Impairment of spermatogenesis in mice lacking a functional aromatase (cyp19) gene


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ABSTRACT It is well established that spermatogenesis is controlled by gonadotrophins and testosterone. However, a role for estrogens in male reproduction recently was suggested in adult mice deficient in estrogen receptor α. These mice became infertile primarily because of an interruption of fluid reabsorption by the efferent ductules of the epididymis, thus leading to a disruption of the seminiferous epithelium [Hess, R. A., Bunick, D., Lee, K. H., Bahr, J., Taylor, J. A., Korach, K. S., and Lubahn, D. B. (1997) Nature (London) 390, 509–512]. Despite the demonstration of the aromatase enzyme, which converts androgens to estrogens, and estrogen receptors within the rodent seminiferous epithelium, the role of aromatase and estrogen in germ cell development is unknown. We have investigated spermatogenesis in mice that lack aromatase because of targeted disruption of the cyp19 gene (ArKO). Male mice deficient in aromatase were initially fertile but developed progressive infertility, until their ability to sire pups was severely impaired. The mice deficient in aromatase developed disruptions to spermatogenesis between 4.5 months and 1 year, despite no decreases in gonadotrophins or androgens. Spermatogenesis primarily was arrested at early spermiogenic stages, as characterized by an increase in apoptosis and the appearance of multinucleated cells, and there was a significant reduction in round and elongated spermatids, but no changes in Sertoli cells and earlier germ cells. In addition, Leydig cell hyperplasia/hypertrophy was evident, presumably as a consequence of increased circulating luteinizing hormone. Our findings indicate that local expression of aromatase is essential for spermatogenesis and provide evidence for a direct action of estrogen on germ cell development and thus fertility.

It is well known that androgens and gonadotrophins play crucial roles in spermatogenesis and in the development and function of the male reproductive system. Recent clinical studies of two men who possess natural mutations of the aromatase gene (aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis; the product of the cyp19 gene) suggest that estrogens may have a vital role in processes such as bone formation (1, 2). However, the role of estrogens in male reproduction still remains to be elucidated.

Recently an indirect role for estrogen in spermatogenesis was demonstrated in adult knockout mice lacking a functional estrogen receptor (ER) α isofrom (ERKO) (3). The testes in these mice were atrophic with major disruptions to the seminiferous epithelium. They also presented reduced numbers of sperm with abnormal morphology and a compromised fertility (4). The most prominent characteristic of the testicular phenotype was the grossly dilated seminiferous tubule lumen, observed as early as 20 days (4). Subsequent studies on the efferent ductules of ERKO mice (3) demonstrated that ERα played a crucial role in the reabsorption of luminal fluid in the efferent ductules, such that in the ERKO mice an excess of fluid accumulated in the testis. This subsequently resulted in a back pressure on the seminiferous tubules, which adversely affected the seminiferous epithelium and spermatogenesis. Therefore it was suggested that the disruption to spermatogenesis in the ERKO mice was an indirect effect caused by abnormal fluid reabsorption (3).

The localization of ERs within the testis has been somewhat controversial. Nevertheless, it is recognized that ERα is present only in the Leydig cells (5) and the efferent ductules (5, 6) of the rat. ERβ, however, has been demonstrated in rat Sertoli cells (7–9), late spermatocytes, and early round spermatids (8–10) and in mouse Leydig cells and elongated spermatids (11). Although aromatase activity in Sertoli cells and Leydig cells is well documented (12, 13), albeit at different stages of development, aromatase also has been demonstrated in spermatocytes and spermatids of the mouse (14) and rat (15, 16). Thus, the localization of ERβ and aromatase within the seminiferous epithelium raises the possibility that estrogen may play a direct role in spermatogenesis that is yet to be resolved.

We have investigated spermatogenesis in knockout mice that lack a functional aromatase enzyme (ArKO) because of targeted disruption of the cyp19 gene (17), but these mice are unable to convert C19 steroids (androgens) to C18 steroids (estrogens). We evaluated the testicular phenotype by using stereological techniques to quantify changes in volumes of testicular compartments and used the optical dissector approach to enumerate Sertoli and germ cells. We found that mice lacking a functional aromatase gene initially are fertile (17), but develop progressively disrupted spermatogenesis despite no decreases in gonadotrophins or androgens. Our findings indicate that testicular expression of aromatase is essential for spermatogenesis and provide evidence for a direct action of estrogen on male germ cell development.

MATERIALS AND METHODS

Generation of ArKO Mice. The strategy used for targeted disruption of the cyp19 gene and a preliminary description of the phenotype already have been presented (17). Briefly, exon IX of the mouse cyp 19 gene was selected for disruption because the coding region sequence between the EcoRV (bp 1047) and XhoI (bp 1210) sites present in this exon is highly

Abbreviations: ER, estrogen receptor; ERKO, ER knockout; ArKO, aromatase knockout; w/t, wild type; TUNEL, terminal deoxynucleotidyl tranferase-mediated dUTP-biotin nick end labeling; LH, luteinizing hormone.

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ArKO, volume of Leydig cells was determined in 3 compartments (22). Cross sections of seminiferous tubules also 16 fine points was used to assess the volume of testicular magnification, and a point grid with a series of four major and middle, and embedded sections and then in half. One-half of each piece was dehydrated and embedded in methacrylate resin according to the manufacturer’s instructions (Technovit 7100, Kulzer, Wehrheim, Germany). Sections (2 μm and 25 μm) were cut on a supercut microtome (Reichert), stained with periodic acid-Schiff (19), and counterstained with haematoxylin. For the terminal deoxynucleotidyl tranferase-mediated dUTP-biotin nick end labeling (TUNEL) assay, portions of fixed testis were dehydrated and embedded in paraffin, and 5-μm sections were prepared. Immunocytochemical localization of 3β hydroxysteroid dehydrogenase staining was performed on 5-μm paraffin sections as described (20).

Stereology. Stereological analysis of the testes was performed as described (21). Briefly, sections were examined under an BX-50 Olympus microscope (Tokyo). The image was captured by a Pulnix TMC-6 video camera (Pulnix America, Sunnyvale, CA) coupled to an Amiga 2000 Pentium computer and projected onto the computer screen by using a Screen Machine II fast multimedia video adaptor (Hamburg, Germany). The computer program CASTGRID VI.10 (Olympus, Munich) was used to generate point grids and unbiased counting frames. Two-micrometer sections were examined under ×10 magnification, and a point grid with a series of four major and 16 fine points was used to assess the volume of testicular compartments (22). Cross sections of seminiferous tubules also were measured (90 diameters per animal). The absolute volume of Leydig cells was determined in 3β hydroxysteroid dehydrogenase-stained 5-μm sections by using a point grid as described above.

The optical dissector approach was used to quantitate the number of cells per testis as described (21, 22). Briefly, germ cells were classified into four major groups: spermatogonia, spermatocytes, round spermatids, and elongated spermatids. Fields to be counted were selected by a systematic uniform random sampling scheme as reviewed by Wreford (22). At least 100 cells of each type were counted per animal. Cells were counted in 25-μm sections, under 100× oil immersion, in two unbiased sample counting frames. One was 2,302 μm², in which spermatogonia were counted, and enclosed in this was a 230-μm² frame in which the remaining types were counted. Sertoli cell nuclei were counted at 100× in at least 150 fields with a frame of 1,381 μm².

Serum Hormone Assays. All luteinizing hormone (LH) and follicle-stimulating hormone samples were measured in one assay, as described (23). Serum testosterone was measured by using a double antibody RIA (23).

TUNEL Assay. Apoptotic cells were detected by using a modification of the TUNEL method as described (24). Digoxigenin (DIG)-labeled deoxy-UdTP (5 μM) and TdT (25 units/μl) (all reagents from Boehringer Mannheim) were used. Incorporated DIG was detected with an antidigoxigenin-alkaline phosphatase (Amersham Pharmacia) (1:100 in Tris buffer). Apoptotic cells were visualized with the chromogen 3,3’-diaminobenzidine tetrahydrochloride (Sigma).

Statistical Analysis. All data was analyzed by F test to determine normality, and the appropriate t test was applied at the level of 5% (P < 0.05). Data are expressed as the mean ± SD.

RESULTS

ArKO Testicular Phenotype. Male ArKO mice at the age of 12–14 weeks are fertile with normal-appearing testicular morphology (17). However, by the age of 4.5 months, four ArKO animals had testes with normal morphology, but one animal displayed grossly dysmorphic seminiferous tubules and disrupted spermatogenesis. By 1 year of age, all four w/t animals showed normal testicular morphology (Fig. 1a); however, all seven ArKO mice showed evidence of disrupted spermatogenesis and a reduction in testis weight (P < 0.05, Table 1). The site of spermatogenic disruption appeared to be early spermiogenesis, and degenerating round spermatids and multinucleated cells frequently were seen in the cauda epididymis (Fig. 2a). In these tubules, elongated spermatids were not seen, suggesting that round spermatids did not complete elongation and spermiation. Six of the 1-year-old ArKO mice had tubules displaying spermiogenic arrest, but some normal tubules were also present (Fig. 1c), suggesting a heterogeneity in the disruption. However, one 1-year-old and the one 4.5-month-old ArKO mouse had no elongated spermatids present in the testis, suggestive of complete spermiogenic failure in the testes of these animals. An interesting feature of the tubules displaying early spermiogenic arrest was that abnormal acrosomes often were observed, in which uneven spreading over the nuclear membrane was apparent, and more than one acrosomal granule was frequently visible (Fig. 1d). All 1-year-old ArKO animals showed Leydig cell hyperplasia/hyper trophy (Fig. 1e).

Histological examination of the epididymes (Fig. 2) showed a general reduction in the number of sperm visible within the cauda epididymis of 1-year-old ArKO mice, with some animals showing a complete absence of sperm (Fig. 2b). Interestingly, three animals with elongated spermatids present in the testis showed no sperm in the cauda epididymis. No sperm were present in the epididymis of the 4.5-month-old ArKO mouse with disrupted spermatogenesis. In the two animals that had a complete absence of elongated spermatids in the testis and epididymis, degenerating round germ cells were observed in the epididymis (Fig. 2c).

Quantitative Histomorphometry. To quantify changes in the composition of the testis of ArKO mice, quantitative histomorphometry was performed by using stereological methods (21, 22), which revealed a marked decrease in the volume of seminiferous epithelium in 1-year-old ArKO mice as compared with w/t mice (Table 1). No changes in seminiferous tubule luminal volume were seen, in contrast to the ERKO mouse, in which dilated lumens were obvious (3). One-year-old ArKO animals were shown to have a significant increase in the volume of Leydig cells as compared with w/t (Table 1).

Sertoli and Germ Cell Numbers. Quantitative cell numbers revealed that the numbers of Sertoli cells were unchanged in ArKO vs. w/t of both ages (millions per testis): 4.5 months old, 4.0 ± 0.9 vs. 4.4 ± 1.8; 1 year, 3.3 ± 0.6 vs. 3.6 ± 0.9, mean ± SD. Spermatogonia, spermatocytes, round spermatids, and elongated spermatids also did not differ between the 4.5-month-old w/t and ArKO animals (Fig. 3a). In 1-year-old animals, the numbers of spermatogonia (P = 0.06) and spermatocytes (P = 0.08) were unchanged in ArKO vs. w/t (Fig. 3b). However, there was a significant decrease in the numbers of both round (P = 0.001) and elongated spermatids (P = 0.004) in 1-year-old ArKO animals compared with w/t (Fig. 3b). It is pertinent to note that no changes in any cell numbers were evident between w/t animals at 4.5 months compared with 1 year (data not shown).
TUNEL Assay. To determine whether the decline in round spermatid number was attributable to an increased incidence of apoptosis, TUNEL assays were performed. In tubules exhibiting spermiogenic arrest, adluminal cells showing apoptotic labeling often were observed (Fig. 4b). These observations, together with the appearance of degenerating and multinucleated round spermatids, suggest an increase in cell death in the adluminal compartment of the seminiferous epithelium of the ArKO animals.

Serum Hormone Levels. Elevations in serum LH and testosterone levels in male ArKO mice aged 12–14 weeks have been described (17), and animals in the current study exhibited similar trends in LH levels (data not shown). Serum testosterone levels in the 4.5-month-old and 1-year-old animals were variable as described (17), and no significant increase or decrease was evident (not shown), although testosterone levels were elevated in the mice aged 12–14 weeks (17). Furthermore, serum follicle-stimulating hormone levels in the ArKO mice were unchanged compared with wild type mice of either age group (wild type vs. ArKO (ng/ml): 4.5 months old, 9.0 ± 1.7 vs. 9.9 ± 1.5; 1 year, 10.4 ± 0.5 vs. 10.1 ± 0.7, mean ± SD), and therefore changes in follicle-stimulating hormone could not have contributed to the spermatogenic disruption observed in the ArKO mice.

Observations on ArKO Breeding Rates. In preliminary observations into ArKO fertility, male ArKO mice (n = 3)

<table>
<thead>
<tr>
<th>Mice</th>
<th>Testis weight, mg</th>
<th>Volume lumen, mm³</th>
<th>Volume epithelium, mm³</th>
<th>Volume interstitium, mm³</th>
<th>Volume Leydig cells, mm³</th>
<th>Tubule diameter, μm</th>
<th>Tubule length, m</th>
</tr>
</thead>
<tbody>
<tr>
<td>w/ t, 4 1/2 mth (n = 6)</td>
<td>137.4 ± 6.8</td>
<td>10.2 ± 3.0</td>
<td>106.1 ± 7.7</td>
<td>16.7 ± 3.8</td>
<td>ND</td>
<td>236.1 ± 7.0</td>
<td>2.8 ± 0.3</td>
</tr>
<tr>
<td>ArKO, 4 1/2 mth (n = 5)</td>
<td>113.4 ± 37.2</td>
<td>8.2 ± 2.8</td>
<td>85.1 ± 29.3</td>
<td>15.2 ± 7.4</td>
<td>ND</td>
<td>229.1 ± 34.9</td>
<td>2.4 ± 1.0</td>
</tr>
<tr>
<td>w/ t, 1 yr (n = 4)</td>
<td>134.5 ± 12.9</td>
<td>16.9 ± 7.4</td>
<td>96.0 ± 10.1</td>
<td>15.8 ± 1.4</td>
<td>3.78 ± 0.43</td>
<td>247.2 ± 11.9</td>
<td>2.4 ± 0.3</td>
</tr>
<tr>
<td>ArKO, 1 yr (n = 7)</td>
<td>101.5 ± 27.3*</td>
<td>12.4 ± 7.1</td>
<td>63.6 ± 19.1*</td>
<td>21.4 ± 8.2</td>
<td>7.50 ± 2.56*</td>
<td>214.0 ± 31.3</td>
<td>2.0 ± 0.8</td>
</tr>
</tbody>
</table>

There were no differences in the absolute volumes of the various compartments of the testis in the 4.5-month-old ArKO mice compared to wild type. In 1-year-old ArKO mice the absolute volume of seminiferous epithelium was decreased by 34% (P < 0.05), and the absolute volume of Leydig cells was increased by 50% (P < 0.05). Testicular weight was decreased in the 1-year-old ArKO mice (P < 0.05). Data expressed as mean ± SD. ND = not determined.

*P < 0.05.
were mated at 7 months of age for an average of 6 months, and no litters were sired. This observation was in contrast to heterozygous animals \((n = 3)\), who were mated at the same age and all sired an average of five litters. These observations suggest that fertility in male ArKO mice is severely compromised.

**DISCUSSION**

The findings presented here indicate a direct role for estrogen in male germ cell development. Although ERαKO animals were infertile because of an interruption in fluid reabsorption by the epididymis and thus probably physical disruption to the seminiferous tubules, ArKO animals show a specific defect in the development of spermatids during spermiogenesis. Disruptions to spermatogenesis were evident from 18 weeks of age, and all seven 1-year-old ArKO mice showed evidence of disrupted spermatogenesis. Quantitation of cell numbers demonstrated that Sertoli cell and earlier germ cell populations were unchanged compared with w/t, whereas significant decreases were seen in round and elongated spermatid number in ArKO animals. In addition, ArKO animals displayed an increased frequency of germ cell apoptosis, some abnormalities in early acrosome development, Leydig cell hyperplasia/

hypertrophy, as evidenced by an increase in the absolute volume of Leydig cells per testis, and finally a reduced ability to sire litters. Our findings suggest that estrogen plays a hitherto unsuspected role in spermatid differentiation and spermatogenesis.

The finding that ArKO animals show disruptions to spermiogenesis is supported further by studies in which significant reductions in round and elongated spermatid populations, but not earlier germ cells, were found in adult male bonnet monkeys administered an aromatase inhibitor for 150 days (25). Furthermore, disruptions to spermatogenesis also were noted in adult male rats administered an aromatase inhibitor for 19 weeks (26). In 1987, Tsutsumi et al. (27) injected adult male rats with a partially purified ovarian protein that was isolated because of its ability to inhibit granulosa cell aromatase activity. Round spermatid degeneration and a reduction in elongated spermatid number also were noted in these studies (27, 28), lending further support to our findings.

Disruptions to germ cell development in ArKO animals is not surprising given the localization of aromatase in the seminiferous epithelium. Rodent spermatocytes, round and elongated spermatids (14–16), and Sertoli (13) and Leydig cells (13, 14, 16) express the aromatase enzyme. Studies by

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**FIG. 2.** Morphology of cauda epididymis in 1-year-old animals. (a) One-year-old w/t cauda epididymis with an abundance of sperm. (b) The lumen of the 1-year-old ArKO mouse showing absence of sperm; three of the seven have no elongated spermatids visible in the cauda. (c) The ArKO animals that have undergone spermiogenetic arrest show evidence of degenerating germ cells, presumably round spermatids (arrowheads). (Scale bar: 20 μm.)

**FIG. 3.** Germ cells numbers (millions per testis) in ArKO and w/t mice at the two age groups. (a) The numbers of spermatogonia, spermatocytes, round spermatids, and elongated spermatids did not differ in the 4.5-month-old ArKO mice as compared with the w/t mice. (b) No significant decreases were seen in spermatogonia \((P = 0.06)\) and spermatocyte populations \((P = 0.08)\) in 1-year-old ArKO mice; however, there were significantly less round and elongated spermatids present in the epithelium of the ArKO mice \((P < 0.05)\). Data expressed as mean ± SD.
Nitta et al. (14) appeared to demonstrate that spermatids had more intense immunostaining and higher aromatase activity than any other testicular cells. The localization of aromatase within the spermatids of the rodent seminiferous epithelium is in line with our findings that mice deficient in aromatase show a loss of round and elongated spermatids.

There is also good evidence to suggest that ERs are present within the seminiferous epithelium. ERβ has been reportedly localized to rodent Sertoli cells (7–9), late spermatocytes and early round spermatids (8–10), and Leydig cells and elongated spermatids (11). ERα primarily is expressed in the Leydig cells (5) but not in germ cells. The defect in spermiogenesis in the ArKO mice was characterized by increased apoptosis, formation of multinucleated cells, and sloughing of cells into the lumen, and in some cases the epididymis. We and others (14, 16) hypothesize that estrogen may be synthesized by germ cells, which may act in a paracrine fashion on Sertoli cells causing them to release factors to specifically regulate germ cell development. Aromatase activity in the Leydig cells also could contribute to regulation of germ cell development (13, 15). Alternatively, estrogen could be synthesized by the germ cells and act in an intracrine fashion, providing a local source of estrogen involved in controlling the complex process of spermatogenesis.

In the ArKO mice we observed some evidence of abnormal acrosomal development such as multiple acrosomal vesicles and uneven spreading over the nuclear surface. It is interesting to note that immunocytochemical localization of aromatase P450 shows strong positive labeling of early round spermatids, particularly in the Golgi (14). Therefore local aromatase expression could be important for proper acrosomal formation; however, this hypothesis requires further investigation.

The phenotype of the male ArKO mouse differs from that of the ERKO mouse in which infertility is evident from the onset of puberty (3, 4). Moreover, in the ERKO mouse, the lumina of the seminiferous tubules are extremely dilated, in contrast to the ArKO mouse in which the luminal volume is unchanged (Table 1). The primary defect in the ERKO mouse has been reported to be a failure of fluid transport across the epithelium at the efferent ductules, resulting in a back pressure that is detrimental to the spermatogenic process (3). Thus, the disruption to spermatogenesis in the ERKO mouse appears to be primarily a mechanical one. In ArKO animals there was no evidence of abnormal fluid reabsorption by the efferent ductules. It is interesting to note that Hess and colleagues (3) demonstrated that w/t isolated efferent ducts treated with an antagonist of both ERα and ERβ did not swell like tissues isolated from ERKO animals. Because the antagonism of ERα and ERβ in w/t tissues could be considered analogous to the absence of estrogen in ArKO animals, this observation is consistent with the apparent lack of an effect on fluid reabsorption in ArKO mice.

ArKO animals showed evidence of Leydig cell hyperplasia/hypertrophy, presumably as a consequence of increased circulating LH levels, as was noted in previous studies in rats (29). Because Leydig cells have high levels of aromatase, which may be important for Leydig cell function (16, 29), we cannot exclude the possibility that Leydig cells in ArKO animals may overexpress or underexpress factors that contribute to the disruption of spermatogenesis. Therefore, spermatogenic disruption in ArKO mice may be caused by a deficiency of aromatase in the Leydig cell, the seminiferous epithelium, or both.

We do not believe, however, that the disruption to spermatogenesis in ArKO animals is a direct consequence of high LH/testosterone or LH-induced Leydig cell hyperplasia. Previous studies have shown that mice given a 5α-reductase inhibitor for 83 weeks (30) have similar Leydig cell hypertrophy/hyperplasia and similar serum LH and testosterone levels to ArKO animals. However the 5α-reductase inhibitor-treated animals showed normal seminiferous epithelial morphology and spermatogenesis. These observations support our contention that spermatogenic disruption in ArKO animals is not directly caused by increased androgens or hypertrophic Leydig cells.

We are unclear as to why the disruption to spermatogenesis takes months to develop or why all tubules are not affected. Regarding the latter, heterogeneity in spermatogenic disruption is apparent in other knockout models (4, 31). Furthermore, this variability of response after administration of an aromatase inhibitor also has been noted (26). The progressive nature of the phenotype may be intrinsic to the mechanisms of action of estrogen in the adult seminiferous epithelium. It is interesting to note that female ArKO mice also show a progressive phenotype (unpublished work). An alternative hypothesis to explain the delayed phenotype is that the diet of the mice in this study may contribute sufficient exogenous estrogens to maintain normal spermatogenesis in young animals. Standard mouse chow contains as much as 25% of soy meal, and thus contains significant quantities of isoflavones, phytoestrogens with potential estrogenic activity. We currently are raising mice on a soy-free diet to investigate whether this potential source of phytoestrogens is a contributing factor in the slow onset and variability of the phenotype. Our preliminary studies suggest that the phenotype of ArKO mice is more advanced in animals on a soy-free diet in comparison to animals on normal diet (unpublished data).

Alternative endogenous ligands, which may bind to and activate the ER, also could be important in the delayed phenotype (32). In addition, alternative ligands for ERs could contribute to the difference in the phenotype between ArKO
and ERαKO mice (3). It is believed that growth factors, such as epidermal growth factor, can activate the ER, thus participating in cross talk between the growth factor signaling pathways and steroid receptors (33).

Another ArKO mouse phenotype recently was described in which exon 1 and 2 were lacking (34). These 10- to 18-week-old male mice had sperm present in their epididymis; however, they were infertile and shown to have modified sexual behavior. No behavioral abnormalities were noted in 12-week-old male ArKO mice generated by our group (17), and the behavior of older animals has yet to be assessed. More studies are needed to compare the phenotypes of these different ArKO mice. Recently, the phenotype of the ERβKO mouse was reported (35). Fertility of males was assessed between 6 and 12 weeks of age, and no changes in breeding rates were seen. As indicated previously (17), there is no change in the seminiferous epithelium, we suggest that the actions of estrogen on male germ cell development are a consequence of paracrine and indeed intracrine interactions. Our results together with observations that aromatase deficiency in men and mice results in a marked bone phenotype (1, 2) highlight the hitherto unsuspected role of estrogen as a locally acting male hormone.

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