The effects of oestrogen receptors α and β on testicular cell number and steroidogenesis in mice

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Abstract

Oestrogen plays an important role in testicular function. This study used mice null for oestrogen receptor α (ER α) or β (ER β) to investigate which receptor mediates the effects of oestrogen within the testis. Groups of ER α knockout mice (α ERKO) and ER β knockout mice (β ERKO) and wild-type littermates ($n=5-8$) were killed at 11 weeks *post partum*. One testis was fixed in Bouin's fluid for stereology and the other frozen for testosterone measurement. Trunk blood was collected for testosterone RIA. The optical disector combined with the fractionator methodology was used to estimate Leydig, Sertoli and germ cell numbers. At all times, the knockout animals were compared with their wild-type littermates. The physical disector quantified cells stained immunohistochemically for the apoptotic marker active caspase-3 and Hoechst staining was used to identify nuclear fragmentation. The mean Leydig cell volume was measured using the point sampled intercept method. The Leydig cell number per testis was significantly increased in β ERKO mice but not in α ERKO mice. Plasma and testicular testosterone concentrations were increased in α ERKO mice but no changes were observed in β ERKO mice. Hypertrophic Leydig cell changes were observed in α ERKO mice, and a decreased mean cell volume was seen in β ERKO mice. No difference in Sertoli cell number per testis was observed in any of the groups. The spermatogonial cell number per testis was increased in β ERKO mice. Immunohistochemistry identified increased numbers of active caspase-3-labelled germ cells per testis in α ERKO mice but not β ERKO mice. Hoechst staining supported these findings. There was significant germ cell loss in α ERKO mice. This study suggests that ER β may be involved in regulation of Leydig cell proliferation and testosterone production in the adult mouse testis.

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Introduction

It is now evident that oestrogen plays an important role in male reproductive function (see reviews by Couse & Korach 1999, O'Donnell *et al.* 2001). Oestrogen is produced within the testis by the aromatisation of testosterone (Nitta *et al.* 1993) and has been shown to affect the major cell types within the organ. Administration of exogenous oestrogen during development has been shown to reduce Sertoli cell numbers in the adult (Atanassova *et al.* 1999) but the effect of endogenous oestrogen in this process is less clear. Oestrogen disrupts development of fetal Leydig cells (Delbes *et al.* 2005) and can also act directly on the Leydig cell to inhibit testosterone production (Zhai *et al.* 1996). Oestrogen has also been shown to inhibit gonocyte development in the fetal testis (Delbes *et al.* 2004) and in the adult to enhance spermatogenesis by inhibiting apoptosis of human post-meiotic germ cells (Pentikainen *et al.* 2000).

The effects of oestrogen are mediated by two distinct receptors: oestrogen receptor α (ER α) and β (ER β), both

of which are found within the testis. ER β appears to be the more abundant being localised in the mouse to the Leydig and Sertoli cells as well as spermatogonia and elongated spermatids (Rosenfeld *et al.* 1998, Jefferson *et al.* 2000). ER α has been identified in adult Leydig cells (Zhou *et al.* 2002) and are thus the only cells in the mouse testis reported to express both ERs.

While the localisation of the ERs is becoming clearer, the biological functions mediated by each receptor are less well established. Studies of the ER α knockout mouse (α ERKO) have demonstrated that although the testes of these animals begin to develop normally, by 20 days *post partum* there is dilation of the lumen of the seminiferous tubules and rete testis. A reduced fluid reabsorption in the efferent ducts (Hess 2000) results in testicular disruption and loss of germ cells in the tubules culminating in subsequent infertility (Lubahn *et al.* 1993). How these changes influence Sertoli cell numbers remains unknown. Male ER β knockout mice (β ERKO), on the other hand, are reported to have

normal fertility, even though the β ERKO females have reduced fertility when compared with their wild-types (Krege *et al.* 1998).

As well as abnormalities in the seminiferous epithelium the inactivation of ER α also affects testosterone production. α ERKO mice exhibit increased follicle-stimulating hormone (FSH) levels and also have raised circulating concentrations of luteinizing hormone (LH; Lindzey *et al.* 1998) and testosterone (Akingbemi *et al.* 2003). Leydig cells in the α ERKO mice are hypertrophic and have been shown to produce more testosterone than cells from wild-type mice (Akingbemi *et al.* 2003).

The effects of ER β on the seminiferous epithelium and Leydig cells are less clear. Delbes *et al.* (2004) have demonstrated that ER β may affect proliferation of gonocytes during development but found no effect on the number or testosterone production of fetal Leydig cells at 2 days *post partum*.

The aim of this study was to investigate the effects of loss of the ER β receptor on cellular makeup of the testis in the adult mouse and to determine whether lack of ER α affects Sertoli cell number.

Materials and Methods

Animals

Adult α ERKO and β ERKO mice were kind donations from Prof. Allan Herbison (Department of Physiology, University of Otago). ERKO mice and wild-type littermates were generated by mating C57BL/6J heterozygous mice expressing the disrupted ER α (Lubahn *et al.* 1993) or ER β gene (Krege *et al.* 1998). Genotyping of the offspring was performed on tail-tip DNA using PCR amplification (Couse *et al.* 2003).

Mice were housed under 12 h light:12 h darkness cycles and given food and water *ad libitum*. Experiments were performed with approval from the University of Otago Animal Ethics Committee in accordance with the New Zealand Animal Welfare Act of 1999.

Tissue collection

Male α ERKO mice ($n=8$) and their wild-type littermates ($n=8$), and male β ERKO mice ($n=8$) and their wild-type littermates ($n=5$) were killed at 11 weeks *post partum* by CO₂ inhalation. The testes were removed and one fixed overnight in Bouin's fluid and then stored in 70% alcohol until processing for histological analysis and the other flash-frozen in liquid nitrogen and stored at -80°C . Trunk blood was collected and plasma and testicular testosterone concentrations measured by RIA (Yeung *et al.* 1988).

Cell number estimation using the optical disector

A fractionated sample combined with the optical disector has been shown to be a precise method to estimate testis cell number (Wreford 1995). Each testis was fractionated according to the smooth fractionation method (Gundersen 2002) into ~ 12 pieces from which four blocks were selected randomly. Each selected block was sectioned exhaustively into 30 μm sections and one section from each selected randomly and stained using the periodic acid Schiff (PAS) technique (Clermont & Perey 1957). Microscope images were captured on Spot analysis software (Diagnostic Instruments Inc., Sterling Heights, MI, USA) using a 100 $\times$ oil free objective (NA 0.95) on an Olympus BX-51 microscope with a Spot RT colour camera attachment. A 100 mm $\times$ 100 mm square reference frame (Howard & Reed 1998) was placed on the computer screen over the captured microscope images. The disector was imaged through the z-axis initially incorporating a 2 μm guard zone that randomly and systematically traversed the entire section. The optical disector method was used to count the nucleus of each cell (Gundersen 1986, Gundersen *et al.* 1988).

The testicular cells were identified by the following criteria (see Fig. 1): Leydig cells were located within the interstitial tissue and identified by their round to ovoid cell margins with a single identifiable nucleus. Sertoli cells were identified by their irregular tripartite nucleolus (Russell *et al.* 1990) and their location within the lower third of the seminiferous tubule. Spermatogonia were located adjacent to the basement membrane and did not exhibit PAS staining but rather a particulate appearance of their nuclei (Leblond & Clermont 1952).

Figure 1 Histology of the testis. The testis cells are annotated inside the disector frame. Cells that appeared completely within the frame or intersect the dashed inclusion lines were counted. Cells that intersect the solid exclusion lines were not counted. L, Leydig; S, Sertoli; A, spermatogonia; C, spermatocytes. The round and the elongated spermatids are identified on the left and right respectively.

Spermatocytes were larger, contained clumped chromatin and meiotic chromatin. Spermatids had characteristic PAS staining of the acrosome in both round and elongating cells (Russell *et al.* 1990). On an average, 68 ± 6 Leydig cells; 82 ± 6 Sertoli cells; 48 ± 3 spermatogonia; 549 ± 165 spermatocytes; 1538 ± 556 spermatids; 150 ± 11 caspase-positive cells and 118 ± 28 Hoechst-positive cells were counted in the disectors for each animal.

Point counting

Sections selected for stereology were also used to determine the tissue constituents in the α ERKO and β ERKO mice and their wild-type littermates. Six images from each animal were captured at $10\times$ magnification, and using a randomly placed 20 mm grid the percentage (Vv) of seminiferous tubules and interstitial tissue was determined.

Measurement of apoptotic germ cells

To investigate whether there was an increased rate of regulated cell death in the β ERKO mice during and after meiosis and to determine if the cellular loss seen in the α ERKO was due to active caspase-3 activity, the number of cells exhibiting immunostaining for activated caspase-3 was compared between the knockout and wild-type mice using a method developed by Fenwick & Hurst (2002). Briefly, sections ($5\ \mu\text{m}$) were deparaffinised in xylene and rehydrated through graded alcohols into distilled water. Antigens were unmasked by boiling for 20 min in 0.1 mol Tris-HCl buffer (pH 10) with 5% (w/v) urea (Shi *et al.* 1996). Peroxidase activity was quenched with 0.3% H_2O_2 (v/v) in methanol. Sections were blocked with a 1:200 dilution of donkey serum for 10 min and then incubated in rabbit anti-active caspase-3 polyclonal antibody overnight at 4°C (Cell Signaling, Boston, MA, USA). Negative control sections were incubated with an equivalent concentration of rabbit immunoglobulin G (IgG). The sections were incubated in biotinylated anti-rabbit IgG (Amersham Pharmacia Biotech) at a dilution of 1:200 for 30 min and then streptavidin-biotinylated horseradish peroxidase complex (Amersham Pharmacia Biotech) at a dilution of 1:200 for 30 min. Immunoreactivity was visualized with 3-amino-9-ethylcarbazole (Sigma-Aldrich Co.) and counterstained with Harris Haematoxylin.

Slides were viewed under an Olympus BX-51 microscope. Images were captured using the Spot imaging system. The physical disector (Sterio 1984) was used with pairs of serial sections mounted on the same slide and with systematic random sampling across the sections. At least 100 immunopositive cells were scored within the disector reference frames.

Hoechst staining was used as another approach to study and count the numbers of apoptotic cells. Morphological criteria identified apoptotic cells with nuclear fragmentation, blebbing and chromatin condensation. Apoptotic cells stained with Hoechst were intensely bright and irregularly stained. Normal cells were lightly regularly stained and residual bodies remained unstained. Cells were scored as apoptotic if they exhibited a bright fluorescent staining as well as defined morphological criteria. Tissue from randomly selected blocks were cut ($5\ \mu\text{m}$) and dewaxed. After equilibrating in PBS for 5 min, the Hoechst stain (H-3570, Molecular Probes, Eugene, Oregon, USA) was applied ($10\ \mu\text{g}/\text{ml}$ in PBS) for 10 min in the dark. The slides were washed in PBS for 5 min and then coverslipped using Vectorshield mounting media (H-1000, Vector Laboratories, Burlingame, CA, USA). The physical disector method was used to count Hoechst stained cells in a manner identical to the method used for active caspase-3 immunostaining.

Figure 2 Comparison of (a) body weight and (b) testicular weight between α and β oestrogen receptor knockout mice. Bars represent mean $\pm$ S.E.M. α WT (α wild-type littermates) $n=8$, α ERKO body weight $n=8$, α ERKO testis weight $n=7$, β WT (β wild-type littermates) $n=5$, β ERKO $n=8$. ** $P<0.005$, *** $P<0.001$ when compared with wild-type mice.

Mean Leydig cell volume

It has previously been described that α ERKO mice have hypertrophic Leydig cells and increased circulating testosterone levels (Akingbemi *et al.* 2003). In this study, the optical disector determined that there were changes in Leydig cell numbers in β ERKO mice. To determine if there were changes in the Leydig cell volume of the knockout mice when compared with their wild-type littermates, the point sampled intercept method was used (Gundersen *et al.* 1988).

Using the same images that were randomly captured for Hoechst staining, the Leydig cell outlines were clearly identified due to their distinct morphology. A sine weighted frame was placed on the image and the intercept test grid was placed so that one of the intercept lines passed through the bottom left corner as well as a randomly selected number.

Using Spot analysis software and following the intercept grid as a guide, a line was drawn across the chosen cells. One hundred cell intercepts were measured for each animal.

Testosterone RIAs

Testosterone was extracted from plasma with ether according to the method of Pierantoni *et al.* (1984) and from tissue using the method of Fink *et al.* (2005). Testosterone was measured in the extracted tissue and plasma samples (Yeung *et al.* 1988). The interassay variation was 6.3% and the intraassay variation was

2.8% for the testicular testosterone and 7.6% for the plasma testosterone RIAs.

Statistical analyses

All statistical analyses were performed using GraphPad Prism 4.0 (GraphPad Software Inc., San Diego, CA, USA). Data are expressed as the mean $\pm$ S.E.M.

When comparing the knockout animals with their wild-type littermates, the Student's unpaired *t*-test was used to determine significance. The significance level was set at $P < 0.05$. When determining significance across all groups, the ANOVA was used with a *post hoc* Bonferroni correction test with the significance level set at $P < 0.05$.

The coefficient of error equation was used to test variability of the observations to ensure adequate sampling (Gundersen & Jensen 1987). This equation was used for the optical disector, the physical disector and point counting.

Results

No significant differences in body weight between the α ERKO (*t*-test; $P = 0.42$) or β ERKO mice (*t*-test; $P = 0.77$) and their respective wild-type littermates were seen. There was, however, a significant difference in body weight between the two groups of wild-type littermates (Bonferroni, $P < 0.001$; Fig. 2a). Testis weight was significantly reduced in the α ERKO mice when compared with the wild-type controls (*t*-test;

Figure 3 Haematoxylin- and eosin-stained sections of testis showing (a) seminiferous tubule disruption with severe cellular loss in the α ERKO mice. In atrophic tubules, Sertoli cells are seen lining the basement membrane. (b) β ERKO testes showing normal tubules with increased Leydig cell numbers. (c) Testis sections from the α ERKO wild-type littermates and (d) from the β ERKO wild-type littermates showed normal morphology. Bars = 50 μ m.

$P=0.0022$; Fig. 2b) but no difference in testis weight was seen between the β ERKO mice and their wild-type controls (t -test; $P=0.24$).

Histological sections from wild-type littermates of both the α ERKO and the β ERKO exhibited normal testicular histology with full spermatogenesis (Fig. 3). The testes of the α ERKO male mice showed disrupted seminiferous tubules with a partial or complete loss of germ cells. In the most severely affected tubules, Sertoli cells were seen lining the otherwise empty tubules. The testes from the β ERKO mice appeared to show a normal histology with complete spermatogenesis. However, within the interstitial tissue there appeared to be increased numbers of Leydig cells.

Stereology

When the volume contributed by seminiferous tubules and interstitial tissue was estimated using tissue point counting, no significant differences were seen between the α ERKO and the β ERKO mice and their respective wild-type littermates in the percentage of seminiferous tubules (92.9–93.9%) and the interstitial tissue (6.1–7.1%), despite the severe cellular loss in some but not all α ERKO tubules.

Figure 4 The total number of (a) Leydig cells and (b) Sertoli cells per testis in α and β oestrogen receptor knockout mice. Bars represent mean $\pm$ S.E.M. α WT $n=8$, α ERKO $n=8$, β WT $n=5$, β ERKO $n=8$. ** $P<0.01$ when compared with β wild-type mice.

The fractionator method and optical disector were used to evaluate the effect of loss of ER α and ER β on the individual cell types within the testis. The number of Leydig cells per testis was significantly increased (t -test; $P=0.0078$) in β ERKO mice when compared with their wild-type littermates (Fig. 4a) with $\sim 40\%$ more Leydig cells observed in the β ERKO mice. No significant difference was observed in the number of Leydig cells per testis in the α ERKO when compared with their respective wild-types (t -test; $P=0.79$). There were no differences seen in Sertoli cell numbers per testis in the β ERKO (t -test; $P=0.099$) or the α ERKO mice (t -test; $P=0.27$) when compared with their wild-type littermates (Fig. 4b).

Figure 5 The total number of (a) spermatogonia, (b) spermatocytes and (c) spermatids per testis from α and β oestrogen receptor knockout mice. Bars represent mean $\pm$ S.E.M. α WT $n=8$, α ERKO $n=8$, β WT $n=5$, β ERKO $n=8$. * $P<0.05$, ** $P<0.01$ when compared with β wild-type mice.

Figure 6 Immunohistochemistry for active caspase-3 (a–c). (a) Active caspase-3 apoptotic cells seen in the testis sections from α ERKO mice. Testis sections from the β ERKO mice (b) and both the α ERKO and β ERKO wild-type littermates (not shown) had only occasional active caspase-3 immunoreactive cells. Arrows indicate immunopositive cells. The rabbit IgG control (c) showed no immunopositive staining. Bars = 50 μ m. Hoechst stained testis sections (d–f) allowed identification of apoptotic cells using morphological criteria as indicated by arrows. (d) There was a significant increase in cell numbers in the testis sections from α ERKO mice. Both the β ERKO mice (e) and the wild-type littermates (f) showed only the occasional apoptotic cell. Bars = 10 μ m.

A significant loss of all germ cells per testis was seen in the α ERKO mice; spermatogonia (*t*-test; $P=0.024$), spermatocytes (*t*-test; $P=0.0106$) and spermatids (*t*-test; $P=0.008$; Fig. 5) compared to their wild-type littermates. Conversely, spermatogonial numbers per testis were significantly increased in the β ERKO mice (*t*-test; $P=0.033$). No significant change in the number of spermatocytes per testis (*t*-test; $P=0.43$) or spermatids per testis (*t*-test; $P=0.12$) was observed in the β ERKO mice when compared with the β wild-type animals.

In all groups, active caspase-3 was immunolocalised only to the spermatocytes of the testes (Fig. 6a–c). There were no significant differences in the number of cells per testis showing immunoreactivity for active caspase-3 cells (*t*-test; $P=0.63$) between the β ERKO and wild-type mice. The numbers of active caspase-3 immunopositive cells per testis in the α ERKO were, however, increased significantly (*t*-test; $P=0.0008$) when compared with their wild-type littermates (Fig. 7a).

Hoechst staining was used to strengthen the results seen in the active caspase-3, as this stain identifies apoptotic cells by nuclear morphology. Predominantly, spermatocytes were seen as Hoechst-positive but one or two round spermatids per section were also seen. Both cell types were included in the total number of apoptotic

cells (Fig. 6d–f). Once more, there were no significant differences in the number of cells per testis, showing reactivity for Hoechst between the β ERKO and wild-type mice (*t*-test, $P=0.95$). The numbers of Hoechst-positive cells per testis in the α ERKO were significantly increased (*t*-test; $P=0.046$) when compared with their wild-type littermates (Fig. 7b).

Testosterone concentrations

Plasma and testicular testosterone concentrations (Fig. 8) were significantly increased (*t*-test; $P<0.0001$ plasma; $P=0.0013$ testes) in the α ERKO mice when compared with their wild-type littermates. Despite the increased number of Leydig cells per testis in the β ERKO mice there was no significant increase in testicular testosterone (*t*-test; $P=0.81$) or plasma testosterone (*t*-test; $P=0.51$) concentrations in these animals, when compared with the wild-type mice.

Mean Leydig cell volume

Leydig cell volumes were significantly increased in the α ERKO mice (*t*-test, $P=0.0003$). Moreover, Leydig cell volumes were significantly decreased in the β ERKO mice (*t*-test, $P=0.0171$; Fig. 9) when compared with their wild-type littermates.

Figure 7 (a) The total number of apoptotic spermatocytes indicated by active caspase-3 immunostained cells per mouse testis between the α and the β oestrogen receptor knockout mice. Bars represent mean $\pm$ S.E.M. α WT $n=8$, α ERKO $n=8$, β WT $n=5$, β ERKO $n=8$. *** $P<0.001$ when compared with α wild-type mice. (b) The total number of apoptotic cells as indicated by Hoechst staining per testis of the α and β ERKO mice and their respective wild-types. Bars represent mean $\pm$ S.E.M. α WT $n=3$, α ERKO $n=3$, β WT $n=3$, β ERKO $n=3$. * $P<0.05$ when compared with α wild-type mice.

Discussion

This study confirms earlier reports that there is a major disruption of spermatogenesis in the adult α ERKO mice (Lubahn *et al.* 1993) and that apparently testicular morphology is unaltered following loss of the ER β (Krege *et al.* 1998). However, our data show that inactivation of the ER β gene does affect the cellular composition of the testis (Table 1) and demonstrates that confining a study to qualitative assessment and sperm output may not provide an accurate picture of testicular morphology.

The results show that the number of adult Leydig cells per testis was unaffected by the inactivation of the ER α , however, loss of ER β resulted in a 40% increase. This is in contrast with fetal Leydig cells where it has been shown that loss of neither ER α nor β affects the number of cells in the mouse 2 days *post partum* (Delbes *et al.* 2004, 2005). Fetal Leydig cells express ER α but have not, as yet, been shown to express ER β (Jefferson *et al.* 2000), which may explain the lack of effect of ER β on these

Figure 8 Testosterone concentrations in (a) plasma (b) testis in the α and the β oestrogen receptor knockout mice. Bars represent mean $\pm$ S.E.M. α WT $n=8$, α ERKO $n=8$, β WT $n=5$, β ERKO $n=8$. *** $P<0.0001$ when compared with wild-type mice.

cells. Our data suggest that activation of ER β may normally have an inhibitory effect on the regulation of adult Leydig cell number.

As well as affecting Leydig cell number, oestrogen also affects the steroidogenic capacity of the Leydig cell. Studies using aromatase-deficient mice have shown that these animals have elevated circulating testosterone and LH and hyperplastic Leydig cells (Robertson *et al.* 1999). Our data confirm earlier studies that the inhibitory effect of oestrogen on testosterone production may be

Figure 9 Mean Leydig cell volume in α and β ERKO mice and their respective wild-types. Bars represent mean $\pm$ S.E.M. α WT $n=8$, α ERKO $n=8$, β WT $n=5$, β ERKO $n=8$. *** $P<0.001$, * $P<0.05$ when compared with wild-type mice.

Table 1 Summary of the effects of loss on the testis of oestrogen receptor α (ER α) and β (ER β) when compared with wild-type mice.

Parameter	α ERKO	β ERKO
Testis weight	↓	=
Leydig cell number per testis	=	↑68%
Leydig cell volume	↑	↓
Plasma testosterone	↑	=
Number of Sertoli cells per testis	=	=
Number of spermatogonia per testis	↓28%	↑38%
Number of spermatocytes per testis	↓42%	=
Number of spermatids per testis	↓45%	=
Germ cell apoptosis per testis	↑	=

mediated by ER α since α ERKO mice display similar changes to mice deficient of oestrogen (Oliveira *et al.* 2002). On the other hand, β ERKO mice, despite having increased Leydig cells per testis, have normal plasma and testicular testosterone concentrations and circulating LH levels (Temple *et al.* 2003). At the cellular level these Leydig cells had a smaller volume suggesting a decrease in testosterone production by each individual cell. Since these changes occur in the presence of normal LH levels this suggests that activation of ER β , unlike ER α , may normally mediate a direct stimulatory effect on testosterone production in the adult Leydig cell. Such an effect is not observed in fetal Leydig cells which lack ER β (Delbes *et al.* 2004).

In the rat, there is evidence that administration of exogenous oestrogens and/or xenoestrogens during development may inhibit Sertoli cell number in the adult (Atanassova *et al.* 1999). The evidence for a role of oestrogen in Sertoli cell proliferation in the mouse is less clear. Sertoli cell numbers were unchanged in the aromatase-deficient mouse (Robertson *et al.* 1999). Similarly, no effect on Sertoli cell number per testis was seen in either the α ERKO or the β ERKO mice in the present study when compared with their respective wild-type littermates. This is perhaps surprising since ER α mRNA and protein are expressed in Sertoli cells and, while ER β mRNA expression has yet to be demonstrated, the protein has been reported in these cells (Zhou *et al.* 2002). The reported effects of exogenous oestrogens on Sertoli cell number in the rat (Atanassova *et al.* 1999) may thus be a species specific effect or reflect the fact that Sertoli cell proliferation is only inhibited by supraphysiological levels of oestrogen. The latter is supported by Robertson *et al.* (2002) who demonstrated that a soy containing diet may decrease Sertoli cell number in the wild-type but not the aromatase knockout mouse.

The effects of the loss of the ER α receptor on germ cells have been previously described (Lubahn *et al.* 1993). Gonocyte number was found to be normal in the neonatal α ERKO mice (Delbes *et al.* 2005) but spermatogenesis progressively became disrupted due to the increased tubular pressure relating to reduced fluid reabsorption in the efferent ducts (Hess 2000). Our findings of increased caspase-3-positive staining and

fragmented pyknotic nuclei using the Hoechst stain confirm that perturbations of the efferent ducts increase the rate of apoptosis in germ cells.

The effects of inactivation of ER β in the adult mouse have not previously been described. Delbes *et al.* (2004) have demonstrated that an increased number of gonocytes are present in the 2-day-old β ERKO mice and suggested that this increase results from an increase in cell proliferation and decreased apoptosis. A similar increase in the number of spermatocytes per testis was observed in 11-week-old mice in this study. There were, however, no significant increases in the number of meiotic germ cells in these mice. The lack of an increased rate of germ cell apoptosis and the presence of normal circulating FSH concentrations in the β ERKO mice (Temple *et al.* 2003) suggests that while there may be a larger pool of spermatogonia persisting in the adult mouse the number being recruited into spermatogenesis at any one time is not affected by the presence or absence of ER β . Further studies are required to test this hypothesis.

Conclusion

In conclusion, this study demonstrates that in the mouse ER β is involved in the regulation of adult Leydig cell number and may also affect the steroidogenic capacity of individual Leydig cells. In addition, the disrupted endocrine profile in the α ERKO mouse resulted in hypertrophic Leydig cells and profound cellular loss through active caspase-3 activation in the spermatocytes.

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