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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 ERff and ERb in the penis to identify compartments/cells responsive to estrogen, using immunohistochemistry, Western blotting, in situ hybridization, and RT-PCR analyses. Expressions of ERa and ERb 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 ERff were localized to the mesenchyme and subepithelial stroma and later (postnatal d 2) to the corpus spongiosus, corpus cavernosus, and urethral epithelia. ERb 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, ERα and ERβ, members of the steroid/thyroid hormone receptor superfamily (4, 5).

Both ERα and ERβ are localized in the testis and excurrent ducts of various species (6–8). The most intense signal of ERα 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 ERβ 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 ERα and ERβ 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

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Abbreviations: AR, Androgen receptor; DES, diethylstibesterol; ER, estrogen receptor; NGF, nerve growth factor; NO, nitric oxide; PVDF, polyvinylidene diflouride 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 μg 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 ERα and ERβ 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 ERα cDNA (accession number Y00102) (4) and 45 to 90 of ERβ 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 0.5×10^9 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), 4× standard saline citrate (SSC; 1× 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 μg/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 2× SSC containing 0.1% sarkosyl and twice at 55 C for 40 min in 0.1× SSC containing 0.1% sarkosyl. The sections were either exposed to Hyperfilm-βmax (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 (OD_{260}/OD_{280}), and RNA quantity was estimated at OD_{260}.

RT-PCR was performed by using primers derived from the rat ERα and ERβ 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: ERα sense primer, 5'-AATTCTGACAATCGACGC-3' and antisense primer, 5'-CTTCAACATTCTCCCTCCTC-3' (334 bp; Ref. 17); and ERβ sense primer, 5'-CTCGGGGTCTGAGTGCAGCTCAAC-3' and antisense primer, 5'-CCGAGAGCCTTCAAGGCTATACAAG-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'-GTGGGGCGCCCCAGGCACCA-3', and antisense, 5'-GTCCTTAATGTCACGCACGATTTC-3'; Ref. 19).

Characterization of antisera

The studies of ERα immunoreactivity were carried out preliminarily with three antisera against ERα 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 ERα was used for these studies. Other antisera against ERα used in preliminary studies include an affinity-purified rabbit polyclonal antibody raised against a peptide at the C terminus of the mouse ERα 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 ERα protein code C1355 (Upstate Biotechnology, Inc., Lake Placid, NY). For identification of ERβ, we used the antiserum coded PA1-311, which was raised in rabbit against the amino acid residues 55–70 in the rat ERβ protein (Affinity BioReagents, Inc., Golden, CO). We confirmed that the PA1-311 showed more specific immunolabeling in comparison with other ERβ antisera tested here, a mouse antihuman ERβ monoclonal antibody code MAB463 (Chemicon International, Temecula, CA), and a goat antimouse ERβ 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 ERα antiserum was adsorbed with the ERβ peptide and then applied to tissue sections (the ERα immunostaining appeared normal). Likewise, adsorbing ERβ antiserum with the ERα protein did not diminish immunostaining for ERβ.

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 manufacture's instructions. The samples processed without primary antibodies served as negative controls. For double-label immunofluorescence staining for ERα and ERβ, the sections were incubated with ERα mouse monoclonal antibody, followed by Cy3-conjugated antimouse IgG, and then incubated with ERβ 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 diflouride 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 ERα; 1:2000 dilution for ERβ) in TTBS containing 1% albumin. After extensive washing with TTBS, the PVDF was incubated with horseradish peroxidase-conjugated antimouse or antirabbit 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 ± 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 ERα mRNA in undifferentiated and homogenous mesenchymal cells of the primordial penis at fetal d 17, the earliest age examined (Fig. 1A). The epithelium lacked ERα mRNA signals (data not shown). During the perinatal period, the stroma surrounding the urethral epithelium and basal epithelium expressed pronounced signals of ERα mRNA (Fig. 1, B–G).

The corpus spongiosus and penile urethral epithelium expressed intense signals of ERα mRNA during postnatal d 2–7 (Fig. 1, B–G); the signal intensity decreased in a proximodistal 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 ERα mRNA in the basal region than the distally located stratified epithelium (Fig. 1, B–E). However, in adults ERα mRNA signals in the basal region were primarily limited to the regions within and close to glans penis (Fig. 1H). Moderate signals of ERα 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).

ERβ 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). ERβ 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 ERα mRNA. Furthermore, unlike ERα mRNA, ERβ 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 ERα 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, ERβ 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 ERα was found in the penis spongiosus, whereas ERβ distributed predominantly in the urethra and neurovascular bundle (including dorsal blood vessels and dorsal nerve). The penis cavernosus appeared to express ERα and ERβ 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, ERα and ERβ antibodies preadsorbed with the synthetic peptide did not reveal any immunostaining (Figs. 3H and 4G). Furthermore, using the antisera against ERα and ERβ employed in this study, the specificity in the immunohistochemical reaction was ascertained in the adult female rat ovary and oviduct (Fig. 5, A–C). ERα was found to be localized in thecal and interstitial gland cells but not in granulosa cells of follicles. In the oviduct, nuclear ERα staining was observed in luminal epithelium and muscle cells. On the other hand, ERβ was predominantly detected in granulosa cells of follicles and in epithelium of the oviduct. The immunodetection of ERα and ERβ 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, ERα and ERβ, 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. ERα 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 ERα mRNA. In contrast, ERβ 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 ERα 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 ERβ 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 ERβ transcripts. ICI alone had no effect on penile expressions of ERα and ERβ 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 ERα, in the rat ovary

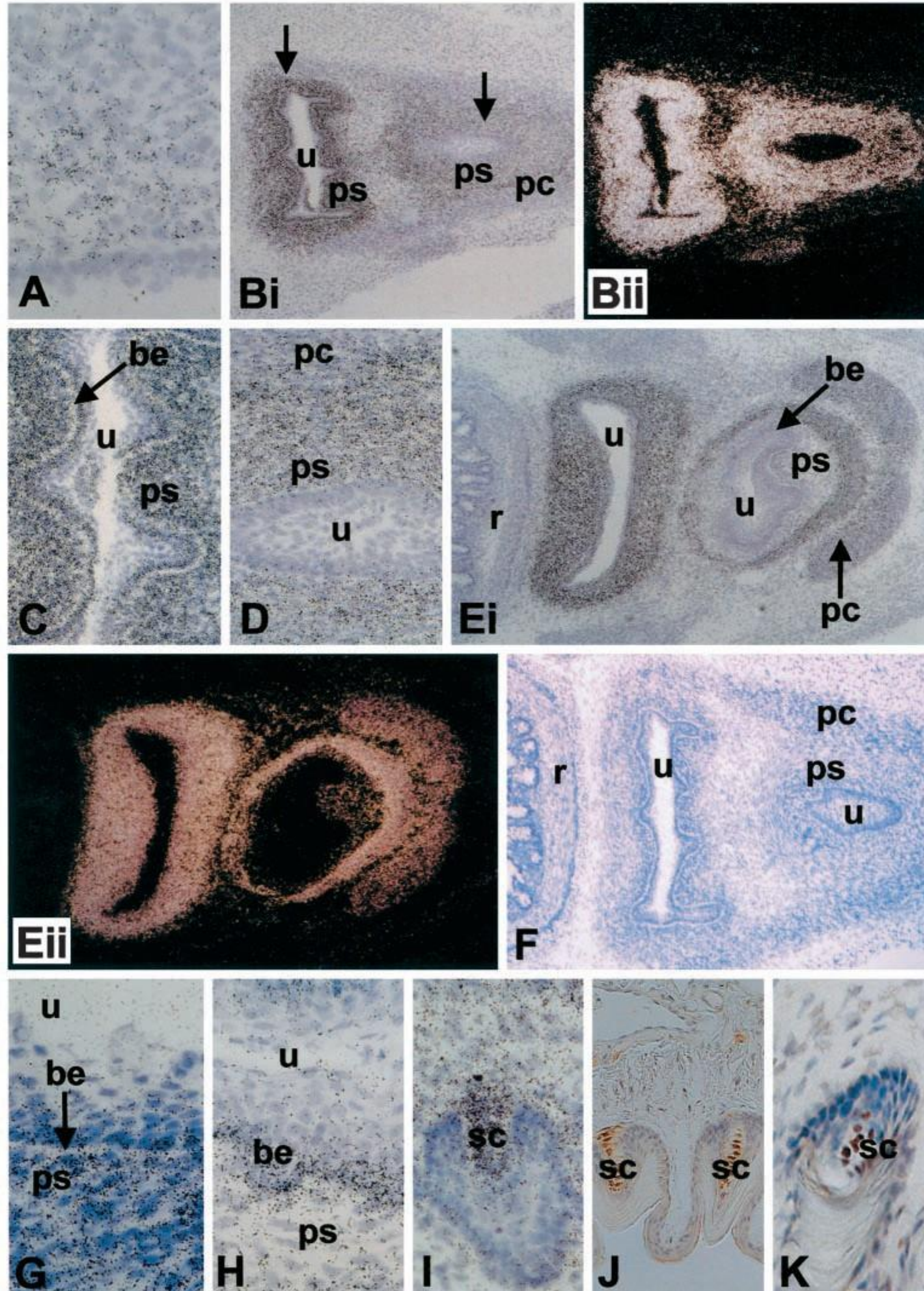

FIG. 1. *In situ* hybridization analysis showing gene expressions of ER*a* in the rat penis. A, Distinct ER*a* mRNA signals were seen in the mesenchyme of the developing penis at fetal d 17. B–E, The transverse sections of the primordial penis exhibited more pronounced signals of ER*a* mRNA in the proximal 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*a* 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*a* mRNA in the basal epithelial layer (be). Strong ER*a* 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*a* 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).

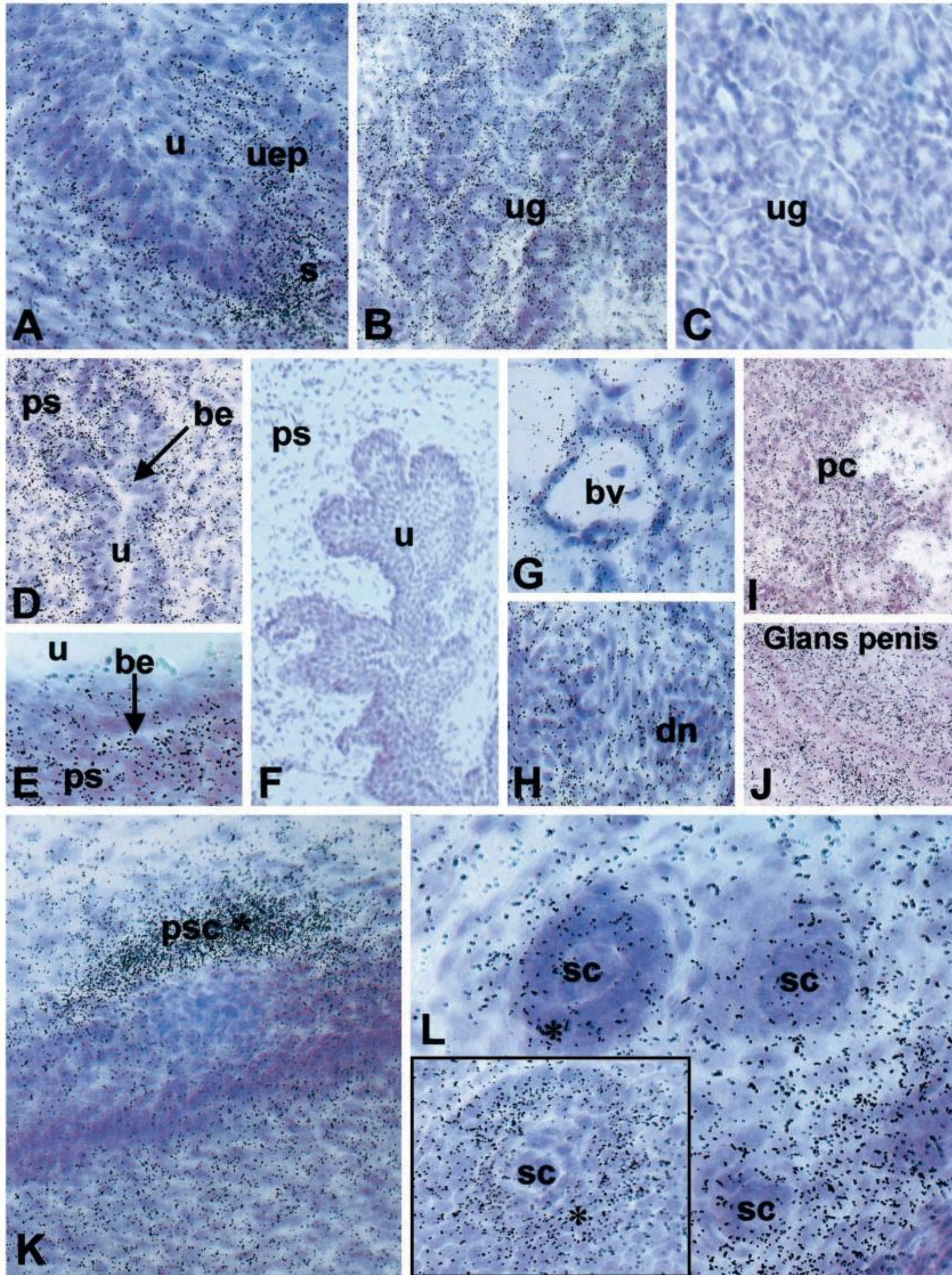

FIG. 2. *In situ* hybridization analysis showing gene expression of ERβ in the rat penis. A, Moderate ERβ mRNA signals are scattered in the stroma (s) of the developing glans penis at postnatal d 2. u, Urethra; uep, urethral epithelium. The signals were modestly seen in the epithelium of urethral glands (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 ERβ mRNA expression (*) was clearly found in cells surrounding the sensory corpuscles (sc) at postnatal d 14 (*inset*). Note that no grain (ERβ 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).

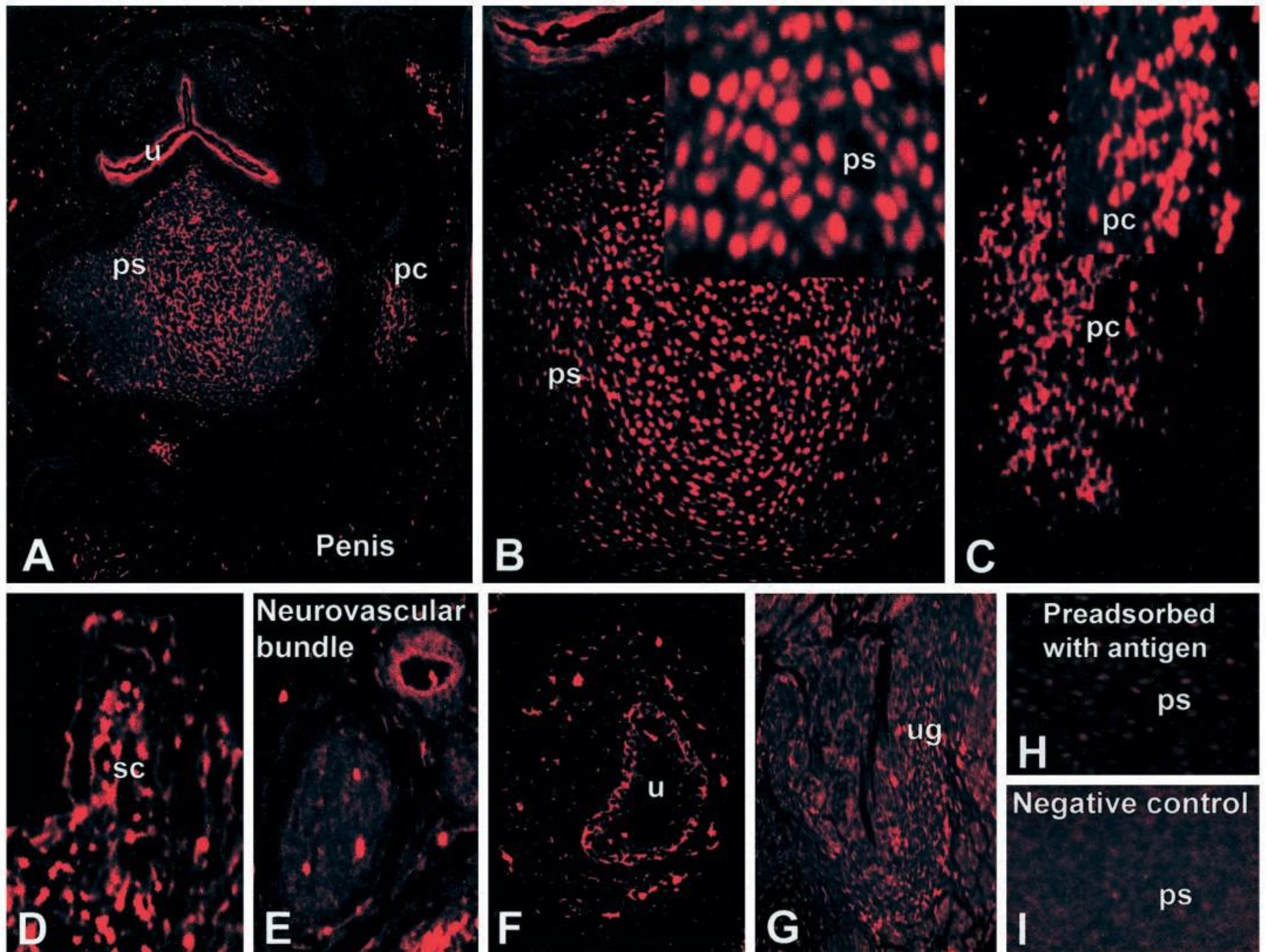

FIG. 3. Immunofluorescent findings for ERα in the rat penis. A, Penile ERα distribution in postnatal d 1 rat (low magnification, X100). Positive staining *(red)* was found in the penis spongiosus (ps), urethra (u), and penis carvenosus (pc). B and C, Immunoreactivity was found in the penis spongiosus (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 ERα was found at lower levels in the penis with increasing age. Densitometric quantification of the signal revealed that the penile ERα protein levels at 1, 8, and 35 wk of age were 46 ± 3%, 16 ± 2% ($P < 0.001$ *vs.* 1 wk), and 14 ± 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 ERβ 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 ERβ protein was 105 ± 4% at 1 wk, 71 ± 5% ($P < 0.001$ *vs.* 1 wk) at 8 wk, and 59 ± 5% ($P < 0.001$ *vs.* 1 wk) at 35 wk of age (n = 5).

Pretreatment with DES caused a marked decrease in ERα and ERβ protein expressions to 28 ± 3% (n = 5, $P < 0.001$) and 32 ± 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 ± 2% for ERα, 98 ± 3% for ERβ, n = 5, $P < 0.001$). ICI alone did not affect penile expressions of ERα (100 ± 2%, n = 5) and ERβ (100 ± 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 ERα and ERβ mRNA transcripts and proteins in the male rat penis. Expressions of ERα and ERβ 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 ERα than ERβ. *In*

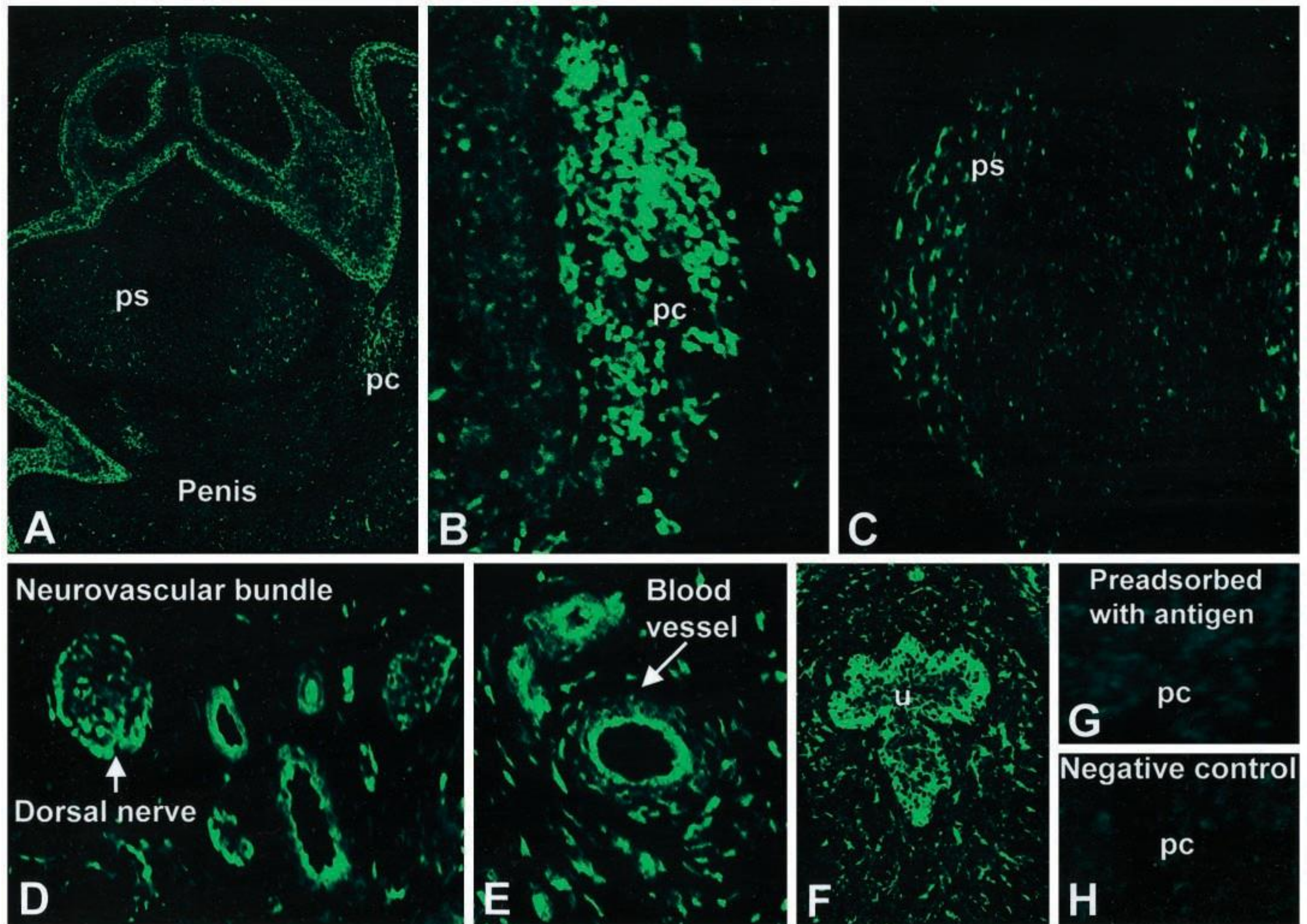

FIG. 4. Immunofluorescenct findings for ER[) in the rat penis. A, Penile ER[) distribution in postnatal d 1 rat (low magnification, X100). B and C, Immunoreactivity found in carvenosus (pc) of body penis much more strongly than in the penis spongiosus (ps) (high magnification, X400). D, Positive staining 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 ERa and ER[) protein in the rat penis as a single band. ERa and ER[) 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 ERa and 55–63 kDa for ER[), noted in other tissues (23–26). To date, various isoforms of ER[) have been reported, namely ER[)2, ER[)cx, and ER[) variants, which are altered by a deletion within the DNA binding domain (ER[)103 and ER[)203) (25, 27, 28). Because for ER[) detection we used PA1-311, which was raised against the N-terminal part of rat ER[) (29), we cannot entirely rule out the possibility that this antibody may have recognized C-terminally truncated variants such as ER[)2 and ER[)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

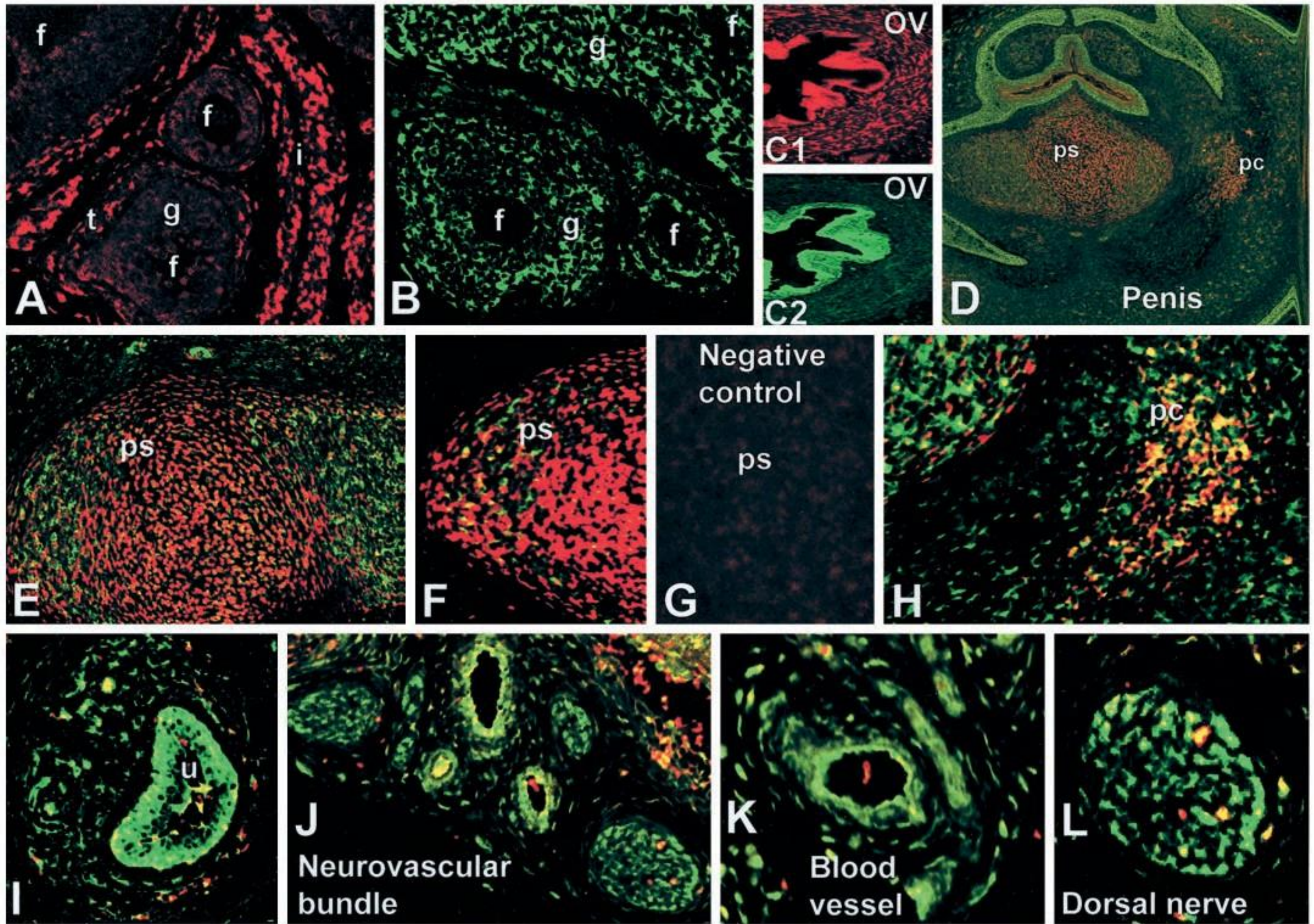

FIG. 5. Immunohistological localization of ERα (*red*) and ERβ (*green*) in the rat ovary (A–C) and penis (D–L). A, In the adult female rat ovary, ERα was immunolocalized to thecal cells (t) and interstitial gland cells (i) but not to granulosa cells (g) of follicles (f). B, In the same ovary, ERβ was predominantly detected in granulosa cells (g) of follicles (f). C, Intense ERα staining (C1) was detected in luminal epithelium and muscle cells of the oviduct (ov), whereas ERβ (C2) was expressed mainly in epithelium of the oviduct (ov). D, Immunofluorescence double labeling for ERα and ERβ in 1-d-old rat penis (low magnification, X100). E and F, ERα was expressed more abundantly than ERβ 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 ERα and ERβ were present in the penis cavernosus (pc) (high magnification, X400). I, ERβ was more dominant in the urethra than ERα (magnification, X100). J–L, ERβ 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). Androstenedione, 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 ERα and ERβ 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 ERα and ERβ

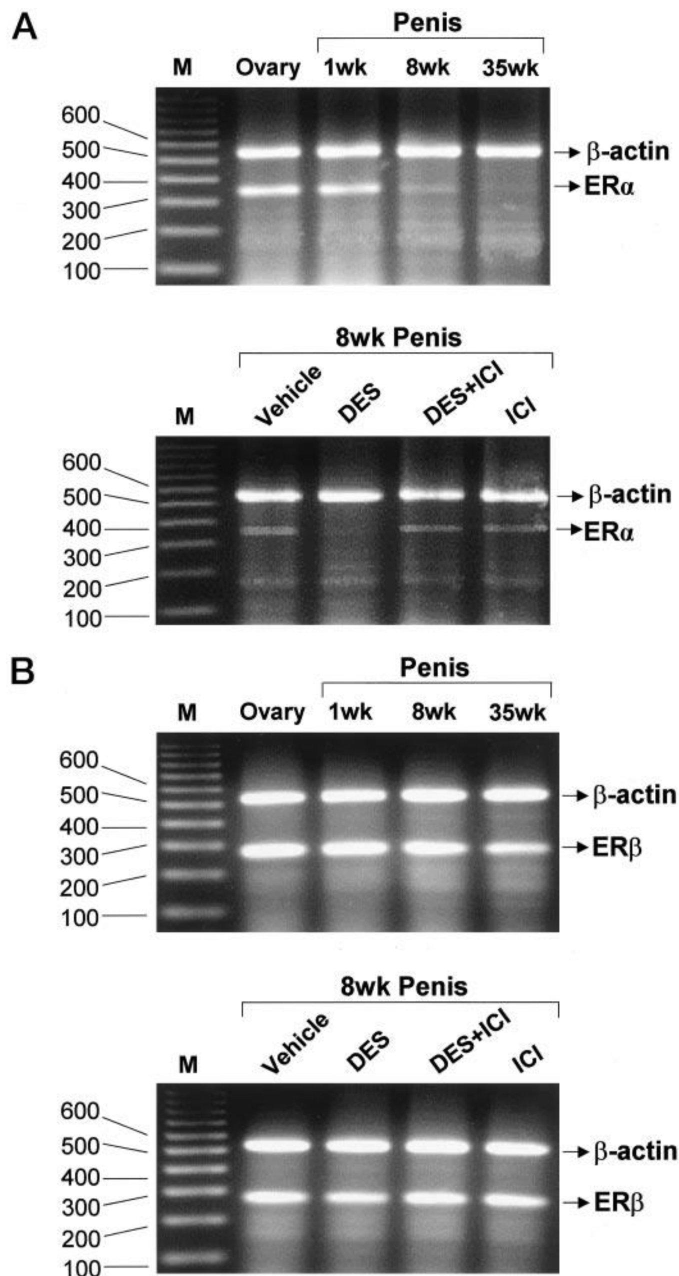

FIG. 6. RT-PCR analysis showing gene expressions of ERa (A1) and ER[) (B1) in the penile tissues of 1-, 8-, and 35-wk-old rats (lanes 2–4). The ovary from the adult female rat (lane 1) was used as positive control. The standard molecular weight used was 100 bp marker. [)-Actin mRNA was used as internal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

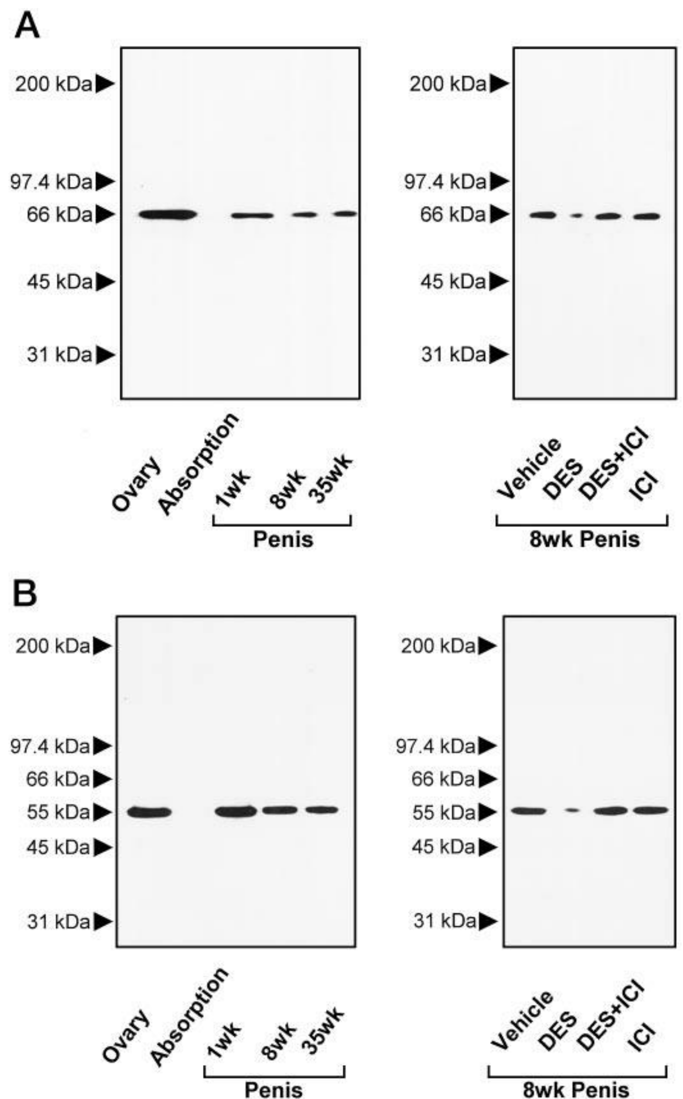

FIG. 7. Immunoblot analysis for ERa (A1) and ER[) (B1) in the penile tissues of 1-, 8-, 35-wk-old rats (lanes 3–5). The ovary from the adult female rat (lane 1) was used as a positive control. No band was seen when the antibody had been preadsorbed with antigen. *Lower panels* (A2, B2) show changes in the blots for ERa and ER[) 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[)-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, 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, ERa 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[) 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 ERβ 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.

ERα was localized to the sensory corpuscle of glans penis, with ERβ 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 ERα and ERβ 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 ERα-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 ERα 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 ERα immunoreactive. We thus assume that estrogen, working through ERα, 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 17β-estradiol, working through ERα and ERβ 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 ERα and ERβ appear to differentially modulate neurites (54).

In summary, this study is the first to demonstrate the presence of ERα and ERβ 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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