



Evidence For A Potential Role Of Estrogen In The Penis: Detection Of Estrogen Receptor-A And -B Messenger Ribonucleic Acid And Protein

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Abstract

Body tissues are traditionally classified as estrogen targets based on both the response to the hormone and the presence of estrogen receptors (ERs). We undertook the study on expression of ER α and ER β in the penis to identify compartments/cells responsive to estrogen, using immunohistochemistry, Western blotting, in situ hybridization, and RT-PCR analyses. Expressions of ER α and ER β in the rat penis were age dependent at both mRNA and protein levels, with the most intense signals being observed during the perinatal period and declining thereafter with age. Initial signals (fetal d 17) of ER α were localized to the mesenchyme and subepithelial stroma and later (postnatal d 2) to the corpus spongiosus, corpus cavernosus, and urethral epithelia. ER β was initially detected by postnatal d 2 and was localized diffusely in corpus spongiosus and cavernosus in immature rats. In the adult, both ERs were concentrated largely to the urethral epithelia and vascular and neuronal structures. The present study provides the first evidence for ER expression in the penis. Thus, our data add the penis to the list of estrogen-responsive tissues in males and provide a base and insight for future studies aimed at investigating a functional role of estrogen in the penis, especially in development.

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ALTHOUGH THE PRESENCE of estrogen in the male gonad has been well documented for more than 50 yr (1), its role in regulating male reproductive events only recently has gained appreciation (2, 3). Estrogen is largely produced in the testis and adrenal gland in quantities overall far less than in the female (1). It exerts its influence on male reproductive cells by acting principally through two estrogen receptor (ER) subtypes, ER α and ER β , members of the steroid/thyroid hormone receptor superfamily (4, 5).

Both ER α and ER β are localized in the testis and excurrent ducts of various species (6–8). The most intense signal of ER α is in the epithelia of the efferent duct and initial segment of the epididymis, sites responsible for sperm concentrations (7). The concentrations of estrogen in the luminal fluid of these ER-rich tissues are reportedly higher than those of plasma estradiol in the female (9), implying that estrogen may have profound effects on the functions of such tissues. In male rats, the most intense expression of ER β is in the epithelia of the accessory glands, most notably the prostate gland, with medium signals being in the testis and vas deferens (6, 8).

To date, there is no evidence directly linking estrogen to normal penile development and function. However, a number of studies report that male offspring of wildlife and laboratory animals as well as that of humans exposed to

estrogen-like endocrine disruptors [e.g. diethylstibesterol (DES)] during development exhibit abnormal reproductive organs including stunted penises (10, 11). Also, mothers with significant exposure to phytoestrogens are more likely to give birth to boys with hypospadias (12). Furthermore, when the estrogen antagonist, tamoxifen, is given to neonatal rats, it permanently disrupts differentiation of the os penis and completely erases epidermal projections and keratinization in the glans penis (13, 14). Surprisingly, neonatal castration does not suppress formation of os penis, although the size is less than controls (13). Taken together, these observations strongly suggest a potential involvement of estrogen in penile development and function. Because most estrogenic effects are classically mediated by ERs, demonstration of ERs in the penile tissue would directly implicate importance of estrogen in penile physiology. Therefore, the present study was undertaken to explore mRNA and protein expressions of ERs in the rat penis.

This study represents the first report that ER α and ER β are expressed in the rat penis at both mRNA and protein levels in an age-dependent manner, and these results provide evidence that estrogen may have important functions in penile tissues.

Materials and Methods

Animals and treatments

Healthy perinatal and adult male as well as adult female Wistar rats were employed. Male rats were sampled at prenatal d 17, 18, and 20 and postnatal d 1, 2, 3, 5, 7, 9, 15, 24, 32, 60, and 70 ($n = 10$ animals per age group). For RT-PCR and Western blot experiments, male rats were used

Abbreviations: AR, Androgen receptor; DES, diethylstibesterol; ER, estrogen receptor; NGF, nerve growth factor; NO, nitric oxide; PVDF, polyvinylidene difluoride filter; SSC, standard saline citrate; TBS, Tris-buffered saline; TTBS, TBS-Tween buffer.

at 1, 8, and 35 wk of age ($n = 10$ for each). In some experiments, 8-wk-old male rats had been treated with sc injection of either 2 μ g DES (Sigma, St. Louis, MO), 1 mg of the ER antagonist ICI 182,780 (AstraZeneca, Osaka, Japan) or DES plus ICI twice per week for 3 wk ($n = 6$ for each). Age-matched control male rats had received an equivalent volume (0.1 ml) of sesame oil, which was used as vehicle for DES and ICI alone. On the day that the gonadal tissues were harvested, the animals were killed by exsanguination under anesthesia with gaseous diethyl ether. All procedures were in accordance with the regulations laid down by the Hokkaido University School of Medicine Animal Care and Use Committee.

In situ hybridization

Three nonoverlapping ^{35}S -labeled antisense oligonucleotide probes (45 mer in length) for each of ER α and ER β mRNAs were used in preliminary studies to select the most sensitive probe for later use. All three probes produced almost the same signal, but the probe described later produced the most robust and consistent signal. The selected probes were complementary to nucleotide residues 301 to 346 of ER α cDNA (accession number Y00102) (4) and 45 to 90 of ER β cDNA (accession no. U57439; Ref. 5). The oligonucleotides were labeled with ^{35}S -dATP, using terminal deoxyribonucleotidyl transferase (Promega Corp., Madison, WI) at a specific activity of 0.5 $\times 10^9$ dpm/ μg DNA.

Tissues (penis or ovary) were rapidly removed and frozen in liquid nitrogen. Cryostat sections, 15–20 μm in thickness, were prepared and mounted on glass slides precoated with 3-amino-propyltriethoxysilane. The *in situ* hybridization protocol used has been described in detail previously (15). Briefly, tissue sections were fixed in 4% paraformaldehyde for 10 min and acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine-HCl (pH 8.0) for 10 min. Slide-mounted sections were prehybridized for 2 h in a buffer containing 50% formamide, 0.1 M Tris-HCl (pH 7.5), 4X standard saline citrate (SSC; 1X SSC = 150 mM NaCl and 15 mM sodium citrate), 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA, 0.6 M NaCl, 0.25% SDS, 200 $\mu\text{g}/\text{ml}$ tRNA, 1 mM EDTA, and 10% dextran sodium sulfate. Hybridization was performed at 42 C for 10 h in the prehybridization buffer supplemented with 10,000 cpm/ μl ^{35}S -labeled oligonucleotide probes. The slides were washed at room temperature for 20 min in 2X SSC containing 0.1% sarkosyl and twice at 55 C for 40 min in 0.1X SSC containing 0.1% sarkosyl. The sections were either exposed to Hyperfilm- β /max (Amersham Pharmacia Biotech, Buckinghamshire, UK) for 4 wk or dipped in NTB2 nuclear track emulsion (Kodak, Rochester, NY) and exposed for 4–8 wk. The specificity of *in situ* hybridization was confirmed by the disappearance of signals when excess doses of the corresponding nonlabeled (^{35}S -dATP) antisense oligonucleotides (cold) were added to the labeled antisense oligonucleotides (hot) hybridization fluid. Consistent ER mRNA signals above background levels were considered positive and were scored, subjectively, as strong, moderate, or weak.

RNA extraction and RT-PCR analysis

Total RNA was extracted from penises and ovaries by the guanidinium thiocyanate-phenol-chloroform method used routinely in our laboratory (16). RNA purity was determined by the ratio of OD measured at 260 and 280 nm (OD₂₆₀/OD₂₈₀), and RNA quantity was estimated at OD₂₆₀.

RT-PCR was performed by using primers derived from the rat ER α and ER β sequences. The oligonucleotide sequence pairs used for gene amplification in this study generated PCR products of expected sizes that have been sequenced to verify their identities: ER α sense primer, 5'-AATTCTGACAATCGACGC-3' and antisense primer, 5'-CTTCAA-CATTCTCCCTCTC-3' (334 bp; Ref. 17); and ER β sense primer, 5'-CTCGGGTCTGAGTCAGCTCAAC-3' and antisense primer, 5'-CCGAGGCCCTCAAGCTATAAAG-3' (285 bp; Ref. 18). cDNA was reverse transcribed from 1 μg total RNA according to the manufacturer's instructions. The cDNA was subjected to 32 cycles of PCR amplification consisting of 60 sec at 94 C, 90 sec at 55 C, and 120 sec at 72 C. After amplification, the resulting PCR products were visualized on 3% agarose gels stained with ethidium bromide. A 100-bp marker (Bio-Rad Laboratories, Inc., Hercules, CA) was used as the standard molecular weight. The gels were dried, and the products were quantified using an Image Analyzer (Bio-Rad Laboratories, Inc.). To standardize the

amount of the target molecule, the amount of β -actin mRNA, a ubiquitously expressed housekeeping gene, was determined using the primer pair (sense, 5'-GTGGGGCGCCCAGGCACCA-3', and antisense, 5'-GTCCTTAATGTCACGCACGATTTC-3'; Ref. 19).

Characterization of antisera

The studies of ER α immunoreactivity were carried out preliminarily with three antisera against ER α from different sources. In our hands, the antiserum coded NCL-ER-6F11, which is a mouse monoclonal antibody against prokaryotic recombinant protein corresponding to the full-length α form of the ER molecule (Novocastra Laboratories, Newcastle upon Tyne, UK), produced the most consistent robust signal with immunofluorescence; thus, this antiserum for ER α was used for these studies. Other antisera against ER α used in preliminary studies include an affinity-purified rabbit polyclonal antibody raised against a peptide at the C terminus of the mouse ER α protein code MC-20 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and a rabbit antiserum raised against the last 15 amino acids of the rat ER α protein code C1355 (Upstate Biotechnology, Inc., Lake Placid, NY). For identification of ER β , we used the antiserum coded PA1-311, which was raised in rabbit against the amino acid residues 55–70 in the rat ER β protein (Affinity BioReagents, Inc., Golden, CO). We confirmed that the PA1-311 showed more specific immunolabeling in comparison with other ER β antisera tested here, a mouse antihuman ER β monoclonal antibody code MAB463 (Chemicon International, Temecula, CA), and a goat antimouse ER β polyclonal antibody code Y-19 (Santa Cruz Biotechnology, Inc.).

Controls included omission of the primary antiserum, omission of the secondary antibody, adsorption of the primary antiserum with its respective antigen (Panvera, Madison, WI; the ratio of antigen to antibody = 10:1), and cross-adsorption controls. For the latter, the ER α antiserum was adsorbed with the ER β peptide and then applied to tissue sections (the ER α immunostaining appeared normal). Likewise, adsorbing ER β antiserum with the ER α protein did not diminish immunostaining for ER β .

Immunofluorescence staining

For immunohistochemical determination of ERs, tissue specimens were fixed in 4% buffered formalin solution, dehydrated, and then embedded in paraffin. The preparations were cut in 4- μm sections transversely, deparaffinized, and treated for 20 min with citrate buffer (10 mM citric acid, pH 6.0) in a microwave oven (750 W) before immunostaining. To prevent nonspecific staining by the secondary antibody, the sections were blocked by nonimmune serum (1% bovine albumin in Tris) for 30 min at room temperature. After overnight incubation at 4 C with primary antibodies, the sections were rinsed in phosphate buffer solution and then exposed to the fluorescence secondary antibody, Cy3-conjugated AffiniPure antimouse IgG or fluorescein-conjugated AffiniPure goat antirabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 2 h according to the manufacturer's instructions. The samples processed without primary antibodies served as negative controls. For double-label immunofluorescence staining for ER α and ER β , the sections were incubated with ER α mouse monoclonal antibody, followed by Cy3-conjugated antimouse IgG, and then incubated with ER β rabbit polyclonal antibody, followed by fluorescein-conjugated antirabbit IgG. The coverslips were mounted with Immunon (Thermo Shandon, Pittsburgh, PA). Immunofluorescence images were observed under a laser scanning confocal imaging system (MRC-1024, Bio-Rad Laboratories, Inc.).

Western blot analysis

After penises and ovaries had been removed and rinsed in sterilized water on ice, the tissues were minced with scissors, homogenized, and then centrifuged at 1000 g for 15 min to pellet any insoluble material. The protein concentration of supernatant was determined by the method of Lowry *et al.* (20) with BSA as standard. Samples (10 μg) were run on SDS-PAGE, using 8% polyacrylamide gel, and electrotransferred to polyvinylidene difluoride filter (PVDF) membrane. To reduce nonspecific binding, the PVDF was blocked for 60 min at room temperature in Tris-buffered saline (TBS; 20 mM Tris-HCl, 500 mM NaCl, pH 7.5) containing 1% albumin. Thereafter, the PVDF was washed for 5 min three

times in TBS-Tween buffer (TTBS; 20 mm Tris-HCl, 500 mm NaCl, 0.05% Tween 20, pH 7.5) and incubated overnight at 4°C with specific antibodies (1:100 dilution for ER α ; 1:2000 dilution for ER β) in TTBS containing 1% albumin. After extensive washing with TTBS, the PVDF was incubated with horseradish peroxidase-conjugated antimouse or anti-rabbit antibody (Bio-Rad Laboratories, Inc.) diluted at 1:6000 in TTBS containing 1% albumin at room temperature for 60 min. Then the PVDF was washed for 5 min twice in TTBS and washed for 5 min in TBS. The blots were visualized using the enhanced chemiluminescence detection system (Amersham), exposed to x-ray film for 5 min, and analyzed by NIH image software produced by Wayne Rasband. The results are expressed as percent of the band obtained with ovary in each experiment. To check for protein loading/transfer variations, all blots were stained with Ponceau Red (washable, before incubation with antibodies) and with Coomassie brilliant blue (permanent, after the enhanced chemiluminescence detection system). Intensity of total protein bands per lane was evaluated by densitometry. Negligible loading/transfer variation was observed between samples.

Statistical analysis

The data are presented as means \pm sem. Statistical assessment of the data were performed by ANOVA with multiple comparisons by Fisher's protected least significance *t* test. *P* values less than 0.05 were considered significant.

Results

In situ hybridization studies showed moderate and diffuse signals for ER α mRNA in undifferentiated and homogenous mesenchymal cells of the primordial penis at fetal d 17, the earliest age examined (Fig. 1A). The epithelium lacked ER α mRNA signals (data not shown). During the perinatal period, the stroma surrounding the urethral epithelium and basal epithelium expressed pronounced signals of ER α mRNA (Fig. 1, B-G).

The corpus spongiosus and penile urethral epithelium expressed intense signals of ER α mRNA during postnatal d 2-7 (Fig. 1, B-G); the signal intensity decreased in a proximal-distal pattern from postnatal d 7 onward (Fig. 1H). During this period (neonate), the epithelium of the proximal penile urethra, classified as transitional epithelium, had more pronounced signals of ER α mRNA in the basal region than the distally located stratified epithelium (Fig. 1, B-E). However, in adults ER α mRNA signals in the basal region were primarily limited to the regions within and close to glans penis (Fig. 1H). Moderate signals of ER α mRNA were observed in the lamellated sensory corpuscles by postnatal d 14 (Fig. 1I) and slightly decreased in intensity in the adult (data not shown). These neuronal structures, which are located in the glans penis dermal papillae, were identified by their positive immunoreactivity with S100 protein antiserum (Fig. 1J) and had basal immunoreactivity to active caspase 3, a proapoptotic factor (Fig. 1K) and Bcl-2, an antiapoptotic factor (data not shown).

ER β mRNA signals were diversely but modestly observed in the primordial penile corpus spongiosus, corpus cavernosus, and stroma of the glans penis and cells immediate to the glans penis (Fig. 2). Positive signals were also observed in penile spongiosus, cavernosus, epithelium of urethral glands and stroma, blood vessels, and dorsal nerve in the root and body of the penis at different postnatal days (Fig. 2). ER β mRNA also declined in intensity with age, but such an age-dependent change was found at a later age in comparison with the case of ER α mRNA. Furthermore, unlike

ER α mRNA, ER β mRNA was more diffused and much weaker in intensity per unit area. The distribution pattern did not change much with advancement of age.

Immunofluorescence staining for ER α protein showed that its expression was mainly localized to the penis spongiosus, penis cavernosus, basal epithelial layer of urethra, and sensory corpuscle of glans penis and to a lesser extent in neurovascular bundle, dorsal nerve, and blood vessels (Fig. 3). On the other hand, ER β was expressed in penis cavernosus, urethral glands, blood vessels, and dorsal nerve of penile spongiosus (Fig. 4).

Double immunofluorescence studies (Fig. 5, D-L) showed that, in the penis of the 1-d-old rat, the predominant distribution of ER α was found in the penis spongiosus, whereas ER β distributed predominantly in the urethra and neurovascular bundle (including dorsal blood vessels and dorsal nerve). The penis cavernosus appeared to express ER α and ER β equally.

Primary antibodies alone (data not shown) and secondary antibodies alone (Figs. 3I, 4H, and 5G) showed no immunoreactivity with the penile spongiosus or cavernosus, indicating the specificity of the antibodies. Also, ER α and ER β antibodies preadsorbed with the synthetic peptide did not reveal any immunostaining (Figs. 3H and 4G). Furthermore, using the antisera against ER α and ER β employed in this study, the specificity in the immunohistochemical reaction was ascertained in the adult female rat ovary and oviduct (Fig. 5, A-C). ER α was found to be localized in thecal and interstitial gland cells but not in granulosa cells of follicles. In the oviduct, nuclear ER α staining was observed in luminal epithelium and muscle cells. On the other hand, ER β was predominantly detected in granulosa cells of follicles and in epithelium of the oviduct. The immunodetection of ER α and ER β in the rat ovary and oviduct presented here is in general agreement with the findings demonstrated previously by other investigators (21, 22).

Gene expressions of the two receptor subtypes, ER α and ER β , in the penis in different age groups were analyzed by RT-PCR. The classical target organ of estrogen, ovary, which richly expresses both ER subtypes, was used as a positive control tissue. β -Actin mRNA was used as an internal standard and for adjustment of sample-to-sample variations. ER α mRNA was detected in the 1-wk-old rat penis but was barely detectable at 8 and 35 wk of age (Fig. 6A1), indicating that adult and aged rat penises poorly express ER α mRNA. In contrast, ER β mRNA was detected in all ages examined, although its expression showed a trend to decrease with age (Fig. 6B1).

At 8 wk of age, the transcript band for ER α was essentially very faint and became barely detectable when DES was pretreated (Fig. 6B2). The signal appeared weakened when the animals received ICI together with DES. The transcript band for ER β also evidently faded with DES pretreatment (Fig. 6B2). When ICI was used together with DES, it resulted in nearly complete prevention of the DES-induced reduction in ER β transcripts. ICI alone had no effect on penile expressions of ER α and ER β mRNAs.

Immunoblot analysis using NCL-ER-6F11 antiserum showed a single band with a molecular mass of approximately 65 kDa, which was referred to as ER α , in the rat ovary

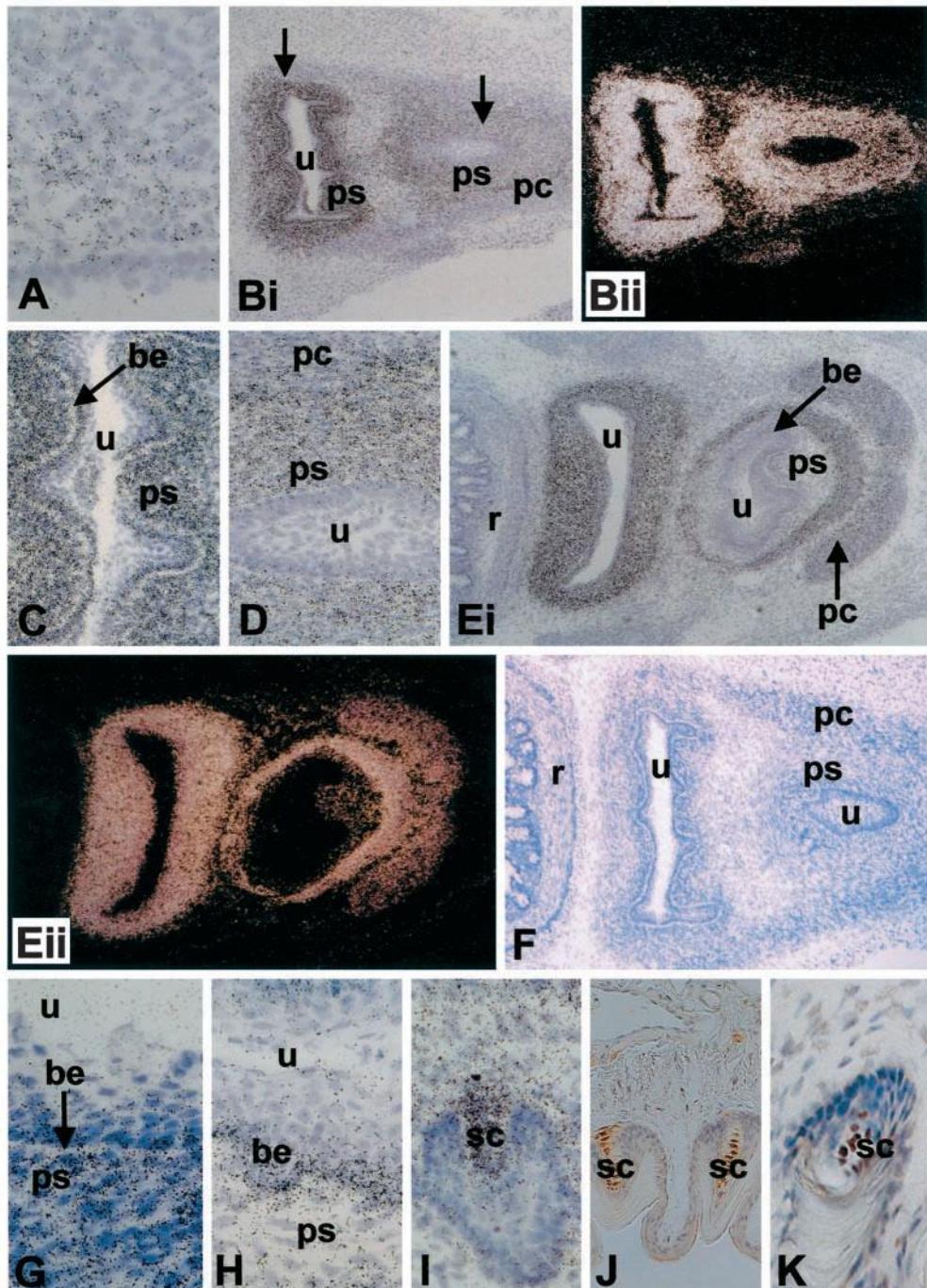


FIG. 1. *In situ* hybridization analysis showing gene expressions of ER α in the rat penis. A, Distinct ER α mRNA signals were seen in the mesenchyme of the developing penis at fetal d 17. B–E, The transverse sections of the primordial penis exhibited more pronounced signals of ER α mRNA in the proximal portion (left arrow in panel Bi) than the distal region (right arrow in panel Bi) at postnatal d 3. Urethral epithelium, especially the basal epithelium (be), and penile spongiosus (ps) of the proximal penis were more strongly labeled than those of the distal penis (D) (pc, penile cavernosus; u, urethral lumen). C and D are higher magnifications (X400) of B, whereas Bi and Bii are bright-field and dark-field images, respectively. Ei and Eii are also bright- and dark-field images of the transverse section of the penis at postnatal d 7 taken at a more rostral or cranial position. F, No grain (ER α mRNA signal) was observed in the transverse section of the penis at postnatal d 3 when excessive cold probe was added to a little amount of the corresponding hot probe during the hybridization process. G–H, The stratified epithelia of the middistal penile urethra at postnatal d 2 (G) and 14 (H) showed significant signals of ER α mRNA in the basal epithelial layer (be). Strong ER α mRNA signals in the spongiosus (ps) were seen at postnatal d 2 (G), which declined at postnatal d 14 (H). I, Distinct and intense signals of ER α mRNA were localized to the lamellated sensory corpuscles (sc) of the glans penis at postnatal d 24. J, Immunohistochemical detection for S100 protein. Positive staining (brown) was found in the lamellated sensory corpuscles (sc). K, Immunohistochemical detection for active caspase 3, a member of caspase superfamily, which initiates apoptotic events. Positive staining (brown) was found in the lamellated sensory corpuscles (sc). Magnification, X200 (X100 for B, E, and F).

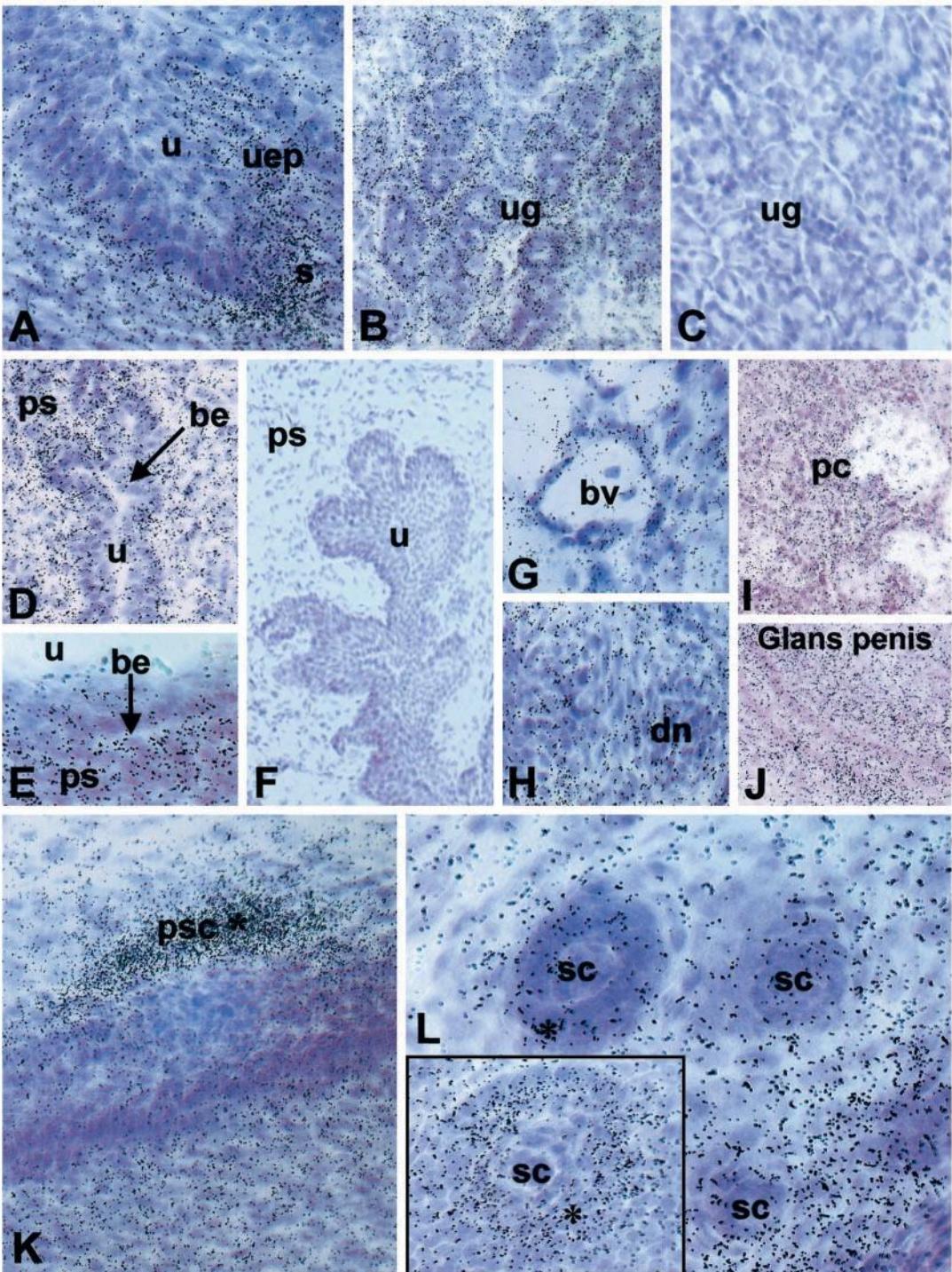


FIG. 2. *In situ* hybridization analysis showing gene expression of *ER β* in the rat penis. A, Moderate *ER β* mRNA signals are scattered in the stroma (s) of the developing glans penis at postnatal d 2. u, Urethra; uep, urethral epithelium. The signals were modestly seen in the epithelium of urethral glands (ug) of the root penis at postnatal d 24 (B) in the urethral epithelium, centering around the basal epithelium (be), and penile spongiosus (ps) at postnatal d 24 (D, E), in the wall of blood vessel (bv), possibly the venule, at postnatal d 24 (G), the dorsal nerve (dn) at postnatal d 24 (H), the penile cavernosum (pc) at postnatal d 14 (I), and the edge of the glans penis at postnatal d 2 (J). K, Highly discrete signals on the edge of the glans penis, which appears possible sites for the sprouting primordial sensory corpuscles (psc*) at postnatal d 5. L, The signals were localized in the surrounding of the sensory corpuscles (sc) at postnatal d 2. Note that moderate *ER β* mRNA expression (*) was clearly found in cells surrounding the sensory corpuscles (sc) at postnatal d 14 (inset). Note that no grain (*ER β*) mRNA signal was observed in the epithelium of urethral glands (ug) of the root penis (C) or in penile spongiosus (ps) and urethra (u) (F) at postnatal d 24 when excessive cold probe was added to a little amount of the corresponding hot probe during the hybridization process. Magnification, X400 (X200 for D, I, and J; X100 for F).

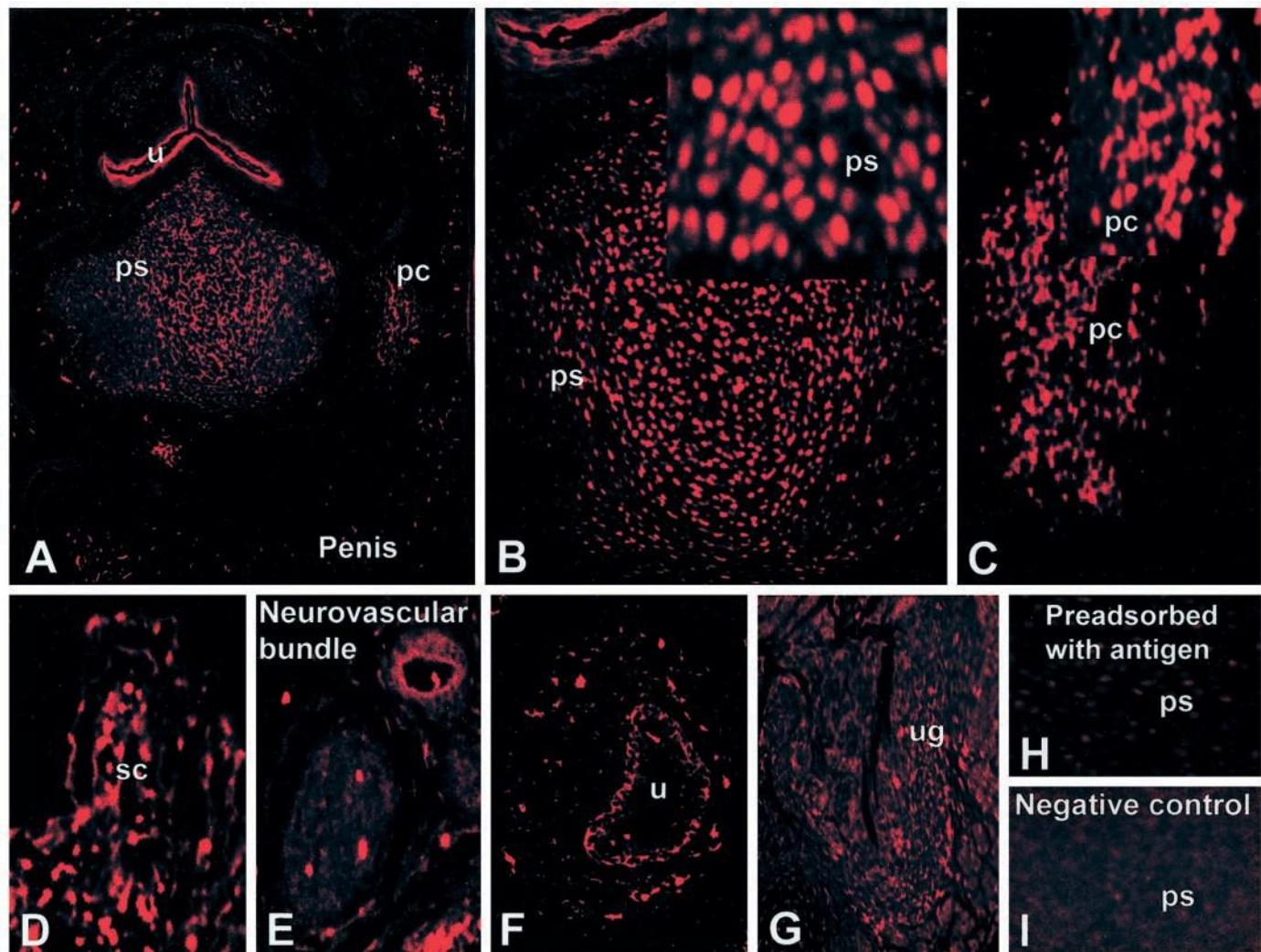


FIG. 3. Immunofluorescent findings for ER α in the rat penis. A, Penile ER α distribution in postnatal d 1 rat (low magnification, X100). Positive staining (red) was found in the penile spongiosus (ps), urethra (u), and penis carvenosus (pc). B and C, Immunoreactivity was found in the penile spongiosus (ps) and penis carvenosus (pc) (high magnifications X200, [inset, X400]). D, Positive staining was also found in the sensory corpuscle (sc) of glans penis (magnification X400). E, Immunoreactivity was less in the neurovascular bundle of body penis (magnification, X200). F and G, Positive staining was found in the urethra (u) and urethral gland (ug) (magnification, X100). H, No staining was observed in the penile spongiosus (ps) when peptide-adsorbed antibody was used (magnification, X200). I, Primary antibody (data not shown) or secondary antibody (data presented) showed no immunoreactivity with the penile spongiosus (ps), indicating the specificity of the antibodies (magnification, X400).

and penis (Fig. 7A1). Immunodetectable ER α was found at lower levels in the penis with increasing age. Densitometric quantification of the signal revealed that the penile ER α protein levels at 1, 8, and 35 wk of age were $46 \pm 3\%$, $16 \pm 2\%$ ($P < 0.001$ vs. 1 wk), and $14 \pm 4\%$ ($P < 0.001$ vs. 1 wk) of the level obtained in the adult rat ovary, respectively ($n = 5$). PA1-311 antiserum detected a approximately 55-kDa protein band referred to as ER β in the rat ovary and penis (Fig. 7B1). The band obtained from the pubertal rat penis was evidently darker than those from the young and aged adult rat penises. Compared with the adult rat ovary (taken as 100%), the penile expression level of ER β protein was $105 \pm 4\%$ at 1 wk, $71 \pm 5\%$ ($P < 0.001$ vs. 1 wk) at 8 wk, and $59 \pm 5\%$ ($P < 0.001$ vs. 1 wk) at 35 wk of age ($n = 5$).

Pretreatment with DES caused a marked decrease in ER α and ER β protein expressions to $28 \pm 3\%$ ($n = 5$, $P < 0.001$) and $32 \pm 3\%$ ($n = 5$, $P < 0.001$), respectively, of vehicle

pretreatment in the 8-wk-old rat penis (Fig. 7, A2 and B2). The decreased expression level of each ER subtype in the penis was completely recovered when ICI was given together with DES ($95 \pm 2\%$ for ER α , $98 \pm 3\%$ for ER β , $n = 5$, $P < 0.001$). ICI alone did not affect penile expressions of ER α ($100 \pm 2\%$, $n = 5$) and ER β ($100 \pm 2\%$, $n = 5$).

Discussion

This study using immunoblotting, RT-PCR, immunohistochemistry, and *in situ* hybridization histochemical techniques has detailed the presence and distribution pattern of ER α and ER β mRNA transcripts and proteins in the male rat penis. Expressions of ER α and ER β in the penis were age dependent at both mRNA and protein levels. Thus, the penile expression levels were declined with advancement of age, although this trend was more marked for ER α than ER β . In

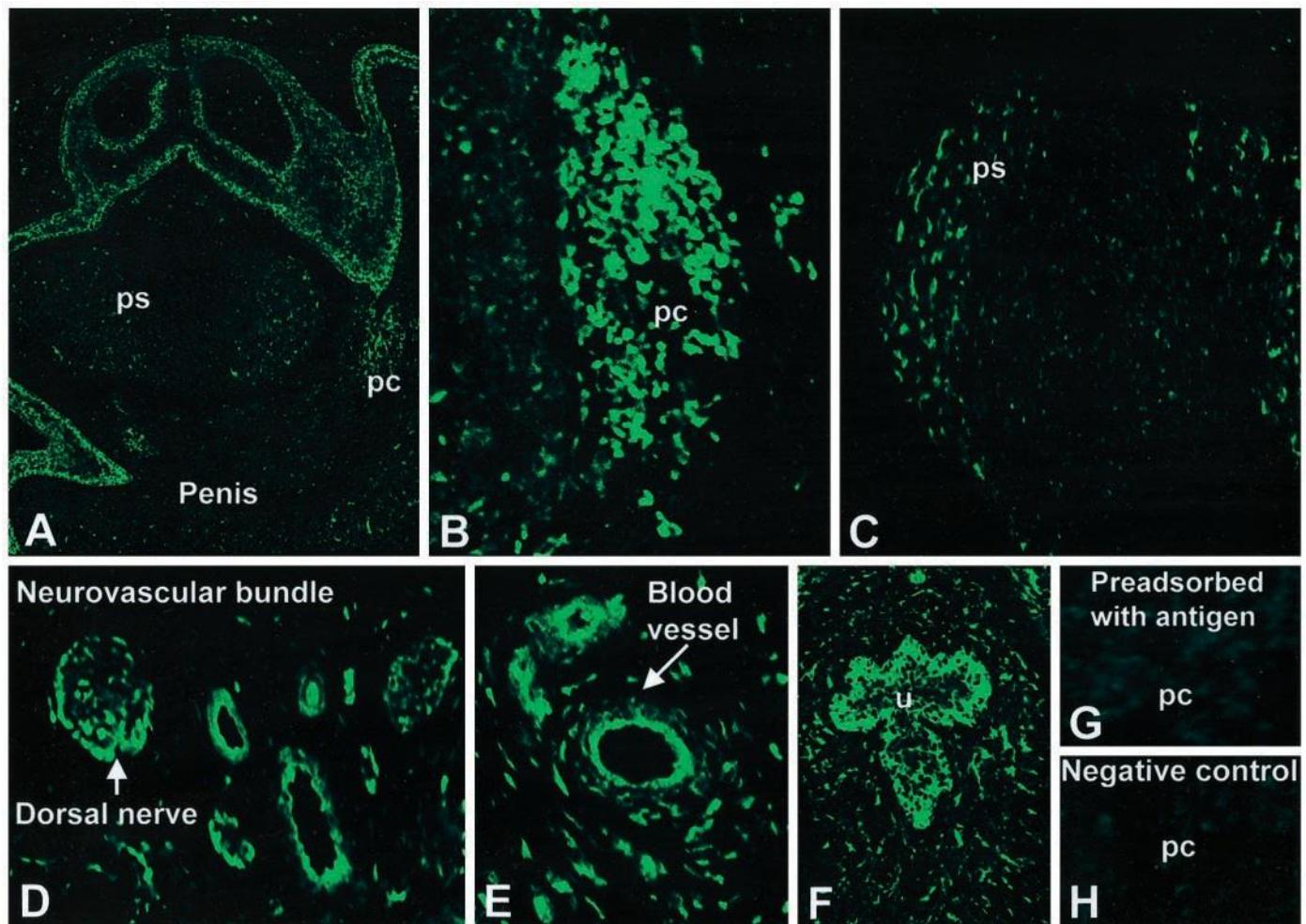


FIG. 4. Immunofluorescent findings for ER β in the rat penis. A, Penile ER β distribution in postnatal d 1 rat (low magnification, X100). B and C, Immunoreactivity found in carvenosus (pc) of body penis much more strongly than in the penis spongiosus (ps) (high magnification, X400). D, Positive staining was seen in the neurovascular bundle of body penis (magnification, X200). E, Positive staining found in the penile artery of root penis (magnification, X400). F, Positive staining found in urethra (u) (magnification, X100). G, No staining was observed in the penile cavernosus (pc) when peptide-adsorbed antibody was used (magnification, X400). H, Primary antibody (data not shown) or secondary antibody (data presented) showed no immunoreactivity with the penile cavernosus (pc), indicating the specificity of the antibodies (magnification, X400).

situ hybridization signals in most penile compartments of the adult rat were less pronounced. This may probably be related to the poor penetration of probes because of the significant presence of fibrous tissues developed in the adult penis. On Western blots, we detected each of ER α and ER β protein in the rat penis as a single band. ER α and ER β proteins migrated as bands around 65 kDa and 55 kDa, respectively. These molecular weights are within the range of reported values, 60–67 kDa for ER α and 55–63 kDa for ER β , noted in other tissues (23–26). To date, various isoforms of ER β have been reported, namely ER β 2, ER β cx, and ER β variants, which are altered by a deletion within the DNA binding domain (ER β 103 and ER β 203) (25, 27, 28). Because for ER β detection we used PA1-311, which was raised against the N-terminal part of rat ER β (29), we cannot entirely rule out the possibility that this antibody may have recognized C-terminally truncated variants such as ER β 2 and ER β cx.

Initial fetal ER signals were localized to the mesenchyme and subepithelia of the primordial penis, in coincidence with

our earlier reports on the ER expression pattern in other developing reproductive tissues of both sexes (8, 30). This expression pattern is also in good agreement with the proposed concept on mesenchyme and epithelia interaction (31). Urogenital mesenchyme under the influence of various factors, most notably estrogen and testosterone, is believed to promote epithelial morphogenesis, proliferation, and differentiation and evoke expression of tissue-specific secretory proteins. It is interesting to note that penile expression of androgen receptors is also age dependent and largely found in the same cells as ERs reported here (32). This would imply that estrogen and androgen may coregulate some penile events, especially in early growth and differentiation. The significance of testosterone in late phallic growth has been revealed by Jost's experiments of late castration (33). Late castration leads to reduced phallic growth of normally masculinized genitals. The conclusion that estrogen may act together with androgen as a regulator of penile development and/or function is supported by the fact that no appreciable

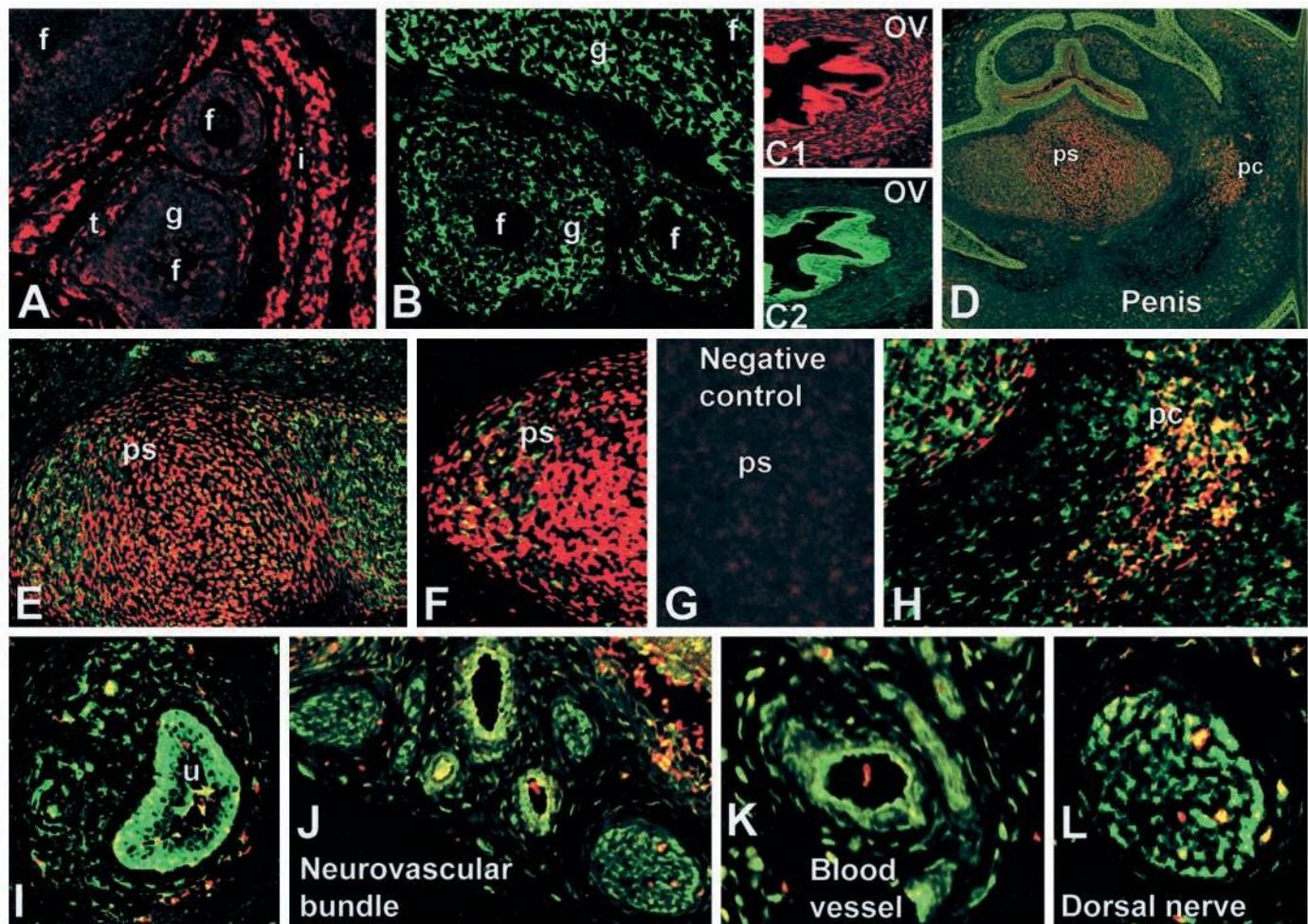


FIG. 5. Immunohistological localization of ER α (red) and ER β (green) in the rat ovary (A–C) and penis (D–L). A, In the adult female rat ovary, ER α was immunolocalized to thecal cells (t) and interstitial gland cells (i) but not to granulosa cells (g) of follicles (f). B, In the same ovary, ER β was predominantly detected in granulosa cells (g) of follicles (f). C, Intense ER α staining (C1) was detected in luminal epithelium and muscle cells of the oviduct (ov), whereas ER β (C2) was expressed mainly in epithelium of the oviduct (ov). D, Immunofluorescence double labeling for ER α and ER β in 1-d-old rat penis (low magnification, X100). E and F, ER α was expressed more abundantly than ER β in the penis spongiosus (ps) (high magnification, X200 and X400). G, Primary antibodies (data not shown) or secondary antibodies used for double labeling (data presented) showed no reactivity with the penile spongiosus (ps), indicating the specificity of the antibodies (magnification, X400). H, Both of ER α and ER β were present in the penis cavernosus (pc) (high magnification, X400). I, ER β was more dominant in the urethra than ER α (magnification, X100). J–L, ER β immunoreactivity was main in the neurovascular bundle, dorsal artery, and dorsal nerve (magnification, X200 for J, X400 for K and L).

differences between penile and clitoral sizes (organs commonly derived from the genital tubercle) from 11 to 14 wk is evident in humans, even though this is the period of maximum sex differences in the plasma testosterone concentration (33). Moreover, the major period of fetal penile growth occurs at a time that fetal plasma testosterone concentration is on the decline and maternal estrogen on the increase (34).

Furthermore, it is interesting to note that greater penile growth in spotted hyenas was observed during the period of low increases in androgen levels, compared with the period of maximum increase, and prepubertal castration had minimal effects on overall penile size, whereas ovariectomy led to a significant reduction in clitoral diameter (35). Androstanedione, a unique male hormone metabolized into either testosterone or estrogen by specific enzymes, is converted to estrogen in humans (and perhaps most mammals) but to

testosterone in spotted hyenas (36). Thus, the penilelike clitoris (pseudopenis) of the spotted hyena female is attributed to their unique pattern of placental steroid metabolism. Based on the data from those studies and the present study, it would be reasonable to suggest that the phallic size may, to some extent, be a function of the estrogen/androgen ratio. Further studies will be needed to identify androgen- and estrogen-regulated genes in the male reproductive system and to address the possible involvement of aromatization, a local conversion of androgen to estrogen, at the penis level, in the gene regulation.

In the present study, penile expressions of ER α and ER β in 8-wk-old rats after treatment with DES for 3 wk were severely diminished at both mRNA and protein levels. Such DES-induced changes were completely prevented by ICI. DES has a 3- to 4.5-fold higher affinity for ER α and ER β

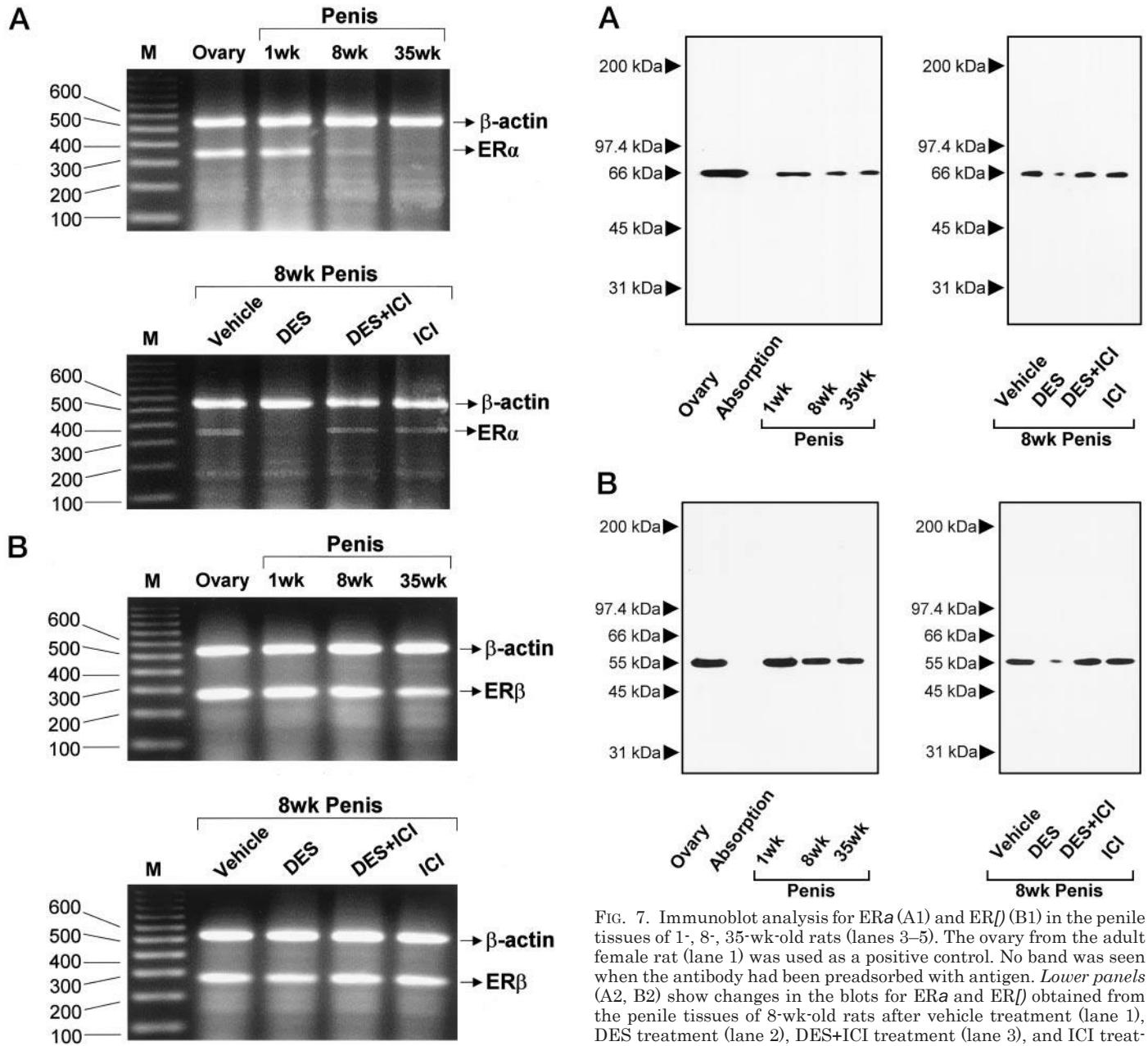


FIG. 6. RT-PCR analysis showing gene expressions of ER α (A1) and ER β (B1) in the penile tissues of 1-, 8-, and 35-wk-old rats (lanes 2–4). The ovary from the adult female rat (lane 1) was used as positive control. The standard molecular weight used was 100 bp marker. β -Actin mRNA was used as internal control. Lower panels (A2, B2) show changes in the gene expressions of ER α and ER β obtained from the penile tissues of 8-wk-old rats after vehicle treatment (lane 1), DES treatment (lane 2), DES+ICI treatment (lane 3), and ICI treatment (lane 4) for 3 wk.

proteins than 17 β -estradiol (17). Thus, this down-regulation of ERs would be the adaptive mechanism that provides the cells with the means of switching off the response in the face of continuous presence of the potent synthetic estrogen. Our findings are consistent with the previous report showing estrogen-induced down-regulation of ER mRNA content in the preoptic medial area of neonatal rats (37). Interestingly,

FIG. 7. Immunoblot analysis for ER α (A1) and ER β (B1) in the penile tissues of 1-, 8-, 35-wk-old rats (lanes 3–5). The ovary from the adult female rat (lane 1) was used as a positive control. No band was seen when the antibody had been preadsorbed with antigen. Lower panels (A2, B2) show changes in the blots for ER α and ER β obtained from the penile tissues of 8-wk-old rats after vehicle treatment (lane 1), DES treatment (lane 2), DES+ICI treatment (lane 3), and ICI treatment (lane 4) for 3 wk.

neonatal treatment of male rats with DES up-regulates androgen receptor (AR) mRNA in the forebrain (38) but suppresses AR immunoexpression in the reproductive tract (39). Furthermore, ER α knockout mice have lower expression of AR immunoreactivity in some areas of the brain (40). Experiments assessing the impact of DES treatment on penile expression of ARs remain to be performed. There may exist a close relationship/interaction between estrogen and androgen and their respective receptors, which may be crucial to the development and function of the penis.

We found the predominant distribution of ER β in the blood vessel lining and walls. The endothelium is known to be the key modulator of vascular integrity by performing a complex array of functions in close association with vascular

smooth muscle cells (41). These functions are integrated and mediated by a complicated system of chemical mediators including nitric oxide (NO) and prostacyclin (41). Estrogen increases endothelial NO synthase and stimulates NO release from endothelial cells, thereby causing vasodilation (42). ERs are expressed in endothelial and vascular smooth muscle cells and functional in vascular tissues (43). Our findings would suggest that estrogen through activation of ER β may play a role in the regulation of penile blood vessel endothelial cells, which repeatedly undergo mechanical and hemodynamic stress, ensuring vascular integrity and, subsequently, vascular tone and erectile function.

ER α was localized to the sensory corpuscle of glans penis, with ER β being in the neurovascular bundle of penile spongiosus and cells surrounding the sensory corpuscles. The glans penis has the highest concentration of sensory nerve fibers (44). It may be intriguing to note that the main efferent parasympathetic pathway supplies vasodilating innervation to the cavernosus bodies, whereas the main sympathetic pathway supplies mostly the vasoconstriction innervation, thus chiefly mediates detumescence (44).

Both ER α and ER β have been identified in neural circuits involving central neurons, autonomic and sensory ganglionic neurons, and spinal cord neurons in areas that have connections with the male and female reproductive systems (45–48). Estrogen reportedly has an array of effects on many different neuronal systems and these effects largely occur in neurons that have demonstrable ERs (49, 50). In ER α -transfected PC12 cells, neural crest derivatives that are nerve growth factor (NGF) dependent, estrogen has cytoprotective effects that involve NGF and the antiapoptotic molecule Bcl-X $_L$, a member of the Bcl-2 family (51). In addition, Sohrabji *et al.* (52) have indicated that estrogen acting through ER α modulates NGF-dependent neuronal plasticity and repair in sensory neurons of dorsal ganglion. We showed that caspase 3 and Bcl-2, which are associated with apoptosis and cell survival, respectively, were colocalized in the sensory corpuscles of glans penis. The sensory corpuscles were mainly ER α immunoreactive. We thus assume that estrogen, working through ER α , may promote cell survival of sensory neurons in the glans penis by regulating expressions of NGF receptors and Bcl-2. Recent works have shown that 17 β -estradiol, working through ER α and ER β in developing dorsal root ganglion neurons, increases the survival of neurons deprived of NGF and promotes the expression of Bcl-X (53), suggesting that both ERs are important in the survival and maintenance of the neurons, although ER α and ER β appear to differentially modulate neurites (54).

In summary, this study is the first to demonstrate the presence of ER α and ER β in the penis and opens up an entirely novel arena in which to explore the roles of estrogen in the male reproductive system. Of particular importance will be studies aimed at delineating the specific functional significance of estrogen *vs.* those of testosterone, a principal classic hormone of the penis.

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References

- Carreau S, Genissel C, Bilinska B, Levallet J 1999 Sources of oestrogen in the testis and reproductive tract of the male. *Int J Androl* 22:211–223
- Meistrich ML, Hughes TJ, Bruce WR 1975 Alteration of epididymal sperm transport and maturation in mice by oestrogen and testosterone. *Nature* 258: 145–147
- Hess RA 2000 Oestrogen in fluid transport and reabsorption in efferent ducts of the male reproductive tract. *Rev Reprod* 5:84–92
- Koike S, Sakai M, Muramatsu M 1987 Molecular cloning and characterization of rat estrogen receptor cDNA. *Nucleic Acids Res* 15:2499–2513
- Kuiper GGJM, Enmark E, Pelto-Huikko M, Nilsson, Gustafsson JA 1996 Cloning of a novel estrogen receptor expressed in the rat prostate and ovary. *Proc Natl Acad Sci USA* 93:5925–5930
- Hess RA, Gist DH, Bunick D, Lubahn DB, Farrell A, Bahr J, Cooke PS, Greene GL 1997 Estrogen receptor (α , β) expression in the excurrent ducts of the adult male rat reproductive tract. *J Androl* 18:602–611
- Hess RA, Bunick D, Lee KH, Bahr J, Taylor JA, Korach KS, Lubahn DB 1997 A role for estrogen in the male reproductive system. *Nature* 390:509–512
- Mowa CN, Iwanaga T 2001 Expression of estrogen receptor α and β mRNAs in the male reproductive system of the rat as revealed by *in situ* hybridization. *J Mol Endocrinol* 26:165–174
- Free MJ, Jaffe RA 1979 Collection of rete testis fluid from rats without previous efferent duct ligation. *Biol Reprod* 20:269–278
- Stillman RJ 1982 *In utero* exposure to diethylstilbestrol: adverse effects on the reproductive tract and reproductive performance of male and female offspring. *Am J Obstet Gynecol* 142:905–921
- Greene R, Burrill M, Ivy A 1939 Experimental intersexuality: modification of sexual development of the white rat with a synthetic estrogen. *Proc Soc Exp Biol Med* 41:169–170
- North K, Golding J 2000 A maternal vegetarian diet in pregnancy is associated with hypospadias. The ALSPAC Study Team. *Avon longitudinal study of pregnancy and childhood. Br J Urol Int* 85:107–113
- Iguchi T, Irisawa S, Uesugi Y, Kusunoki S, Takasugi N 1990 Abnormal development of the os penis in male mice treated neonatally with tamoxifen. *Acta Anat (Basel)* 139:201–208
- Deveci E, Onen A, Tarac O, Yildirim A 1997 The effect of tamoxifen on the neonatal development of the rat glans penis. *Clin Exp Obstet Gynecol* 24: 237–239
- Mowa CN, Iwanaga T 2000 Differential distribution of oestrogen receptor α and β mRNAs in the female reproductive organ of the rats as revealed by *in situ* hybridization. *J Endocrinol* 165:59–66
- Matsuda N, Hattori Y, Gando S, Akaishi Y, Kemmotsu O, Kanno M 1999 Diabetes-induced down-regulation of β -adrenoceptor mRNA expression in rat heart. *Biochem Pharmacol* 58:881–885
- Kuiper GGJM, Carlsson B, Grandien K, Enmark E, Hagglund J, Nilsson S, Gustafsson JA 1997 Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptor α and β . *Endocrinol* 138:863–870
- Kuiper GGJM, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson JA 1996 Cloning of a novel receptor expressed in rat prostate and ovary. *Proc Natl Acad Sci USA* 93:5925–5930
- Gilchrist M, MacDonald AJ, Neverova I, Ritchie B, Befus AD 1997 Optimization of the isolation and effective use of mRNA from rat heart mast cells. *J Immunol Methods* 201:207–214
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ 1951 Protein measurement with the folin phenol reagent. *J Biol Chem* 193:265–275
- Saunders PTK, Maguire SM, Gaughan J, Millar MR 1997 Expression of oestrogen receptor beta (ER β) in multiple rat tissues visualized by immunohistochemistry. *J Endocrinol* 154:R13–R16
- Sar M, Welsch F 1999 Differential expression of estrogen receptor- β and estrogen receptor- α in the rat ovary. *Endocrinology* 140:963–971
- Friend KE, Resnick EM, Ang LW, Shupnik MA 1997 Specific modulation of estrogen receptor mRNA isoforms in rat pituitary throughout the estrous cycle and in response to steroid hormones. *Mol Cell Endocrinol* 131:147–155
- Bhat RA, Harnish DC, Stevis PE, Lytle CR, Komm BS 1998 A novel estrogen receptor β : identification and functional analysis of additional terminal amino acids. *J Steroid Biochem Mol Biol* 67:233–240
- Ogawa S, Inoue S, Watanabe T, Orimo A, Hosoi T, Ouchi Y, Muramatsu M 1998 Molecular cloning and characterization of human estrogen receptor β cx : a potential inhibitor of estrogen action in human. *Nucleic Acids Res* 26:3505–3512
- Nishihara E, Nagayama Y, Inoue S, Hiroi H, Muramatsu M, Yamashita S, Koji T 2000 Ontogenetic changes in the expression of estrogen receptor α and

- β* in rat pituitary gland detected by immunohistochemistry. *Endocrinology* 141:615–620
27. Peterson DN, Tkalcevic GT, Koza-Taylor PH, Turi TG, Brown TA 1998 Identification of estrogen receptor *β*2, a functional variant of estrogen receptor *β* expressed in normal rat tissues. *Endocrinology* 139:1082–1092
28. Moore JT, McKee DD, Slenz-Kesker K, Moore LB, Jones SA, Horne EL, Su JL, Kliewer SA, Lehmann JM, Wilson TM 1998 Cloning and characterization of human estrogen receptor *β* isoforms. *Biochem Biophys Res Commun* 247:75–78
29. Rosenfeld CS, Ganjam VK, Taylor JA, Yuan X, Stiehr JR, Hardy MP, Lubahn DB 1998 Transcription and translation of estrogen receptor-*β* in the male reproductive tract of estrogen receptor-*α* knock-out and wild-type mice. *Endocrinology* 139:2982–2987
30. Mowa CN, Iwanaga T 2001 Developmental changes of the oestrogen receptor-*α* and -*β* mRNAs in the female reproductive organ of the rats – an analysis by *in situ* hybridization. *J Endocrinol* 167:363–369
31. Donjacour AA, Cunha GR 1991 Stromal regulation of epithelial function. *Cancer Treat Res* 53:335–336
32. Takane KK, George FW, Wilson JD 1990 Androgen receptor of rat penis is down-regulated by androgen. *Am J Physiol* 258:E46–E50
33. Jost A 1953 Problems in fetal endocrinology: the gonadal and hypophyseal hormones. *Recent Prog Horm Res* 8:379
34. Feldman KW, Smith DW 1975 Fetal phallic growth and penile standards for newborn male infants. *J Pediatr* 86:395–398
35. Glickman SE, Coscia EM, Frank LG, Licht P, Weldele ML, Drea CM 1998 Androgens and masculinization of genitalia in the spotted hyaena (*Crocuta crocuta*). 3. Effects of juvenile gonadectomy. *J Reprod Fertil* 113:129–135
36. Licht P, Hayes T, Tsai PI, Cunha G, Kim H, Golbus M, Hayward S, Martin MC, Jaffe RB, Glickman SE 1998 Androgens and masculinization of genitalia in the spotted hyaena (*Crocuta crocuta*). 1. Urogenital morphology and placental androgen production during fetal life. *J Reprod Fertil* 113:105–116
37. DonCarlos L, McAbee M, Ramer-Quinn D, Stancil D 1995 Estrogen receptor mRNA levels in the preoptic area of neonatal rats are responsive to hormone manipulation. *Dev Brain Res* 84:253–260
38. McAbee MD, DonCarlos LL 1999 Estrogen, but not androgens, regulates androgen receptor messenger ribonucleic acid expression in the developing male rat fore brain. *Endocrinology* 140:3674–3681
39. McKinnell C, Atanassova N, Williams K, Fisher JS, Walker M, Turner KJ, Saunders PTK, Sharpe RM 2001 Suppression of androgen action and the induction of gross abnormalities of reproductive tract in male rats treated neonatally with diethylstibestol. *J Androl* 22:323–338
40. Wersinger S, Sannen K, Villalba C, Lubahn D, Rissman E, DeVries G 1997 Masculine sexual behavior is disrupted in male and female mice lacking a functional estrogen receptor *α* gene. *Horm Behav* 32:176–183
41. Calles-Escandon J, Cipolla M 2001 Diabetes and endothelial dysfunction: a clinical perspective. *Endocr Rev* 22:36–52
42. Yang S, Bae L, Zhang L 2000 Estrogen increases eNOS and NO_x release in human coronary artery endothelium. *J Cardiovasc Pharmacol* 36:242–247
43. Andersson C, Lydrup M-L, Fernö M, Gustafsson J-Å, Nilsson B-O 2001 Immunocytochemical demonstration of oestrogen receptor *β* in blood vessels of the female rat. *J Endocrinol* 169:241–247
44. Kandeel FR, Koussa VKT, Swerdlow RS 2001 Male sexual function and its disorders, physiology, pathophysiology, clinical investigation, and treatment. *Endocr Rev* 22:342–388
45. Taleghany N, Sarajari S, DonCarlos LL, Gollapudi L, Oblinger MM 1999 Differential expression of estrogen receptor *α* and *β* in rat dorsal root ganglion neurons. *J Neurosci Res* 57:603–615
46. Burke KA, Schroeder DM, Abel RA, Richardson SC, Biggsby RM, Nephew KP 2000 Immunohistochemical detection of estrogen receptor *α* in male rat spinal cord during development. *J Neurosci Res* 61:329–337
47. Papka RE, Storey-Workley M, Shughrue PJ, Merchenthaler I, Collins JJ, Usip S, Saunders PTK, Shupnik M 2001 Estrogen receptor-*α* and -*β* immunoreactivity and mRNA in neurons of sensory and autonomic ganglia and spinal cord. *Cell Tissue Res* 304:193–214
48. Murphy AZ, Hoffman GE 2001 Distribution of gonadal steroid receptor-containing neurons in the preoptic-periaqueductal gray-brainstem pathway: a potential circuit for the initiation of male sexual behavior. *J Comp Neurol* 438:191–212
49. MacLusky NJ, Bowby DA, Brown TJ, Peterson RE, Hochberg RB 1997 Sex and the developing brain: suppression of neuronal estrogen sensitivity by developmental androgen exposure. *Neurochem Res* 22:1395–1414
50. Lombardi G, Zarrilli S, Colao A, Paesano L, Di Somma C, Rossi F, De Rosa M 2001 Estrogens and health in males. *Mol Cell Endocrinol* 178:51–55
51. Gollapudi L, Oblinger MM 1999 Estrogen and NGF synergistically protect terminally differentiated, ER*α*-transfected PC12 cells from apoptosis. *J Neurosci Res* 56:471–481
52. Sohrabji F, Miranda RC, Toran-Allerand CD 1994 Estrogen differentially regulates estrogen and nerve growth factor mRNAs in adult sensory neurons. *J Neurosci* 14:459–471
53. Patron C, Andersson S, Korhonen L, Lindholm D 1999 Estrogen receptor-dependent regulation of sensory neuron survival in developing dorsal root ganglion. *Proc Natl Acad Sci USA* 96:10905–10910
54. Patron C, Pollio G, Vegeto E, Enmark E, de Curitis I, Gustafsson J-Å, Maggi A 2000 Estradiol induces differential neuronal phenotypes by activating estrogen receptor *α* and *β*. *Endocrinology* 141:1839–1845