

## Rat Testicular Germ Cells and Epididymal Sperm Contain Active P450 Aromatase

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**ABSTRACT:** Although testosterone is the principal sex steroid produced by the testis, estrogen is known to be produced by both Leydig and Sertoli cells during different developmental periods. Additionally, evidence is unfolding to suggest that germ cells might also participate in the synthesis of estrogen within the male reproductive tract. We have recently reported that the messenger ribonucleic acid (mRNA) for P450 aromatase (P450arom), the enzyme that converts androgen to estrogen, is synthesized by rat germ cells. Therefore, the present study was conducted to determine which germ cell types synthesize active P450arom and to measure the activity of this enzyme in germ cells throughout spermatogenesis and in maturing sperm during epididymal transit. First, P450arom activity was measured in pachytene spermatocytes, round spermatids, and a mixture of round spermatids, elongating spermatids, and residual bodies using the tritiated water (3H<sub>2</sub>O) assay. Second, sperm isolated from

different regions of the epididymis were assayed for P450arom activity. Sperm isolated from the caput epididymis with attached efferent ductules had the higher P450arom activity, whereas sperm isolated from the corpus and cauda epididymides had lower P450arom activity. The decrease in P450arom activity in cauda sperm was further confirmed by immunocytochemistry. On the basis of these observations, we conclude that rat testicular germ cells from pachytene spermatocytes through elongating spermatids and epididymal sperm contain active P450arom and that sperm lose aromatase activity as they mature during epididymal transit. Therefore, both post-pachytene rat germ cells and epididymal sperm are capable of estrogen synthesis and are an additional, potentially significant, source of estrogen in the male reproductive tract.

Key words: Estrogen, testis, epididymis.

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Previously, it was reported that the primary sources of estrogen in the male reproductive system are Sertoli cells of the immature male (de Jong et al, 1974; Dorrington and Armstrong, 1975; Fritz et al, 1976) and Leydig cells of the adult male (Pierrepoint et al, 1966; Payne et al, 1976; Valladares and Payne, 1979; Valladares and Payne, 1981). However, some researchers have suggested that the seminiferous tubules of adult mammals are also able to synthesize estrogen (de Jong et al, 1974; Pudney et al, 1985). Presently, a growing body of evidence indicates that germ cells are likely participants in the synthesis of estrogen within the adult male reproductive tract (Nitta et al, 1993; Tsubota et al, 1993; Kwon et al, 1995; Janulis et al, 1996). In this regard, we have recently reported that the messenger ribonucleic acid (mRNA) for P450 aromatase (P450arom), the enzyme that converts

androgen to estrogen, is synthesized by rat germ cells (Janulis et al, 1996).

The present study was conducted to determine if adult rat testicular germ cells and epididymal sperm are potential sources of estrogen by ascertaining which specific germ cell types contain active P450arom. We measured the activity of this enzyme in germ cells throughout late spermatogenesis and during epididymal transit using the 3H<sub>2</sub>O assay for P450 aromatase activity. These results were further confirmed by immunocytochemistry.

### Materials and Methods

#### Germ Cell Separation

Testicular mixed germ cell and interstitial cell preparations were obtained by following the initial cell separation (Sta-put) procedure as described by Bellevue et al. (1997a,b). Briefly, testes were removed and decapsulated from five adult (4-6-month-old) Sprague-Dawley rats. Testes were then placed into ice-cold RPMI-1640 medium (Gibco, Grand Island, New York) (50 ml) containing sodium bicarbonate and lactate and adjusted to pH 7.2 by gassing with CO<sub>2</sub>. Collagenase (75 mg) (Type 1A, Sigma, St. Louis, Missouri) was added to the medium; the medium was gassed with CO<sub>2</sub>, covered, and placed in a 34°C waterbath.

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The flask was gently agitated in the waterbath until the testes were dissociated into seminiferous tubules (30-35 minutes). After collagenase digestion, tubules were allowed to sediment and the supernatant was collected. Tubules were repeatedly rinsed with 50 ml of RPMI medium and sedimented as before until the supernatant remained clear. Supernatants containing interstitial cells were pooled, pelleted by centrifugation, and frozen at  $-80^{\circ}\text{C}$ . Interstitial cells were used as a positive control in these experiments, because Leydig cells, a component of interstitial cells, are known to produce estrogen (Pierrepont et al, 1996; Payne et al, 1976; Valladares and Payne, 1979; Valladares and Payne, 1981). Tubules were then incubated in medium with trypsin (50 mg) (Sigma) in a  $34^{\circ}\text{C}$  waterbath (10-12 minutes) and then dispersed into single cells with gentle pipetting. The resulting cell suspension was filtered through fine nylon mesh to remove clumps of cells and tissue.

Further separation of cell types was accomplished by unit gravity sedimentation through a bovine serum albumin (BSA, Sigma) gradient (Bellve et al, 1977a,b). After off-loading the gradient to a fraction collector, fractions containing mainly pachytene spermatocytes and round spermatids were identified by observation under a phase contrast microscope. Fractions containing populations of germ cells (>90% pure) were pooled. The purity and number of pachytene spermatocyte and round spermatid fractions were determined by light microscopy and by counting using a hemacytometer. The major "impurity" in these cell preparations was other germ cells, not Sertoli cells. Additionally, the purity of our germ cell fractionations had been confirmed using reverse transcription-polymerase chain reaction (RT-PCR) to detect P450arom and Leydig cell-specific 3BHSD mRNA (Janulis et al, 1996). No significant contamination of germ cell fractions with Leydig cell was observed. Fractions containing pure populations of germ cells were pooled, pelleted, and frozen.

#### *Epididymal Sperm Isolation*

Epididymides with attached efferent ductules were removed from adult (4-6-month-old) Sprague-Dawley rats. Epididymides were then placed into sterile petri dishes containing ice-cold RPMI-1640 medium (Gibco) containing sodium bicarbonate and lactate and adjusted to pH 7.2 by gassing with  $\text{CO}_2$ . Epididymides were then dissected into three sections: efferent ductules with caput epididymides, corpus epididymides, and cauda epididymides. Sperm were isolated separately from each of the three sections by gently shearing the tissues and rupturing the tubules, allowing sperm to float into the medium. Sperm were filtered on ice through fine nylon mesh to remove epididymal tissue. Sperm were sonicated and microsomal preparations were obtained using differential ultracentrifugation.

#### *3H2O Assay for P450 Aromatase*

##### *Activity*

P450 aromatase activity was measured by the release of I mot of  $^3\text{H}_2\text{O}$  from the aromatization of I mot of 1 $\beta$ -3H-androst-4ene-3, 17-dione (1 $\beta$ -3H-A) (New England Nuclear, Boston, Massachusetts) as described previously by Ackerman et al (1981), with the following modifications: 1 $\beta$ -3H-A (6.11 DPMs/ $\mu\text{mol}$ ) and various cofactors (NADP [10 mM], NADPH (10 mM), glucose-6-PO4 [100 mM], glucose-6-PO4 dehydrogenase [10 units/

nil], and  $\text{MgCl}_2$  [8.1 mg/ml]; Sigma) were prepared in 20 mM potassium phosphate buffer at pH 7.4 and added to microsomal germ cell, interstitial cell, and epididymal sperm preparations. Microsomal fractions were incubated in a shaking waterbath at  $34^{\circ}\text{C}$  for 1 hour, after which samples were treated with an equal volume of charcoal dextran to remove unconverted 1 $\beta$ -3H-A. Microsomal fractions from homogenates of chicken ovary and rat leg muscle were used as positive and negative controls, respectively. Positive control chicken ovary samples were small follicles (< 8 mm) taken from actively laying hens, 30-60 days old. Blank values were obtained from identical incubations in the absence of microsomes. Samples were centrifuged at  $1,800 \times g$  for 10 minutes, and supernatants were removed and mixed with Aquasol-2 (New England Nuclear) in scintillation vials. Samples were counted for 10 minutes in a liquid scintillation counter (Packard, Downers Grove, Illinois). P450 aromatase activity was calculated as fmol/mg protein/hour. This assay was previously validated for the measurement of P450arom activity in germ cells (Nitta et al, 1993). Protein concentrations in microsomal fractions were determined with the Bradford assay (Bradford, 1976).

#### *Western Blot Analysis*

Total cellular protein was extracted from Sta-put isolated pachytene spermatocytes, round spermatids, and a mixture of residual bodies, round spermatids, and elongating spermatids by boiling the cells in standard Laemmli SDS buffer for 10 minutes followed by sonication to shear the genomic DNA (Laemmli, 1970). Proteins were separated on a 10% SDS-polyacrylamide gel, electrophoretically transferred to an Immobilon-P membrane (Millipore, Burlington, Massachusetts), and probed with the same polyclonal P450arom antibody used for immunocytochemistry. Antibody binding was visualized by diaminobenzidine (DAB) after the use of a secondary goat anti-rabbit IgG peroxidase conjugated antibody.

#### *Immunocytochemistry*

Epididymides were collected from 4-6-month-old Sprague-Dawley rats and fixed in Bouin's solution overnight. The isolated tissue was then dehydrated using a series of ethanol solutions and embedded in Paraplast (Brunswick Co., St. Louis, Missouri). Sections (5  $\mu\text{m}$  thick) were placed on glass slides, deparaffinized, and treated with 10% normal goat serum to reduce background staining. Sections were stained for P450arom by immunocytochemistry, using a polyclonal anti-human placental P450arom antibody. This antibody, raised in a rabbit, was generated against human placental P450arom, which had been previously immunoaffinity-purified using a monoclonal antibody to P450arom (Kitawaki et al, 1989; Yoshida and Osawa, 1991). Control sections were treated with normal rabbit serum in place of the primary antiserum at the same concentration (1:200). Additional control sections were treated with antibody that was preabsorbed with affinity-purified human placental P450arom, the same protein used to generate the antibody. All sections were then reacted with an avidin-biotinperoxidase complex (Rabbit ExtrAvidin Staining Kit, Sigma). Peroxidase was visualized with a DAB-H202 solution. These

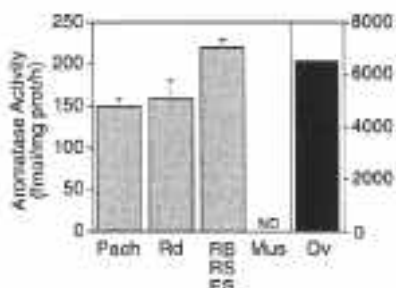


FIG. 1. P450 aromatase activity in Sta-put separated germ cells of the adult rat testis. Data represent means  $\pm$  standard error of the mean from duplicate determinations in a representative experiment. The experiment was repeated three times using five rats each time. Abbreviations are as follows: PACH, pachytene spermatocytes; Rd, round spermatids; RB, RS, and ES, mixture of residual bodies, round spermatids, and elongating spermatids, respectively. Muscle and ovary were used as negative and positive controls, respectively.

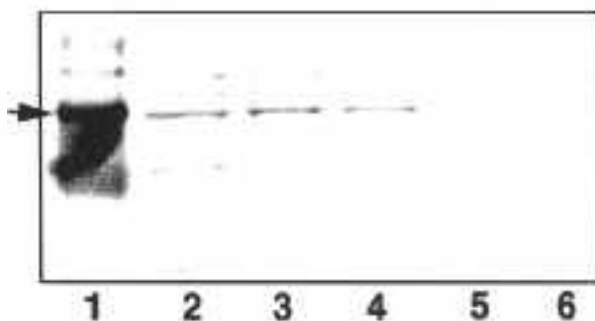


FIG. 2. Western blot analysis of P450 aromatase (P450arom) protein in isolated germ cells of the rat testis. Purified human placental P450arom, a 55 kDa protein (major band seen in blot), was run as a positive control standard (lane 1). Total cellular protein was extracted from pachytene spermatocytes (lane 2), round spermatids (lane 3), a mixture of residual bodies and round and elongating spermatids (lane 4), interstitial cells (lane 5), and chicken muscle as a negative control (lane 6). A major band in each of the germ cell lanes (2-4) and the interstitial cell lane (5) comigrated with the hP450arom protein (lane 1).

techniques have been previously validated in our laboratories (Nitta et al, 1991).

## Results

### P450 Aromatase Activity in Separated Rat Germ Cells

P450arom activity was measured in Sta-put isolated germ cells and interstitial cells to determine if germ cell P450arom was active (Fig. 1). P450 aromatase activities (fmol/mg protein/hour) measured in specific germ cells were as follows: pachytene spermatocytes (Pach),  $150 \pm 10$ ; round spermatids (Rd),  $160 \pm 20$ ; and a mixture of residual bodies and round and elongating spermatids (RB, RS, ES),  $220 \pm 10$ . Ovarian microsomes (Ov) were used as an additional positive control and converted 3H-A to estrone at a rate of  $6,500 \pm 100$  fmol/mg protein/hour. Muscle microsomes (Mus), used as a negative control in this assay, contained no P450arom activity. These results are representative of three separate experiments using five rats each.

### Western Blot Analysis of P450 Aromatase in Separated Rat Germ Cells

Western blot analysis of total cellular protein from Sta-put isolated germ cells was performed to confirm the presence of P450arom in rat germ cells (Fig. 2). Western analysis identified a 55 kDa band in purified human placental P450arom (lane 1), which was run as a positive standard, pachytene spermatocytes (lane 2), round spermatids (lane 3), and a mixture of residual bodies, round spermatids, and elongating spermatids (lane 4). The major band in each of the germ cell lanes (2-4) and the interstitial cell lane (5) comigrated with the hP450arom band. No aromatase band could be detected in the muscle, negative control lane (6).

### P450 Aromatase Activity in Rat Epididymal Sperm

P450 aromatase activity was measured in total sperm isolated from the efferent ductules and the caput, corpus, and cauda epididymis from five rats to determine if the P450arom immunolocalized in efferent ductule and epididymal sperm was active (Fig. 3). The sperm isolations and activity measurements were repeated on three separate occasions, yielding consistent results. P450 aromatase activities (fmol/mg protein/hour) measured in sperm isolated from the caput epididymis with attached efferent ductules, corpus epididymis, and cauda epididymis were  $130 \pm 6$ ,  $60 \pm 8$ , and  $68 \pm 3$ , respectively. Positive (ovarian microsomes) and negative (muscle microsomes) controls were also used. As expected, the controls yielded very high activity levels for ovary and baseline (zero) activity levels for muscle (data not shown).

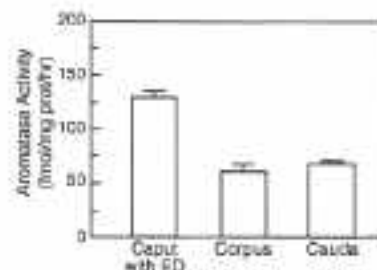


FIG. 3. P450 aromatase activity in rat epididymal sperm isolated from different regions of the efferent ductules (ED) and epididymis (caput, corpus, and cauda). Data represent means  $\pm$  standard error of the mean from duplicate determinations in a representative experiment. The experiment was repeated three times using five rats each time.

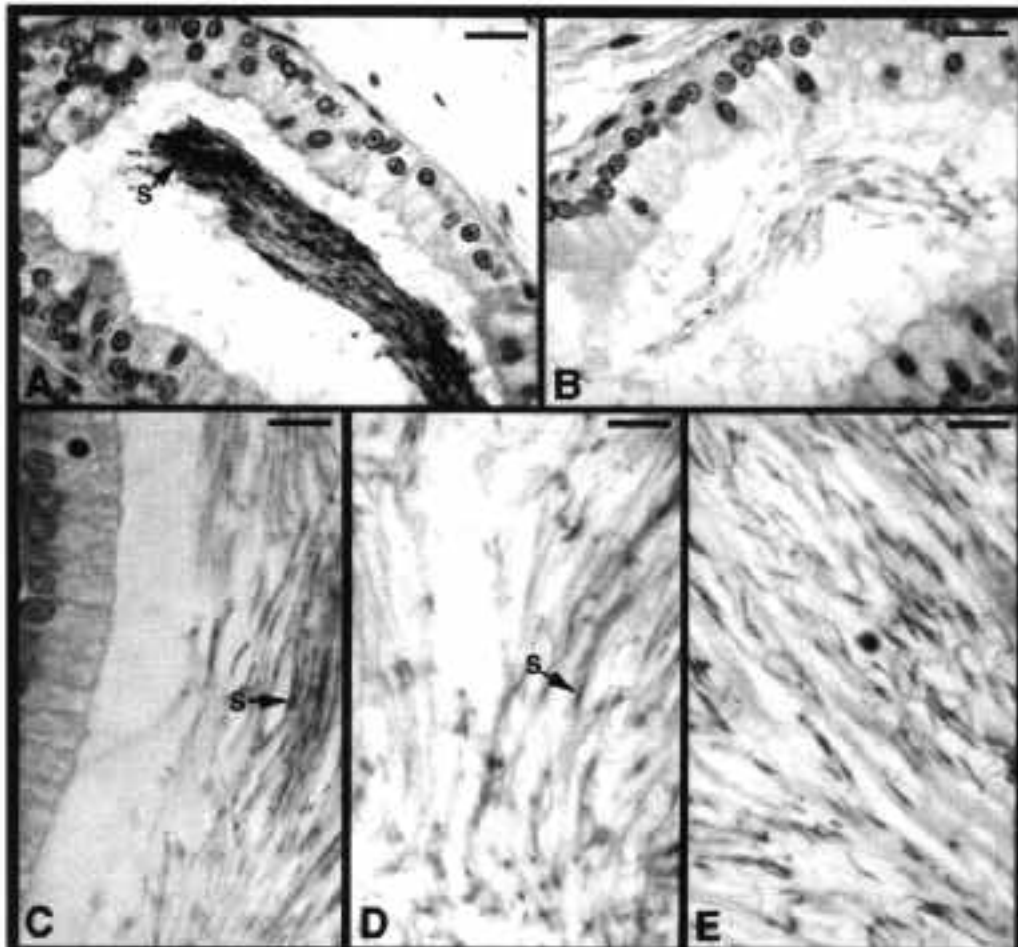


FIG. 4. Immunocytochemical localization of P450 aromatase in the adult rat efferent ductules and epididymis. Immunostaining (a brown-colored reaction product) was strong in sperm tails (s) within the ductule lumen (A). Control sections, treated with normal rabbit serum in place of the primary antiserum at the same concentration, showed no reaction product in the spermatozoa within the lumen or within the ductal epithelium (B). Immunostaining was apparent within sperm in the caput epididymis (C), but staining intensity decreased as sperm moved into the corpus (D) and cauda epididymis (E), although minimal immunostaining in sperm was still apparent. (A, B: bar = 40  $\mu$ m; C, D, E: bar = 30  $\mu$ m).

#### *Immunocytochemical Localization of P450 Aromatase in Epididymal Sperm*

P450 aromatase was localized in sperm present in the efferent ductules (Fig. 4A). In control samples treated with normal rabbit serum in place of the primary antiserum (Fig. 4B) or with antibody that was preabsorbed with affinity-purified human placental P450arom (data not shown), no reaction product was found within the sperm or within the ductal epithelial cells. Although immunostaining was also present in sperm within the caput (Fig. 4C), corpus (Fig. 4D), and cauda epididymis (Fig. 4E), the staining intensity decreased distally down the epididymis. The reaction product appeared more intense along the midpiece of the sperm tail, but also stained the cytoplasmic droplet in caput sperm. At higher magnification

(Fig. 5), the immunocytochemical localization of P450arom can be seen specifically staining the cytoplasmic droplet. Although the sperm tails also stained positive just distal to the cytoplasmic droplet, the sperm heads (light colored in the negative image) are not positive.

#### *Discussion*

The aim of this study was to determine if the P450arom enzyme was present and active in late-stage germ cells and epididymal sperm of the adult rat. We found that 1) P450arom is present in meiotic-postmeiotic germ cells of the adult rat testis and in sperm in the efferent ductules and caput epididymis; 2) P450arom in germ cells and

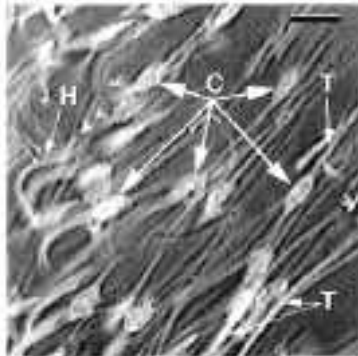


FIG. 5. Immunocytochemical localization of P450 aromatase at higher magnification. Immunostaining was specific for the cytoplasmic droplet (C). The sperm tails (T) also stained positive distal to the cytoplasmic droplet. Sperm heads (H) are not positive but are light colored in a black and white photograph. This is a negative image of the original color photo. The brown precipitate reaction product appears white. Bar = 5  $\mu$ m.

epididymal sperm is an active enzyme that should be capable of estrogen synthesis; and 3) P450arom activity decreases in sperm as they traverse the epididymis, with the highest level of activity present in sperm in the efferent ductules and caput region.

Our results show that germ cells, beginning with pachytene spermatocytes, contain active P450arom. These data suggest that germ cells are capable of synthesizing estrogen during their differentiation within the seminiferous tubule. Western blot analysis indicates that germ cell P450arom is predominately found as a 55 KDa form, with only very minor bands of a slightly larger size detected. As this protein is glycosylated some size variations due to glycosylation differences would be expected. These findings extend previous studies that have shown that germ cells of the adult, mouse contain active P450arom (Nitta et al, 1993). The mouse P450arom activity was higher in a crude mixture of total mouse germ cells than in interstitial cells (Nitta et al, 1993). In contrast, our current studies show P450arom activity was lower in Sta-put separated germ cells than in interstitial cells. This could reflect a real difference in the localization and level of P450arom between mouse and rat. Alternatively, this difference may be due to the longer handling time required for the isolation of separated germ cells as well as artificially inflated protein concentrations in germ cell preparations due to the presence of BSA in fractions collected from the Sta-put system.

The present study shows for the first time that P450arom is present and active in sperm isolated from the efferent ductules and epididymis of the rat, and that it decreases as sperm traverse the epididymis. P450 aromatase activity of sperm isolated from the efferent ductules and caput epididymis was consistently higher than P450arom activity of sperm isolated from the corpus and

cauda epididymis. This decrease in activity was supported by a similar reduction in immunostaining of sperm.

The production of estrogen by testicular germ cells would provide a local source of estrogen within the seminiferous tubule that would be proportional to the number of developing spermatids. This estrogen could act on the Sertoli cell or other cells of the seminiferous tubule during spermatogenesis and/or may cause feedback to the Leydig cell to regulate androgen production within the testis. Alternatively, estrogen produced by maturing germ cells and epididymal sperm may be involved in the regulation of epididymal epithelium. In fact, estrogen is found in higher concentrations in epididymal luminal fluid and tissue than in the blood plasma of many species (Ganjam and Amann, 1976; Eiler and Graves, 1977; Kumari et al, 1980; Claus et al, 1985). For example, in fat caput epididymal tissues, the concentration of estrogen was 25 times greater than in the blood (Kumari et al, 1980). Germ cell and sperm-derived estrogen could potentially act to regulate epididymal function more precisely, because the concentration of estrogen in the efferent ductule and epididymal fluid would depend upon the number of differentiating germ cells and the quantity of maturing sperm traversing this region at any given time.

Although estrogen is present in the testis, seminal fluid, and semen, and estrogen receptors and estrogen binding have been detected in the male reproductive system of several species (Danzo and Eller, 1979; Murphy et al, 1980; Younes and Pierrepont, 1981; Nakhla et al, 1984; Schleicher et al, 1984; Tekpetey and Amann, 1988; Toney and Danzo, 1988; West and Brenner, 1990; Cooke et al, 1991; Iguchi et al, 1991; Greco et al, 1992), the role of estrogen inside the testis is still largely unknown. Although estrogen receptors are found in both Sertoli and Leydig cells (Mulder et al, 1974; de Boer et al, 1976; Benahmed et al, 1982; Nakhla et al, 1984; Terakawa et al, 1985; Iguchi et al, 1991), the highest concentration of estrogen receptors has been reported to be in the efferent ductules of the adult mouse (Schleicher et al, 1984), monkey (West and Brenner, 1990), and goat (Goyal et al, 1997). The presence of estrogen receptors in the testis and epididymis strongly suggests that these tissues are an estrogen target and that estrogen may have a regulatory role in these regions. The estrogen binding cells in these tissues are the same cells reportedly responsible for resorptive activity. The resorptive region of the epididymis has also been designated a "kidney analogue" (Hinton and Turner, 1988), functioning to increase the concentration of sperm within the lumen. In fact, approximately 89-96% of luminal fluid is absorbed in the efferent ductules and initial segment of the caput epididymis (Turner, 1984; Clulow et al, 1994). Therefore, it is plausible that luminal estrogen is involved in fluid resorption in this region.

Moreover, estrogen receptors are required for normal

male reproductive function. Lubahn et al (1993) first reported that the insertional disruption of the estrogen receptor gene (ERKO) in mice resulted in males with decreased fertility. The ERKO mice had decreased testis weights and sperm counts of only 10% of control (Lubahn et al [1993] and personal observation). Subsequent studies of **ERKO mice showed that this mutation results** in sterile males with seminiferous tubule dystrophy (Korach, 1994).

On the basis of observations presented in this study and in previous studies (Pudney et al, 1985; Nitta et al, 1993; Tsubota et al, 1993; Kwon et al, 1995; Janulis et al, 1996), we conclude that adult testicular germ cells and epididymal sperm can synthesize estrogen and that production of estrogen is possibly a site-dependent and timedependent process important to spermatogenesis, sperm maturation, and epididymal function. The presence of estrogen receptors in the testis and epididymis strongly suggests that these tissues are an estrogen target and that estrogen may have a regulatory role in these regions. We hypothesize that estrogen produced by testicular germ cells and maturing epididymal sperm binds to estrogen receptors in the efferent ductules and other epithelial cells of the epididymis (Schleicher et al, 1984; Tekpetey and Amann, 1988; Toney and Danzo, 1988; West and Brenner, 1990; Cooke et al, 1991), thereby assisting in the regulation of physiological function in this region. This study demonstrates that the adult male rat serves as a suitable model for the study of estrogen's role in spermatogenesis and sperm maturation in the male reproductive tract.

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