αERKO males were significantly greater than those of the WT males, while

the testis weights of 18- to 33-week-old Ex3 α ERKO animals were significantly lower than those of WT males (Fig. 1A). In addition, the seminal vesicle weights of 6-week-old Ex3 α ERKO animals were significantly higher than those of WT mice (Fig. 1B). Other studies determined that there were no differences in body weights between WT and Ex3 α ERKO males (Hewitt *et al.* 2010).

Sperm function

Sperm from the cauda epididymis of Ex3 α ERKO males did not appear different morphologically than sperm from WT males at

the light microscope level, but the numbers were significantly lower for Ex3 α ERKO males than WT males for both 6-week-old (1.6 vs 6.5×10^6 /ml) and adult (3.3 vs 21.1×10^6 /ml) mice (Fig. 2A). The use of CASA revealed that the percent of sperm from the cauda epididymis of 6-week-old Ex3 α ERKO males that were motile was significantly less than of sperm from WT mice at time 0 (34% ($n=9$) vs 57% ($n=7$)) and after a 60 min incubation in M2 medium at RT (17% ($n=5$) vs 61% ($n=5$)) (Fig. 2B). However, sperm from 18- to 36-week-old Ex3 α ERKO mice were not motile (data not shown).

Serum hormone levels

Serum testosterone levels were significantly higher ($P<0.001$) in 6-week-old and 10- to 36-week-old male Ex3 α ERKO mice than in WT mice of the same ages (Table 2), while estrogen, progesterone, FSH, and LH levels were not significantly different in adults (Table 2).

Histology

The lumen of seminiferous tubules of 10-day-old Ex3 α ERKO mice varied from mildly (Fig. 3B) to severely (Fig. 3C and D) dilated compared to WT mice (Fig. 3A), while all tubules showed marked dilation in 20-day-old Ex3 α ERKO mice (data not shown). At 6 weeks of age, the seminiferous tubules (Fig. 3F), rete testis (Fig. 3G), and efferent ductules (Fig. 3H) in Ex3 α ERKO mice were considerably dilated, compared to the seminiferous tubules (Fig. 3E), rete testis, and efferent ductules (data not shown) in WT mice. In most adult Ex3 α ERKO males (18–36 weeks of age), the testicular interstitium was expanded, and the seminiferous tubules were reduced in diameter and had a low seminiferous epithelium and dilated lumen or lacked a lumen and contained a vacuolated epithelium (data not shown). These features were identical to what was observed in α ERKO males (Eddy *et al.* 1996).

qPCR assay

Previous studies demonstrated that *Cyp17a1* and *Hsd17b3* transcript levels and CYP17A1 and HSD17B3 enzyme activities were higher in the testes of α ERKO mice than in WT mice (Akingbemi *et al.* 2003). Using qPCR, we found similarly that *Hsd17b3* and *Cyp17a1* transcript levels were also elevated significantly in the testes of adult Ex3 α ERKO mice (Fig. 4A and B). While *Cyp19a1* levels were elevated slightly in the testis of Ex3 α ERKO males compared to WT males, they were far lower than the levels in ovaries of WT females (Fig. 4C and D).

Discussion

The original α ERKO mice were generated by insertion of a *Pgk1*-neo targeting and drug selection cassette into the Not1 site of exon 2 of the *Esr1* gene (Lubahn *et al.* 1993). Homozygous α ERKO mice did not express full-length ER α

Figure 2 Sperm counts and motility for WT and Ex3 α ERKO mice. (A) Sperm from the cauda epididymis of 6-week-old and adult WT and Ex3 α ERKO mice were counted. Data are expressed as the mean $\pm$ S.E.M.; (* $P<0.05$). (B) The motility of sperm from the cauda epididymis of 6-week-old WT and Ex3 α ERKO mice was determined using CASA after 0 and 60 min incubation in M2 medium. Data are expressed as the mean $\pm$ S.E.M.; (** $P<0.01$).

Table 2 Serum hormone levels (ng/dl) for 6-week-old and adult Ex3 α ERKO and wild-type (WT) male mice. Values are the mean $\pm$ s.e.m.

	Testosterone	Progesterone	Estradiol	FSH	LH
6-week-old					
Ex3 α ERKO	661 $\pm$ 134.8 ^a (n=12)	ND	ND	17 $\pm$ 1.8 (n=14)	ND
WT	64 $\pm$ 39.3 (n=7)	ND	ND	22 $\pm$ 2.4 (n=10)	ND
Adult					
Ex3 α ERKO	641 $\pm$ 108.6 ^b (n=8)	0.81 $\pm$ 0.07 (n=8)	11.8 $\pm$ 2.0 (n=8)	31 $\pm$ 1.9 (n=11)	0.72 $\pm$ 0.21 (n=14)
WT	93 $\pm$ 45.3 (n=6)	0.55 $\pm$ 0.12 (n=6)	14.3 $\pm$ 2.2 (n=6)	32 $\pm$ 1.1 (n=11)	1.08 $\pm$ 0.17 (n=11)

ND, not determined.

^aSerum testosterone levels in 6-week-old Ex3 α ERKO males were significantly higher than in 6-week-old WT males ($P < 0.001$).^bSerum testosterone levels in adult Ex3 α ERKO males were significantly higher than in adult WT males ($P < 0.001$).

protein, but a truncated form of ER α containing the DNA-binding and ligand-binding domains was expressed at low levels due to alternative splicing (Couse *et al.* 1995, Pendaries *et al.* 2002). Generating mice with a global *Esr1* gene exon 3 deletion (Ex3 α ERKO) and lacking ER α protein (Hewitt *et al.* 2010) allowed us to determine whether the truncated form of ER α contributed to the α ERKO male infertility phenotype. Male Ex3 α ERKO mice mated with WT female mice failed to sire offspring during a 2-month continuous mating trial, the same result seen with α ERKO male mice (Eddy *et al.* 1996). This strongly suggests that the overriding cause of male infertility in α ERKO mice was the absence of full-length ER α .

There were multiple underlying causes for the infertility in α ERKO male mice, including lack of normal mating behavior, dilation of the rete testis and seminiferous tubules, and reduced sperm counts and sperm motility (Eddy *et al.* 1996, Hess *et al.* 1997, Lee *et al.* 2009). The Ex3 α ERKO male mice failed to produce copulatory plugs, consistent with observations that disruption of ER α caused altered mating behavior in adult α ERKO male mice (Eddy *et al.* 1996, Ogawa *et al.* 1997, Couse & Korach 1999, Rissman *et al.*

1999). Young ENERKI males were subfertile (Sinkevicius *et al.* 2009), but lacked the dysmorphology seen in the testis and excurrent ducts of EX3 α ERKO, α ERKO (Eddy *et al.* 1996, Hess *et al.* 1997, Lee *et al.* 2009), ER α KO (Dupont *et al.* 2000), NERKI (Weiss *et al.* 2008), and ACTB-Cre/ER $^{-/-}$ mice (Chen *et al.* 2009a). This suggests that although the ER α in ENERKI mice is unable to bind estradiol, it can partially rescue the male reproductive phenotype seen in mice with other mutant forms of ER α .

Dilation of the seminiferous tubules was apparent in 10-day-old Ex3 α ERKO mice, and the rete testes and efferent ductules were dilated by 6 weeks of age. The seminiferous tubule dilation in α ERKO males was suggested to be either due to increased fluid production in the seminiferous tubules or by reduced fluid reabsorption in the efferent ductules (Eddy *et al.* 1996). The latter possibility seemed more likely because the efferent ductules reabsorb the majority of the fluid coming from the testis (Jones & Jurd 1987, Veeramachaneni *et al.* 1990), and the efferent ductules have higher ER levels than other regions of the male reproductive tract (Schleicher *et al.* 1984, West & Brenner 1990, Cooke *et al.* 1991). This was confirmed by showing that less fluid is secreted from

Figure 3 Histology of testes from 10-day-old and 6-week-old WT and Ex3 α ERKO mice on sections stained with hematoxylin and eosin. Seminiferous tubules in testis of (A) 10-day-old WT and (B–D) Ex3 α ERKO mice. Seminiferous tubules in testis of 6-week-old (E) WT and (F) Ex3 α ERKO mice. (G) Rete testis in 6-week-old Ex3 α ERKO mouse. (H) Efferent ductules in 6-week-old Ex3 α ERKO mouse. Bars: 25 μ m.

Figure 4 Real-time RT-PCR with RNA from testes and ovaries of WT and Ex3 α ERKO mice. The values shown are the average of two replicates of RNA from testes of WT and Ex3 α ERKO mice that were 2–3 months of age and calculated as the ratio of *Hsd17b3* (A), *Cyp17a1* (B), and *Cyp19a1* in testes (C) to *Rpl7* expression levels. The value shown for ovary is the average of two replicates of RNA from ovaries of WT female mice and calculated as the ratio of *Cyp19a1* to *Rpl7* expression levels (D). Data are expressed as the mean $\pm$ S.E.M. (*Hsd17b3*, * $P < 0.01$; *Cyp17a1* and *Cyp19a1*, ** $P < 0.001$). *n*, number of animals ($n = 2$ for ovary).

the testis in α ERKO mice than in WT mice, and that isolated segments of efferent ductules from α ERKO mice are less effective at reabsorbing luminal fluid than segments from WT mice (Hess *et al.* 1997). In addition, the testis weights in Ex3 α ERKO mice were significantly greater than in WT mice at 6 weeks of age, but significantly less at 18 weeks and later. Similar changes observed in α ERKO mice were suggested to be due to the initial accumulation of fluid and subsequent testicular atrophy (Hess *et al.* 1997). A higher pH was observed in the epididymal lumen in ER α KO mice, and the addition of cAMP rescued the defective motility of sperm from the epididymis of these mice (Joseph *et al.* 2010). A principal difference between Ex3 α ERKO and α ERKO males was that seminiferous tubule dilation occurred as early as 10 days of age in Ex3 α ERKO mice, but not until later in α ERKO mice (Eddy *et al.* 1996), suggesting that tubule dilation due to fluid accumulation begins even earlier in Ex3 α ERKO mice that are null for truncated ER α variants.

Sperm counts and sperm motility declined with age and were significantly lower in Ex3 α ERKO mice than in WT mice, comparable to what was observed in α ERKO mice (Eddy *et al.* 1996). An age-dependent decline in sperm counts was also seen in ACTB-Cre/ER α ^{-/-} (Chen *et al.* 2009a), NERKI (Weiss *et al.* 2008), and ENERKI mice (Sinkevicius *et al.* 2009). Sperm motility was relatively unchanged with age in NERKI mice, suggesting that the hormone responsiveness is regulated by a gene-tethering mechanism (Jakacka *et al.* 2002). The age-dependent infertility in

ENERKI males was reminiscent of results in ArKO mice with a targeted disruption in the aromatase (*Cyp19a1*) gene. This might be expected because even though a full-length ER α is present in both ENERKI and ArKO mice, ArKO mice do not produce estrogen, and the ER α in ENERKI mice cannot bind estrogen. Sperm motility was not evaluated in ACTB-Cre/ER α ^{-/-} mice, but sperm counts, sperm motility, and fertility were normal in most ArKO males at 4.5 months (Robertson *et al.* 1999, 2001), and fertility was reduced considerably by 3 months in ENERKI mice (Sinkevicius *et al.* 2009).

There was a significant increase in serum testosterone levels in Ex3 α ERKO males compared to WT males. This was also observed in α ERKO (Eddy *et al.* 1996, Lindzey *et al.* 1998, Akingbemi *et al.* 2003), ACTB-Cre/ER α ^{-/-} (Chen *et al.* 2009a), NERKI (McDevitt *et al.* 2007, Weiss *et al.* 2008), and ENERKI mice (Sinkevicius *et al.* 2009). Although there was a slight increase in the level of *Cyp19a1* transcripts in the testis of Ex3 α ERKO mice compared to WT mice, it was well below the level found in the ovary of WT mice. In addition, there was not a significant difference in serum estradiol levels in Ex3 α ERKO male mice compared to WT mice. This suggests that the increased levels are due to the increased CYP17A1 and 17 β HSD3 levels that result in a feed-forward effect on steroidogenesis. We observed similar elevated levels of serum testosterone in α ERKO females and expression of these enzymes in thecal cells, and found that estrogen and ER α participate in a short-loop feedback gonadal regulation (Taniguchi *et al.* 2007). The elevated testosterone levels presumably were responsible for the increased seminal vesicle weights seen in Ex3 α ERKO, α ERKO (Eddy *et al.* 1996, Lindzey *et al.* 1998), and ACTB-Cre/ER α ^{-/-} mice (Chen *et al.* 2009a).

The CYP17A1 enzyme is essential for the synthesis of androstenedione, and the HSD17B3 enzyme converts androstenedione to testosterone. We found that the *Cyp17a1* and *Hsd17b3* mRNA levels were increased significantly in testes of Ex3 α ERKO mice. This was also seen in α ERKO mice (Akingbemi *et al.* 2003). This increase in *Hsd17b3* was consistent with the increased serum testosterone levels in Ex3 α ERKO and α ERKO males, the reduced Leydig cell steroidogenic activity caused by DES treatment (Samuels *et al.* 1964, Majdic *et al.* 1996), the reduced CYP17A1 and estrogen levels in Leydig cells lacking estrogen sulfotransferase (Tong *et al.* 2004), and the elevated estrogen levels in transgenic AROM⁺ mice overexpressing human aromatase (Strauss *et al.* 2009). Crossing AROM⁺ and α ERKO mice resulted in expression of normal levels of *Cyp17a1* and *Hsd17b3* mRNA in testes of offspring lacking full-length ER α , while expression of these transcripts remained low in offspring lacking ER β from crosses of AROM⁺ and β ERKO mice. These results strongly suggest that estrogen regulates Leydig cell steroidogenesis through ER α (Strauss *et al.* 2009). In addition, the ovaries of α ERKO females contained cells similar to Leydig cells and elevated levels of *Cyp17a1* and *Hsd17b3* mRNA, and the serum testosterone levels of these mice were comparable to the levels in WT males (Couse *et al.* 2003,

2006, Taniguchi *et al.* 2007). Taken together, these results provide strong evidence that estrogen regulates Leydig cell steroidogenesis intragonadally through ER α (Akingbemi *et al.* 2003, Strauss *et al.* 2009).

Serum FSH and LH levels were not significantly different between Ex3 α ERKO and WT males. The FSH levels were significantly higher in some studies in α ERKO (Lindzey *et al.* 1998) and NERKI male mice (McDevitt *et al.* 2007, Weiss *et al.* 2008), but not in other studies in α ERKO (Eddy *et al.* 1996, Akingbemi *et al.* 2003, Strauss *et al.* 2009), NERKI (Weiss *et al.* 2008), or ENERKI males (Sinkevicius *et al.* 2009). Moderate but significantly higher LH levels were seen in some studies in α ERKO males (Lindzey *et al.* 1998, Akingbemi *et al.* 2003, Strauss *et al.* 2009), and elevated but not significantly higher levels of LH were seen in other studies in α ERKO (Eddy *et al.* 1996), NERKI (Weiss *et al.* 2008), ENERKI (Sinkevicius *et al.* 2009), and ACTB-Cre/ER α ^{-/-} males (Chen *et al.* 2009a), compared to WT males. The modest increases in LH levels in males contrast with the high LH levels found in α ERKO females (Couse *et al.* 2003). This is consistent with the suggestion that while ER α has a critical negative feedback role in regulating LH secretion in the female mouse, this may be mediated predominantly by the androgen receptor and largely independent of ER α in the adult male mouse (Lindzey *et al.* 1998).

Full-length ER α was not detected in α ERKO mice, but variant transcripts produced by utilization of a cryptic splice site in the neo sequence resulted in expression of a truncated form of ER α lacking the N-terminal A/B domain and containing the DNA- and ligand-binding domains (Couse *et al.* 1995, Dupont *et al.* 2000, Pendaries *et al.* 2002). These observations raised doubts whether the α ERKO mouse was a genuine null mutant and cast further doubts whether the reproductive phenotype of α ERKO males (Eddy *et al.* 1996) was solely due to the absence of full-length ER α . However, *Esr1* transcripts lacking the DNA-binding domain were observed in ER α KO (Dupont *et al.* 2000), ACTB-Cre/ER α ^{-/-} (Chen *et al.* 2009a), and Ex3 α ERKO mice (Hewitt *et al.* 2010), and the males were infertile. In addition, ER α protein was not detected with antibodies to the N-terminal or C-terminal regions in ACTB-Cre/ER α ^{-/-} (Chen *et al.* 2009b), the N-terminal region in ER α KO (Dupont *et al.* 2000), or the N-terminal region in Ex3 α ERKO mice (Hewitt *et al.* 2010). The results of the present study along with those observed in ER α KO (Dupont *et al.* 2000) and ACTB-Cre/ER α ^{-/-} (Chen *et al.* 2009a) mice strongly suggest that the presence of a truncated ER α does not substantially affect the male reproductive phenotype.

In summary, these studies provided data that were not reported previously for male mice that are null for ER α due to a global deletion of exon 3 of *Esr1* gene. The following characteristics were not reported for ER α KO mice (Dupont *et al.* 2000, Joseph *et al.* 2010): 1) testis and epididymis weights were significantly higher in 6-week-old mice and significantly lower in adult ER α -null mice than those of WT mice; 2)

epididymal sperm counts were significantly lower in 6-week-old and adult ER α -null mice than those of WT mice; 3) motility of sperm from the cauda epididymis of ER α -null mice was significantly lower upon release and after 1 h than that of sperm from WT mice; 4) testosterone levels were significantly higher in 6-week-old and adult ER α -null mice than those of WT mice; and 5) expression of *Hsd17b3* and *Cyp17a1* was elevated significantly in the testis of ER α -null mice compared to WT mice. The following characteristics were not reported for ACTB-Cre/ER α ^{-/-} mice (Chen *et al.* 2009a,b): 1) testis and epididymis weights for 10-day-old, 20-day-old, and 6-week-old ER α -null mice; 2) combined seminal vesicle and coagulating gland weights for 6-week-old ER α -null mice; 3) motility of sperm from the cauda epididymis of 6-week-old ER α -null mice; and 4) levels of expression of *Hsd17b3*, *Cyp17a1*, and *Cyp19a1* in testes of ER α -null mice.

Declaration of interest

The authors declare that there are no conflicts of interest that could be perceived as prejudicing the impartiality of the research reported.

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