Journal of Andrology, Vol. 23, No. 6, November/December 2002
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Localization of Androgen and Estrogen Receptors in Adult Male Mouse Reproductive Tract

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ABSTRACT: There is considerable variation, both within and between species, in reports of nuclear steroid receptor localizations in the male reproductive tract. In this study, androgen receptor (AR) and estrogen receptors ERα and ERβ were visualized by immunohistochemistry in adult male mice reproductive tracts, including testes, efferent ductules; initial segment, caput, corpus, and cauda epididymides; and vas deferens. Antibody specificity was demonstrated by Western blot and antibody competition. In testis, AR was expressed in Leydig cells, Sertoli cells, and most peritubular cells, but not in germ cells; Sertoli cells showed more intense staining in stages VI–VII; ERα was present in Leydig and some peritubular cells; ERβ was in Leydig, some peritubular, all Sertoli and germ cells except in spermatids and meiotic spermatocytes. In efferent ductules, AR was strongly expressed in ciliated and nonciliated epithelial cells and in stromal cells; ERα was strongly expressed in ciliated and nonciliated epithelial cells; stromal cells were negative; and ERβ was strongly expressed in ciliated and nonciliated epithelial cells and also in stromal cells. In epididymis, AR was strongly expressed in all epithelial cells (not in intraepithelial lymphocytes); ERα was strongly expressed in apical, narrow, and some basal cells of the initial segment, and in caput, principal cells of the caput, clear cells of the distal caput through cauda; stromal cells were negative in the initial segment, but more stromal cells were stained from caput to cauda; ERβ was strongly expressed in most of epithelial cells of the epididymis, but stromal cells were inconsistently stained. In vas deferens, AR was weakly expressed or absent in principal cells but moderately stained in basal cells, smooth muscle cells of stroma were stained intensely, ERα was absent in epithelial cells but present in a subepithelial smooth muscle layer, and ERβ was strongly expressed in all epithelial cells and most stromal cells. This study demonstrates that the reproductive tracts of male mice differ considerably from those of rats in expression of ARs and ERs and that caution is needed when extrapolating nuclear steroid receptor data across mammalian species.

Key words: Efferent ductule, epididymis, steroid hormone receptors, testis, vas deferens.

J Androl 2002;23:870–881

Androgen receptors (ARs) in male reproductive tissues are well recognized for their importance in the regulation of factors that maintain spermatogenesis and ensure the production of a physiological environment that permits spermatozoa maturation in the epididymis. However, estrogen receptors (ERs) are also present throughout the male reproductive tract (Hess et al, 2001a) and it is now accepted that estrogens (Hess et al 1997a, 2001a; Sharpe, 1998; Couse and Korach, 1999; Hess, 2000; O'Donnell et al, 2001) as well as androgens (Ezer and Robaire, 2002) are important in male reproductive tract biology. Both subtypes of ER, α and β, are present in males (Fisher et al, 1997; Hess et al, 1997b; Couse and Korach, 1999; Nie et al, 2002), but ERβ appears more abundant and in a greater number of cell types in the male reproductive system (Saunders et al, 1998, 2001).

The mouse is one of the most extensively used animals in biomedical research and a clear understanding of cellular localizations of AR and ER is necessary for appropriate interpretation of experiments focused on male reproduction. However, few studies have documented these steroid receptors by immunohistochemistry in this species (Iguchi et al, 1991; Zhou et al, 1996; Rosenfeld et al, 1998) and cell specificity is lacking in these reports.

A recent histological examination of the male reproductive tract in the ERα knockout mouse (αERKO) revealed several abnormalities in the epididymis (Hess et al, 2000). These abnormalities were cell-specific and corresponded to cells previously shown to bind ^{3}H-estradiol in mice (Schleicher et al, 1984). Thus, based on these observations, the presence of ERα in the mouse epididymal epithelium would be predicted. However, reports of steroid receptor localizations have not been consistent across species nor among species. Some studies have shown strong expression of ERα in both testis and epi-

Supported by National Institutes of Health grants HD35126 (to R.A.H.) and CA18119 (to B.S.K.).

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Received for publication March 6, 2002; accepted for publication June 6, 2002.

didymis, whereas other species have reduced expression in the testis and sometimes no expression in the epididymis (Hess et al, 2001a, 2002). Our laboratory showed epithelial expression in certain regions of the rat epididymis (Hess et al, 1997b), whereas another laboratory, using different antibodies, found ERα expressed only in the efferent ductules, with no expression in the epididymis (Fisher et al, 1997). A similar inconsistency has been noted in reports of ERβ localization in the testis of several species (van Pelt et al, 1999; Pelletier et al, 2000; Makinen et al, 2001; Saunders et al, 2001).

Because of the significance of nuclear steroid receptors to our understanding of reproductive biology, we thought it important to examine in detail the comparative expression of androgen and estrogen receptors in the reproductive tracts of important mammalian species. In the present study, the pattern of immunohistochemical expression was examined in mice using antibodies to AR, ERα, and ERβ. Antibody specificity was demonstrated by antibody competition and Western blot analysis.

Materials and Methods

Animals

Male reproductive tract tissues from 6 adult C57BL/10J mice (60–90 days of age) were used for immunohistochemistry. Four to 5 adult mice were used for Western blot analysis. Animal experiments were approved by the institutional animal care and use committees of the respective universities and were conducted in accordance with the National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals.*

Tissue Processing

Animals were anesthetized and perfused with 10% neutral buffered formalin (NBF) for 20 minutes. After tissues were postfixed in NBF overnight at 4°C, they were transferred to 70% ethanol and embedded in paraffin. Sections were cut at 5 μm thickness and then dried at 37°C overnight. Tissues evaluated were the testis, efferent ductules, epididymis, and vas deferens.

Single Receptor Staining

Tissues were stained for receptors as described previously (Nie et al, 2002). To unmask the receptor protein, sections were microwaved in a 0.01 M citrate buffer solution pH 6.0 for 20 minutes. Tissues were respectively incubated with AR-specific antibody PG21 at 1:500 diluted with phosphate-buffered saline (PBS), ERα-specific antibody NCL-ER-6F11 (Novocastra, Newcastle upon Tyne, United Kingdom) at 1:1000 dilution, and ERβ-specific antibody S-40 at 1:500 dilution for 12 hours at 4°C. Previous studies have described the generation of the S-40 ERβ and AR antibodies (Prins et al, 1991; Saunders et al, 2000). Three other ERβ antibodies were tested in this study to determine which to use for optimum results. The antibodies included a rabbit anti-rat ERβ polyclonal antibody PA1-310 (Affinity BioReagents, Golden, Colo), and 2 mouse anti-human monoclonal antibodies F-12 and E-12 (Choi et al, 2001). Antibody bindings were visualized by using the avidin-biotin complex (ABC Kit, Vector Laboratories, Burlingame, Calif), and the diaminobenzidine (DAB) chromogen. Hematoxylin (Sigma Chemical Company, St Louis, Mo) was applied as a counter stain. Sections incubated without the primary antibody but with PBS were used as negative controls for color development on the same slide. Images were captured with a Spot II digital camera (Diagnostic Instruments, Sterling Heights, Mich) and compiled using Adobe Photoshop software (Adobe Systems, San Jose, Calif).

Double Receptor Staining

Colocalization of ERα and ERβ in the efferent ductules and the head of the epididymis was examined by double staining. After antigen retrieval and blocking with 10% normal rabbit serum, sections were incubated sequentially in the following solutions, with a PBS rinse in between: ERα (NCL-ER-6F11) mouse antibody (1:50), fluorescein isothiocyanate (FITC)-conjugated anti-mouse immunoglobulin (Ig) G (1:100; Sigma), ERβ, S-40, sheep antibody (1:250), and Texas red–conjugated anti-sheep IgG (1:100; Vector Laboratories). Sections incubated without the primary antibody were used as the negative control. Tissues were examined with a fluorescence microscope with suitable filters for FITC and Texas red, and images were captured with the Spot II digital camera. In Adobe Photoshop, the individual images for ERα (FITC-green) and ERβ (Texas red) were combined using the overlay tool. Cells that contained both receptors stained various shades of yellow-green to bright yellow.

Antibody Competition

AR21 (AR peptide), AR462 (AR unrelated peptide), recombinant human ERα protein (Panvera, Madison, Wis), and peptide P4 (the antigenic peptide for S40) were used to perform antibody competition, respectively. Briefly, 10-fold to 15-fold molar excess of protein or peptide were incubated together with related antibodies overnight at 4°C, then were used as primary antibody as described before for immunohistochemistry. Efferent ductules and corpus epididymides were used for the competition tests of 3 antibodies.

Western Blot Analysis

Testis and epididymis were extracted for detection of AR protein. Mouse epididymis and uterus (a positive control) were extracted for detection of ERα protein. Mouse epididymis was extracted for examination of ERβ protein. Human recombinant ERβ was used as the protein standard for ERβ (Panvera). The method was modified based on a previous publication (Choi et al, 2001). Protein samples were separated on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a nitrocellulose membrane. Nonspecific binding was blocked with 5% dried milk in Tris-buffered saline (TBS; 50 mM Tris-Cl, pH 7.5/150 mM NaCl) containing 0.05% Tween-20 for 1.5 hours and then the membranes were incubated overnight at room temperature with 1:500 diluted AR antibody PG21, 1:50 diluted ERα antibody NCL-ER-6F11 (Novocastra, England), and 1:2000 ERβ antibody S-40 in TBS containing 1%–2% dried milk and 0.2% Tween-20. After washing in TBS containing 0.05% Tween-20, the membranes were incubated

with horseradish peroxidase-conjugated secondary antibodies for 1 hour in the same buffer used for primary antibody incubation. Peroxidase-conjugated secondary antibodies included goat anti-rabbit IgG diluted at 1:2000 (Zymed, San Francisco, Calif), goat anti-mouse IgG diluted at 1:4000 (Pierce, Rockford, Ill), or rabbit anti-sheep IgG diluted 1:2000 (Sigma). Antibody bindings were visualized by using diaminobenzidine (DAB) chromogen as substrate.

Results

Immunohistochemistry

All positive reactivity was noted as predominantly nuclear staining and there was no nonspecific staining when tissues were treated with PBS instead of primary antibodies in control sections. Scoring of staining intensity was classified as negative (no staining), weak (+), moderate (++), or strong (+++). Staining for ERα in efferent ductules was defined as a baseline strong staining. Evaluation of epididymal cell types was divided into epithelial and stromal categories. Stromal cells contained smooth muscle or peritubular smooth muscle cells, connective tissue cells (including fibroblasts), and vascular endothelium. The only cell type in stroma that was specifically identified was the peritubular smooth muscle cell or the myoid cell (in testis). The peritubular myoid cells were confined to cells underlying the seminiferous tubule. Peritubular smooth muscle cells were identified as the cells lying immediately beneath the excurrent ductal epithelium. The ERβ antibodies gave identical nuclear staining patterns in the excurrent ducts (from efferent ductules to vas deferens); however, stain intensity was somewhat better with S40, and therefore it was used for the illustrations. The results of immunostaining are summarized in Table 1.

Testis

AR immunoreactive staining was strong in most Leydig cells and in approximately 95% of peritubular myoid cells (Figure 1, a and m), regardless of proximity to different stages of spermatogenesis (Table 2). Sertoli cells showed stage-specific staining, with the most intense staining of nuclei found in stages VI–VII and the least amount of staining in stages I–III and VIII–XII (Table 2). All germ cells were AR-negative.

ERα immunostaining was intense in many Leydig cell nuclei, as well as some peritubular myoid cells (Figure 1, b and o). Sertoli cells and germ cells in all stages of spermatogenesis were ERα-negative. ERβ was expressed in Leydig cells and most peritubular myoid cells. Within the seminiferous tubule, ERβ staining was found in Sertoli cells and spermatogonia at all stages of spermatocytes (Figure 1, c and q). Spermatocytes were also ERβ-positive, except for spermatocytes in meiotic division.

Table 1. *Androgen and estrogen receptors in the male reproductive tract of adult mouse*

	AR	ERα	ERβ
Testis			
Leydig cell	+++	+++	+++
Peritubular	+++/−	+++/−	+/−
Sertoli cell	+++/+*	−	++
Germ cell	−	−	++/−§
Efferent ductule			
Epithelium	+++	+++	+++
Stroma	+++	−	+++/−
Initial segment			
Epithelium	+++	−/+++†	+++
Stroma	+/−	−	+/−
Caput epididymis			
Epithelium	+++	++/+++‡	+++
Stroma	+++/−	−/+++	−
Corpus			
Epithelium	+++	−/+++¶	+++
Stroma	+++/−	−/+++	++/−
Cauda epididymis			
Epithelium	+++	−/+++¶	+++
Stroma	+++/−	+++/−	+++/−
Vas deferens			
Epithelium	+/++‖	−	+++
Stroma	++	+++/−	+++

* Staining intensity scores were as follows: −, negative; +, weak staining; ++, moderate staining; +++, strong staining. Staining is stage-dependent, with most intense staining in VI–VII.
† Some apical, narrow, and basal cells strongly positive.
‡ Apical cells were more intensely positive.
§ All germ cells positive, except for spermatids and meiotic spermatocytes.
‖ Basal cells were moderately positive.
¶ Clear cells were intensely positive.

Efferent Ductules

Ciliated and nonciliated epithelial cells exhibited intense staining for AR (Figure 1, d and n), ERα (Figure 1, e and p), and ERβ (Figure 1, f and r). Among stromal cells, AR was strongly positive in peritubular smooth muscle and most other cell types. ERα was negative in most stromal cells but weakly positive in a few. Most stromal cells were strongly positive for ERβ.

Initial Segment of the Epididymis

In this region, AR and ERβ shared a similar strong staining pattern. AR (Figure 1, g and s) and ERβ (Figure 1, i and w) were strongly positive in nuclei of all epithelial cells, including apical, narrow, basal, and principal cells. There appeared to be no difference in staining intensity between principal and basal cells. Intraepithelial lymphocytes (halo cells) were negative (observed, but not illustrated). Some apical and narrow cells were also intensively positive for ERα, but other nuclei of these cell types stained weakly positive. Principal cells were weakly positive or negative for ERα. Most basal cells were ERα-negative or weakly positive, but an occasional basal cell

showed an intense staining for ERα (Figure 1, h and u). In the stroma, many cells were weakly to moderately positive for AR and ERβ but were essentially negative for ERα.

Caput Epididymis

AR was expressed in all epithelial cells (Figure 1, j and t). Most principal cells were moderately to strongly positive for ERα (Figure 1, k and v) and ERβ (Figure 1, l and x) except for some basal cells, and the strongest ERα immunostaining was distributed in apical cells (Figure 1k, unlabeled arrows). There was a distinct increase in staining intensity for ERα in the caput epididymis compared with the initial segment.

In the stromal area, AR was positive in peritubular cells and some other stromal cells. Most stromal cells were weakly positive to negative for ERα, and ERβ was generally absent in the stroma.

Corpus Epididymis

AR (Figure 2, a and j) and ERβ (Figure 2, c and n) were expressed abundantly in all epithelial cells lining the corpus epididymal duct. In contrast, ERα was present only in clear cells of the epithelium and some peritubular cells of the stroma (Figure 2, b and l). In the stroma, AR and ERβ were positive in some cells but negative in others.

Cauda Epididymis

Epithelial cells stained intensely for AR (Figure 2, d and k) and ERβ (Figure 2, f and o) but ERα staining (Figure 2, e and m) was identical to the corpus epididymis, where only clear cells and some peritubular smooth muscle cells were strongly positive. Many stromal cells were positive for all three receptors.

Vas Deferens

In the epithelium, AR was expressed weakly in epithelial cells but with moderate strength in basal cells (Figure 2, g and p). ERα was absent within the epithelium (Figure 2, h and r), whereas ERβ was abundant in all epithelial cell types (Figure 2, i and t). In the stroma, AR was expressed in smooth muscle and connective tissue cells, but ERα was found only in the outer layer of smooth muscle cells, whereas ERβ was abundant throughout the stroma.

Double Staining

It was obvious from microscopic observation that both ERα and ERβ were colocalized in the same cells in various regions of the male tract. Therefore, we selected to examine the efferent ductules and initial segment of epididymis using colocalization of fluorescent signals to determine whether the same cells express the 2 receptors. Both ERα (green) and ERβ (red) were expressed in epithelial nuclei of the efferent ductules (Figure 3, a and b). Colocalization of the 2 receptors in the same cell was detected by an orange to yellowish color, with variations in color intensities indicating differences in the proportion of the 2 receptors in an individual cell nucleus (Figure 3c). In the overlay view of the proximal efferent ductule epithelium (Figure 3c), some cells remained green, indicating expression of only ERα; some stained intensely yellow, indicating equivalent expression of ERα and ERβ; and an occasional cell stained only red, indicating an expression of only ERβ.

A transition zone from efferent ductules to the initial segment of the epididymis can be viewed in Figure 3, d–f. The one tubule that stained intensely for both ERα and ERβ belongs to the common efferent duct that opens into the initial segment epididymis. This duct shows less variation in overlay staining (Figure 3f) than does the proximal ductules (Figure 3c), indicating nearly equal expression of the 2 receptors in all epithelial cells. In the same photographs, a region of the initial segment is also noted. The only fluorescence detected for ERα in the initial segment was the apical and narrow cells (Figure 3d). ERβ was expressed in all epithelial cell types (Figure 3e). The overlay shows considerable variation in staining of apical and narrow cells (Fig. 3f; note the unlabeled arrows), similar to what was observed with single receptor staining (Figure 1, h and i).

Antibody Competition

AR21 peptide competed away the nuclear staining given by the AR antibody PG21 reaction to efferent ductule (Figure 2q) and epididymal tissues (not shown), whereas the unrelated peptide AR462 was not able to ward off the specific nuclear staining (not shown). Recombinant human ERα protein competed away all nuclear staining given by monoclonal antibody 6F11 reaction (Figure 2s), and peptide P40 also competed away all staining given by the ERβ antibody S40 (Figure 2u).

Western Blot Analysis

A single dominant band of approximately 110 kd was detected for AR in both mouse testis and epididymal extracts (Figure 4a), which is the reported molecular size for AR (Prins et al, 1991). A comigration band of ERα with mouse uterus was shown in mouse epididymal extract. The molecular size of this band was approximately 66 kd (Figure 4b). Mouse uterine extract was used as a positive control for ERα. Two bands were observed on mouse epididymal extract, which also comigrated with the purified human recombinant ERβ (Figure 4c). The molecular weight of the purified protein was 54 kd. One band comigrated with this positive marker, the other band is approximately 59 kd, as reported for the long form of ERβ (Kuiper et al, 1998).

Figure 1. Immunostaining of AR, ERα, and ERβ in mouse testis **(a–c, m, o, q)**, efferent ductules **(d–f n, p, r)**, initial region of epididymis **(g–i, s, u, w)**, and caput epididymis **(j–l, t, v, x)**. S indicates Sertoli cell; M, peritubular myoid cell; Ly, Leydig cell; C, ciliated cells; B, basal cell; Cl, clear cell; E, epithelial cell; Sm, smooth muscle cell; St, stromal cells. Narrow or apical cells **(g–l)** are pointed out with unlabeled arrows. Bar = 25 μm **(a–l)**; bar = 12.5 μm **(m–x)**.

Figure 2. Immunostaining of AR, ERα, and ERβ in mouse corpus epididymis **(a–c, j, l, n)**, cauda epididymis **(d–f, k, m, o)**, and vas deferens **(g–i, p, r, t)**. Peptide competition of AR antibody **(q)**, ERα **(s)**, and ERβ **(u)**. B indicates basal cell; Cl, clear cell; E, epithelial cell; Sm, smooth muscle cell; St, stromal cells. Bar = 25 μm **(a–i)**; bar = 12.5 μm **(j–u)**.

Discussion

This study provides specific cellular localization of androgen and estrogen receptors in the testis and its excurrent ducts in adult male mice. Based on previous studies in other species (Goyal et al, 1997b; Saunders et al, 2001), epithelial expressions of AR and ERβ throughout the male mouse reproductive system was not surprising. However, expression of ERα in a region and cell-specific manner in the mouse demonstrates that species differences exist for ERα expression in the adult male.

Table 2. *Androgen receptors in adult mouse testis*

Location	Cell Type	Intensity of Stain	
Interstitium	Leydig cell	+++	
	Endothelial cell	+/−	
	Smooth muscle	+++	
	Peritubular cell	+++*	
Seminiferous tubule	Sertoli cell	+	Stages I–III
		++	Stages IV–V
		+++	Stages VI–VII
		+	Stages VIII–XII
	Germ cell	−†	

* All stages of spermatogenesis.
† All germ cells were negative.

Testis

Although there is a substantial decrease in AR in the male mouse reproductive tract tissues from birth to adulthood (Gallon et al, 1989), androgens remain the primary steroid hormones for maintenance of male reproductive function in the adult (Ezer and Robaire, 2002), and the common presence of AR in the male would be expected. There is nearly universal agreement across species that in the adult testis, AR is present in Sertoli cells, peritubular cells, and Leydig cells (Sar et al, 1990; Bremner et al, 1994; Vornberger et al, 1994; Van Roijen et al, 1995; Goyal et al, 1997a,b; Suarez-Quian et al, 1999; Pelletier et al, 2000; Zhu et al, 2000). Expression of AR in Sertoli cells supports previous reports that androgens regulate Sertoli cell function and are essential for spermatogenesis (Sharpe, 1994). The present study found a stage-dependent expression of AR in Sertoli cells, similar to other studies in rats and humans (Bremner et al, 1994; Vornberger et al, 1994; Suarez-Quian et al, 1999).

AR was not found in germ cells of mouse testis. However, the presence of AR in testicular germ cells has been controversial, with most studies in other species having indicated a lack of AR, but some studies showing AR-positive spermatogonia (Kimura et al, 1993; Zhou et al, 1996) or stage-specific elongate spermatids (Vornberger et al, 1994). In support of our findings in the mouse, a recent study using spermatogonial stem cell transplant technology demonstrated that germ cells of the mouse testis do not require a functional AR to complete spermatogenesis (Johnston et al, 2001). Therefore, if Sertoli cells in certain stages also express no AR or low concentrations, it is likely that testosterone provides indirect stimulation of spermatogenesis in those stages through the AR-positive peritubular cells. AR expression in Sertoli cells appears to have more androgen dependency than in Leydig and peritubular cells (Zhu et al, 2000).

In the testes of adult mice, we detected ERα in Leydig cells and peritubular myoid cells, whereas Sertoli and germ cells of the seminiferous epithelium were negative. This is consistent with a recent report that the presence of ERα in germ cells of the testis is not required for their development (Mahato et al, 2000). It is surprising that in testes of adult humans, macaques, marmosets, and goats, no ERα immunoexpression was detected in any cell type (Goyal et al, 1997a; Makinen et al, 2001; Saunders et al, 2001). However, in another study of marmosets, ERα was reported in interstitial (Leydig) cells (Fisher et al, 1997). In cats and dogs (Nie et al, 2002) and rats (Sar and Welsch, 2000), ERα has also been detected in the interstitial (Leydig) cells but not in peritubular cells.

In the present study, ERβ was expressed more extensively than ERα from the testis to the vas deferens, both in cell types and in number of positive cells. Its expression in the testis is controversial, with considerable variation across species and even within species. We found in mouse testis, using the S40 antibody, that ERβ was expressed in Sertoli cells, spermatogonia, and spermatocytes (except for cells in meiotic division). Spermatids and spermatozoa were negative. These results are similar to previous studies in several species (Enmark et al, 1997; Rosenfeld et al, 1998; Saunders et al, 1998, 2001; Pelletier et al, 1999, 2000; van Pelt et al, 1999; Makinen et al, 2001; Nie et al, 2002), but contrasts with other reports. In one study of rats, ERβ was found only in Sertoli cells (Pelletier et al, 2000) and in a study of humans, Makinen et al (2001) found that ERβ was expressed only in germ cells. Rosenfeld (1998) found that in mice, ERβ was also expressed in elongated spermatids.

Efferent Ductules

There are few reports of AR localization in efferent ductules, but in all species studied to date, AR appears to be underexpressed in efferent ductule epithelium, compared to the epididymis, except in mice, as reported here. Ungefroren et al (1997) reported no AR messenger RNA or immunohistochemical signal in humans. In rhesus monkeys, AR was expressed but was less abundant in efferent ductule than in caput and corpus epididymis (Roselli et al, 1991). Goats also showed lower expression, with no appearance in ciliated cells and variable staining in nonciliated cells (Goyal et al, 1997a). The function of efferent ductules in fluid reabsorption appears to be similar in all species (Hess, 2002); however, this difference in AR expression in mice compared to other species raises serious questions regarding interspecies comparisons in toxicological studies. Further study of AR expression in this region of the male is needed in other species, and in particular in rats, which are used extensively in toxicology. One study in rats to day 18 postpartum showed AR expression that was abated with perinatal treatment with diethylstilbestrol (McKinnell et al, 2001). Collectively, these data are in agreement with our previous studies showing the importance of ERα abundance in these tu-

bules and the major role of ERα, rather than AR, in regulation of efferent ductule function (Hess et al, 2002).

ERα expression in efferent ductule epithelium has been consistent across all species studied, including rats, mice, roosters, dogs, cats, goats, monkeys, and humans (West and Brenner, 1990; Ergun et al, 1997; Fisher et al, 1997; Goyal et al, 1997a; Hess et al, 1997b; Kwon et al, 1997; Rosenfeld et al, 1998; Saunders et al, 2001; Nie et al, 2002). However, in some species, the ciliated cells were ERα-negative (West and Brenner, 1990; Ergun et al, 1997; Goyal et al, 1997a; Saunders et al, 2001), but in mice, all epithelial cells were ERα-positive in these ductules. ERα showed the highest intensity of reproductive tract staining in the efferent ductules, consistent with our previous studies showing that ERα is responsible for regulating fluid reabsorption in these ducts (Hess et al, 1997a) through the control of epithelial ion transporters (Lee et al, 2001; Zhou et al, 2001). Efferent ductules are responsible for reabsorbing more than 90% of the fluid entering from the rete testis (Clulow et al, 1998). This action of estrogen in the efferent ductules is now recognized as being essential for male fertility (Eddy et al, 1996; Hess et al, 2001b; Oliveira et al, 2001, 2002; Zhou et al, 2001).

The expression of ERβ throughout the excurrent ducts is ubiquitous even though it seems more predominant in epithelia than stroma. The expression profile of ERβ in mouse tract is more similar to AR than it is to ERα. Due to its unclear physiological role in the male, further study is need to clarify ERβ action in the testis and its excurrent ducts (Krege et al, 1998; Couse et al, 1999; Dupont et al, 2000).

Epididymis

AR has been demonstrated by various techniques to be present in the epididymis of numerous species (Younes and Pierrepoint, 1981; Schleicher et al, 1984; Toney and Danzo, 1988; Gallon et al, 1989; Tekpetey et al, 1989; Sar et al, 1990; Cooke et al, 1991; Roselli et al, 1991; Goyal et al, 1997b; Ungefroren et al, 1997; You and Sar, 1998; Pelletier, 2000), and dependence of the epididymis on androgens for structure and function is well known (Ezer and Robaire, 2002). In mice, immunostaining for AR demonstrates a decrease in intensity from the efferent ductule epithelium to the vas deferens, with the caput, corpus, and cauda regions showing equivalent staining, but the vas exhibiting very low expression of AR. Some species variation is seen in the ram, in which there also appears to be regional differences in expression (Carreau et al, 1984; Tekpetey et al, 1989). In the present study, AR was expressed equally in principal and basal cells in mice, in contrast to that of rats (Zhu et al, 2000).

Although AR appears to be dominant in the epididymal epithelium, the αERKO male demonstrated epididymal abnormalities in the absence of a functional ERα (Hess et al, 2000). It is interesting that the same cell types that were abnormal in αERKO clearly show ERα-positive immunoreactivity in the present study, in particular in narrow cells of the initial segment, apical cells of the caput, and in clear cells. Principal cells of the caput were also ERα-positive in mice, but they did not show gross abnormalities in αERKO mice. These data are consistent with an earlier autoradiography study in mice (Schleicher et al, 1984), which showed much higher binding of ^{3}H-estradiol in the initial segment and caput epididymis than in the corpus through vas deferens, with greater binding in apical/narrow cells and in clear cells. What is not understood is that ERβ is abundant throughout the epididymal epithelium of mice, and yet the autoradiographic data show little evidence of equivalent binding of estradiol throughout the epididymal epithelium. Because ERα is absent in principal cells of the corpus, cauda, and vas, ^{3}H-estradiol binding shown in these cells (Schleicher et al, 1984) must represent the presence of ERβ. In adult rats, inconsistent results for epididymal ERα have been reported, apparently due to the different antibodies applied (Fisher et al, 1997; Hess et al, 1997b; Sar and Welsch, 2000; Atanassova et al, 2001). These data are consistent with messenger RNA hybridization studies in situ (Mowa and Iwanaga, 2001). Sar and Welsch (2000) reported all stroma to be positive but only some epithelial cells in the rat were positive. In contrast, the entire epididymis, both epithelium and stroma, were found to be negative in several species, including rats (Fisher et al, 1997; Atanassova et al, 2001), dogs (Nie et al, 2002), goats (Goyal et al, 1998) and marmoset monkeys (Fisher et al, 1997). Other studies have shown variable amounts of staining by the epididymal epithelium with the same antibodies (Saunders et al, 2001; Nie et al, 2002), which suggests there are major species difference for ERα expression in the adult epididymis.

ERβ was expressed throughout the male mouse reproductive tract epithelium, similar to what has been reported in other species (Sar and Welsch, 2000; Atanassova et al, 2001; Saunders et al, 2001). There did not appear to be cell-specific expression of ERβ; however, the epithelium showed more intense staining than did the stroma in all regions, except for the cauda and vas deferens.

Vas Deferens

Although there is considerable evidence for androgen action in the vas deferens (Schindelmeiser et al, 1988; Dassouli et al, 1995; Darne et al, 1997), few reports have described AR presence. The vas deferens epithelium in humans shows only weak staining for AR (Ungefroren et al, 1997), which is similar to our findings in mice and corresponds to data showing a decrease in AR from neonatal to adult ages in rats (Gallon et al, 1989). However,

Figure 3. Colocalization of ERα and ERβ in mouse efferent ductules **(a–c)** and initial segment of epididymis **(d–f)**. Immunostaining of ERα (green) and ERβ (red) are both positive in the nucleus of efferent ductules, but only ERβ in the connective tissue cells. The combined photo overlays **(c** and **f)** show colocalization of the 2 receptors in the same cells (yellow). A transition area from efferent ductules to the head of epididymis **(d–f)** shows one efferent ductule that is stained strongly positive for ERα and also some epithelial cells in initial segment. Most epithelial cells are positive for ERβ. Unlabeled arrows in the inset photo enlargements point to narrow or apical cells in the initial segment of epididymis **(d–f)**. Bar = 25 μm **(a–c)**; bar = 50 μm **(d–f)**.

goat, rat (Goyal et al, 1997b), and human epithelium (Sar et al, 1990) expressed AR with intense immunostaining. In the mouse, there was abundant expression of AR in the stroma, which is consistent with autoradiographic studies (Weaker and Sheridan, 1983).

As observed in most species, (Goyal et al, 1997b; Hess et al, 1997b; Jefferson et al, 2000; Nie et al, 2002), the vas deferens epithelium was ERα-negative. Only the cat vas deferens shows ERα-positive staining (Nie et al, 2002). ERβ is abundant in both the epithelium and stroma of the vas deferens, similar to all species examined (Jefferson et al, 2000; Nie et al, 2002).

Receptors in Stromal Tissue

The interaction of epithelia and stroma has been studied in many tissues (Cunha et al, 1985; Cooke et al, 1986), and there is clear evidence that stroma plays an influential role in determining the fate of epithelial differentiation and function through paracrine regulation (Cooke et al, 1998, 1997; Prins et al, 2001). Our data demonstrate a predominant presence of AR in stroma of the entire excurrent ductal system, and that ERα and ERβ exhibit more variation in expression. For example, there is little expression of ERα in stroma of efferent ductules, but ERβ is expressed as strongly as that of AR. There is a tendency

Figure 4. Western blot analysis of AR, ERα, and ERβ in mouse tissues. **(a)** AR. Mouse testis (1), mouse epididymis (2). **(b)** ERα. Mouse uterus (1), mouse epididymis (2). **(c)** ERβ. Human recombinant protein (1), mouse epididymis (2). Arrow indicates molecular weight of dominant band in tissues for respective antibodies.

for decreased expression of both ERα and ERβ in stroma, going from the head of the epididymis through the vas. Even though ERβ appears to be present in more cell types, ERα maintains much stronger intensity of staining in a cell-specific manner than does ERβ. Therefore, these data suggest that further study is needed not only to determine the interactions between receptors and their specific hormone ligands, but also to determine the interactions between epithelial and stromal cells in the epididymal region.

Conclusions

This study demonstrates that the male mouse reproductive tract is substantially different from that reported in rats for the expression of ARs and ERs. This finding further supports the need to be cautious when extrapolating nuclear steroid receptor data across species in the study of male reproductive tract biology. All epithelial and most stromal cells contained AR, except for the germ cells and some vas deferens cells. ERα was abundant in efferent ductules, similar to all other species, but its presence in specific cell types along the epididymis was novel, because it differs from that seen in rats. ERβ distribution was similar to that of AR, except that ERβ alone was prominent in germ cells and vas deferens epithelium. Many cells expressed all three steroid receptors, including Leydig cells, peritubular myoid cells surrounding the seminiferous tubules, all epithelial cells from the efferent ductules, apical and narrow cells of the initial segment, and clear cells of the epididymis. The coexistence of multiple receptors in the same cells raises important questions regarding steroid hormone interactions and receptor cross-talk in the control of male reproductive tract function.

Acknowledgment

We appreciate the excellent laboratory assistance of Kay Carnes.

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