

# Expression of Functional Estrogen Receptors in Human Fetal Male External Genitalia

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It is generally assumed that male genital development is determined by androgens on a default program leading to female genitalia. Female genitalia virilization is due to high levels of androgens, whereas feminization is linked to reduction or lack of fetal androgen. Excess androgen determines sex reversal in female, whereas excess estrogen does not cause male feminization. In the present study, we investigate the presence of androgen receptors (AR) and estrogen receptors (ER) in human fetal penile tissue and in a cellular model of human fetal penile smooth muscle cells (hfPSMC). By immunohistochemistry, we showed the presence of ER and AR in the developing penile tissue of male fetuses. Besides the pres-

ence of AR, hfPSMC showed ER $\alpha/\beta$  as demonstrated by RT-PCR, Western blot, and binding techniques. These receptors are functionally active because cell stimulation with 17 $\beta$ -estradiol increased progesterone receptor B expression and inhibited hfPSMC growth, both effects being reversed by tamoxifen. Conversely, cell proliferation was stimulated by R1881 and testosterone, an effect enhanced by letrozole. These findings are the first demonstration of the presence of functional ER in differentiating male external genitalia and indicate a possible novel inhibitory role of estrogens in the regulation of the development of these sex structures. (*J Clin Endocrinol Metab* 88: 1815–1824, 2003)

ALTHOUGH THE MOLECULAR mechanisms involved in the development of external genitalia during fetal life are not yet fully elucidated, this process seems to be regulated by a complex balance between male and female sex steroid hormones. Two phases underlie this developmental process. The first, common to both female and male, is not regulated by sex hormones and consists of formation and initial outgrowth of the genital tubercle, probably under control of the distal urethral epithelium (1, 2). During the second phase, morphological differentiation between male and female under the control of sex steroid hormones leads to sexual dimorphism of the external genitalia. Developmental abnormalities in urogenital structures are one of the most common birth defects in humans and occur in part as a consequence of disruption of the normal equilibrium between estrogen and androgen production during the first 12 wk of fetal life (3). In the male, these defects vary from mild to severe hypospadias, characterized by an abnormal urethral opening on the ventral surface of the penis and incomplete formation of the scrotal folds, to various degrees of more severe disorders of sex differentiation, known as male pseudohermaphroditism (3). The incidence of congenital hypospadias in the United States is in the range of 2–6 per 1000 live births, and, as for other male urogenital abnormalities, the incidence of this defect has been increasing in the past few years (4, 5). During the critical temporal window of the first

12 wk of gestation, androgens produced by the male fetus determine the masculinization of fetal external genitalia (6). Indeed, the start of genital differentiation corresponds to the onset production of androgens from fetal Leydig cells (7), and the progressive growth of penis is maintained during pregnancy. Conversely, in the absence of androgen production, passive development of internal and external genitalia is thought to occur in the female fetus. Alterations in androgen function and production in male fetus due to hormone deficiency such as hypogonadotropic hypogonadism, 5 $\alpha$ -reductase type 2 deficiency, or androgen resistance may result in reduced development of external genitalia (8, 9). On the other hand, females exposed during fetal life to an excess of androgen production, often caused by 21-hydroxylase deficiency, have normal internal but masculinized external genitalia (10, 11) with an enlarged clitoris and fused labial folds to an extent depending on timing and duration of exposure (12).

Many pharmacological agents and environmental toxicants can alter the normal balance between sex steroid hormones, leading to a disturbance of sexual differentiation; for this reason they have been collectively called endocrine disruptors (13, 14). Besides the role of antiandrogens in sex reversal of male genital structures, increasing evidence shows the detrimental effects of *in utero* exposure to estrogenic compounds on reproductive organs of male fetuses both in humans (14–18) and in experimental animals (19, 20), suggesting a direct influence of abnormal levels of estrogens not only on internal (21) but also on external genitalia (14). A recent cohort study has definitely demonstrated an increased risk of hypospadias in the sons of women exposed to diethylstilbestrol (DES) in uterus (18), confirming previous

Abbreviations: AR, Androgen receptor(s); CC, corpus cavernosum; DES, diethylstilbestrol; ER, estrogen receptor(s); FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; [ $^3$ H]estradiol, [2,4,6,7,16,17- $^3$ H]estradiol; hfPSMC, human fetal penile smooth muscle cell(s); LNCaP, lymph node carcinoma of prostate; PR, progesterone receptor; T, testosterone.

case reports (22). Although a metaanalysis of 14 studies showed a strict association between first trimester exposure to sex hormones and external genitalia abnormalities only for DES (17), many other compounds are suspected to act as endocrine disruptors in causing urogenital abnormalities. However, little is known about the effect of exposure to estrogens and compounds with estrogenic activity on the development of male external genitalia.

To understand at the molecular level the possible detrimental effects of environmental endocrine disruptors on the development of male external genitalia, we investigated the presence and activity of estrogen receptors (ER) in developing fetal penile tissue. In particular, we studied *in vitro* effects on cellular growth exerted by estrogens and androgens in established cell cultures of penile cells obtained from four fetal penile tissues at 12 wk gestation.

## Materials and Methods

### Chemicals

MEM, DMEM-F12 1:1 mixture, PBS, BSA, glutamine, antibiotics, collagenase type IV, trypan blue, 17 $\beta$ -estradiol, testosterone (T), synthetic androgen R1881, tamoxifen, the aromatase inhibitor, and reagents for immunocytochemistry, for aromatase activity measurement, for SDS-PAGE and peroxidase-conjugated secondary antibodies were from Sigma Chemical Co. (St. Louis, MO). 7 $\beta$ -<sup>3</sup>H androstenedione and [<sup>3</sup>H]R1881 were purchased from NEN Life Science Products (Boston, MA), whereas [2,4,6,7,16,17-<sup>3</sup>H]estradiol ([<sup>3</sup>H]estradiol) was from Amersham Biosciences Italia (Milan, Italy). Fetal bovine serum (FBS) was obtained by Unipath (Bedford, England). The BM enhanced-chemiluminescence system was purchased from Roche Diagnostic (Milan, Italy). The protein measurement kit was from Bio-Rad Laboratories, Inc. (Hercules, CA). Monoclonal rat antibodies against  $\alpha$  and  $\beta$  isoforms of ER (H222 and H226) used for Western blot analysis were a kind gift from Prof. G. Greene (The Ben May Institute for Cancer Research, University of Chicago, Chicago, IL). Mouse monoclonal antibody against ER (clone ER1D5) used for immunohistochemistry was from BioGenex Laboratories, Inc. (San Ramon, CA). Mouse monoclonal anti-progesterone receptor c262 antibody was obtained from StressGen (Victoria, Canada). Monoclonal and polyclonal antibodies to androgen receptor (AR) were from Novocastra (Newcastle, UK) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Forward and reverse primers specific for AR, glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH), and *sry* were from MWG Biotech (Florence, Italy). Forward and reverse primers specific for ER $\alpha$  and ER $\beta$  were kindly supplied by Prof. Gianna Fiorelli (Department of Clinical Physiopathology, University of Florence, Florence, Italy). Plasticware for cell cultures was purchased from Falcon (Oxnard, CA). Disposable filtration units for growth media preparation were purchased from PBI International (Milan, Italy). Letrozole was a gift from Novartis Pharma (Basel, Switzerland).

### Immunohistochemistry

Immunohistochemical studies were performed on deparaffinized and rehydrated tissue sections or cultured cells fixed in 3.7% paraformaldehyde for 15 min, as previously described (23). The specimens were subsequently exposed to 0.3% hydrogen peroxidase-methanol solution to quench endogenous peroxidase activity. Slides were rinsed in tap water, then immersed in 10 mmol/liter citrate buffer (pH 6.0), and microwaved for 40 min at 350 W to enhance antigen exposure. The primary antibody at the appropriate dilution in PBS was added to the slide and incubated overnight at 4 C. Sections were rinsed in PBS, incubated with biotinylated secondary antibodies, and finally incubated with streptavidin-biotin peroxidase complex (LSAB kit, DAKO Corp., Carpinteria, CA). The reaction product was developed with deamino-benzidine tetrahydrochloride or 3-amino-9-ethyl-carbazole as chromogen. Slides were washed in running tap water and counterstained with hematoxylin, followed by dehydration and coverslip mounting. Controls were performed by processing slides lacking the primary anti-

bodies or by staining with the corresponding nonimmune serum. The slides were evaluated and photographed with a Nikon Microphone-FX microscope (Nikon, Tokyo, Japan).

### Cell cultures

Human fetal penile smooth muscle cells (hfPSMC) were obtained from four samples of fetal male external genitalia (11–12 wk gestation) obtained after spontaneous or therapeutic abortion. Legal abortions were performed in authorized hospitals, and certificates of consent were obtained. The Local Ethical Committee gave approval for the use of human material. Briefly, tissues were mechanically dispersed and treated with 1 mg/ml bacterial collagenase for 15 min at 37 C. Fragments were then collected, washed in PBS, and cultured in a 1:1 mixture (vol/vol) of DMEM and Ham's/F12 (DMEM/F-12 1:1 Mix) supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin in a fully humidified atmosphere of 95% air and 5% CO<sub>2</sub>. Cells began to emerge within 24–48 h and were used within the fifth passage. Specific antibodies were used to characterize hfPSMC. They showed positive staining for  $\alpha$  smooth muscle actin, suggesting fibromuscular morphological features. Both chromosomal analysis and PCR for *sry* gene confirmed that these cells were from male fetuses. All experiments, except for immunohistochemistry, shown in Fig. 1, that was conducted on tissue sections from the four fetuses, were performed on these cell cultures using positive controls as indicated in the text.

### PCR

Total RNA was extracted from hfPSMC cultures and from human testis, corpora cavernosa, and uterus, used as positive controls, with RNAasy Mini Kit (QIAGEN, Valencia, CA). RNA concentrations were determined by spectrophotometric analysis at 260 nm. Total RNA (500 ng) was reverse-transcribed for 30 min at 50 C, denatured for 2 min at 95 C, and amplified for 30 cycles with the following parameters: denaturation 45 sec at 95 C, annealing 1 min at 62 C for ER and 60 C for AR, and extension 1 min at 70 C. The oligonucleotide sequences of the pairs of specific primers used for retrotranscription and amplification were as follows: ER $\alpha$  sense primer (exon 1), 5'-GAC CCT CCA CAC CAA AGC ATC TG-3'; ER $\alpha$  antisense primer (exon 4), 5'-CTC CTC TTC GGT CTT TTC GTA TCC-3'; ER $\beta$  sense primer (exon 1), 5'-TAG TGG TCC ATC GCC AGT TAT-3'; ER $\beta$  antisense primer (exon 3, 4), 5'-GGG AGC CAC ACT TCA CCA T-3' (Ref. 23); AR sense primer, 5'-ACT CTG GGA GCC CGG AAG CTG-3'; and AR antisense primer, 5'-AAT GCT TCA CTG GGT GTG GAA-3'. The quality of total RNA used was assessed by performing additional RT-PCR using primers specific for the GAPDH gene: GAPDH sense primer, 5'-CCA TGG AGA AGG CTG GGG-3'; GAPDH antisense primer, 5'-CAA AGT TGT CAT GGA TGA CC-3' (Ref. 24). The contamination of genomic DNA was excluded by performing 35 cycles of amplification without retrotranscription.

Genomic DNA was extracted from hfPSMC by DNeasy Tissue System according to the manufacturer's instructions (QIAGEN). PCRs were performed using the following primers for *sry* gene: sense primer, 5'-GAA TAT TCC CGC TCT CCG GA-3'; antisense primer, 5'-GCT GGT GCT CCA TTC TTG AG-3'. DNA was denatured at 95 C for 5 min and amplified for 35 cycles by the following protocol: denaturation 1 min at 95 C, annealing 1 min at 63 C, extension 1 min at 72 C.

### SDS-PAGE and Western blot analysis

To evaluate the presence of AR, ER $\alpha$ , and ER $\beta$  in hfPSMC, cultured cells grown in RPMI 1640 to 70–80% confluence were washed and scraped in PBS. After centrifugation, pellets were extracted in lysis buffer [20 mmol/liter Tris (pH 7.4), 150 mmol/liter NaCl, 0.25% Nonidet P-40, 1 mmol/liter Na<sub>3</sub>VO<sub>4</sub>, 1 mmol/liter phenylmethylsulfonyl fluoride] on ice for 2 h. After protein measurement, aliquots containing 30  $\mu$ g protein were diluted in reducing 2 $\times$  Laemmli's sample buffer [62.5 mmol/liter Tris (pH 6.8), 10% glycerol, 2% sodium dodecyl sulfate, 2.5% pyronine, and 100 mmol/liter dithiothreitol] and loaded onto 8% and 10% polyacrylamide-bisacrylamide gels. After SDS-PAGE, proteins were transferred to nitrocellulose membranes. Membranes were blocked 2 h at room temperature in 5% milk-TTBS (0.1% Tween 20, 20 mmol/liter Tris, 150 mmol/liter NaCl) or 5% BSA-TTBS, washed in TTBS, and incubated



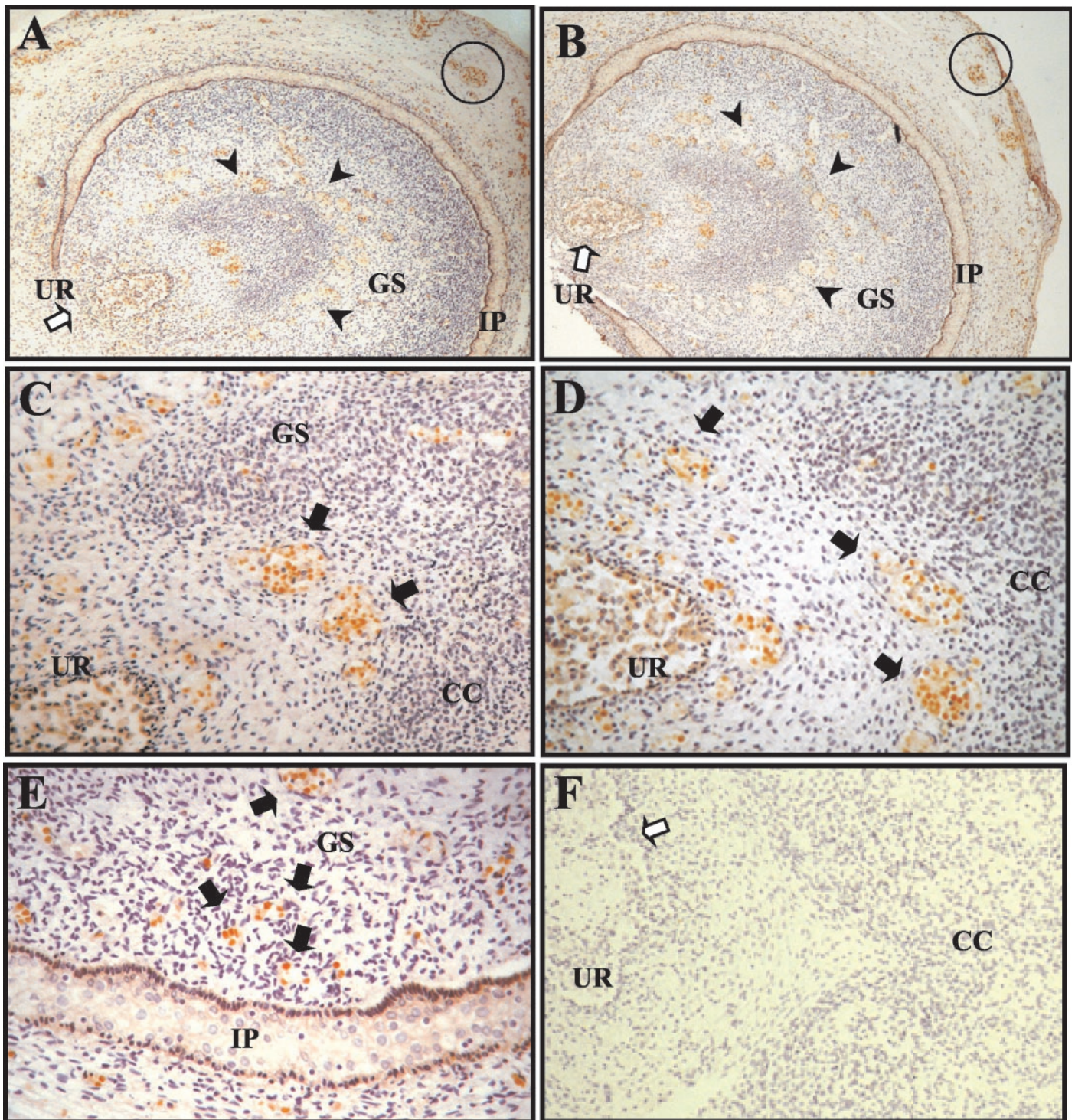


FIG. 1. Immunohistochemistry for ER and AR in human fetal penile tissue. Transverse sections of human male external genitalia (MEG) of a fetus at the 12<sup>th</sup> week of gestation. *White arrows* indicate the forming male urethra (UR); *black arrowheads* in the glans stroma (GS) indicate the condensing connective tissue of the forthcoming CC. Note the inner prepuce (IP) surrounding the forming CC. A and B, Low magnification of the MEG. Immunostaining for ER-positive (A) and AR-positive (B) cells was present in several portions of the MEG, including the skin (*circle*), the UR, and the GS. Positivity for both sex steroid receptors occurred apparently in the same structures. C and D, Panels correspond to A and B, respectively. Note that blood vessels (*black arrows*) surrounding and invading the CC are positive for ER and AR. Stromal cells of the UR were also stained. E, ER immunostaining at the level of the prepuce showing positive endothelial and smooth muscle cells. F, Negative control avoiding primary antibodies. Magnification, A and B,  $\times 80$ ; C–F,  $\times 150$ .



for 2 h with specific primary antibodies (rabbit anti-AR antibody 1:100 in 5% milk-TTBS or rat anti-ER antibodies 1:400 in 2% BSA-TTBS) followed by peroxidase-conjugated secondary IgG (1:4000 either in 5% milk-TTBS or 5% BSA-TTBS). Finally, reacted proteins were revealed by BM enhanced-chemiluminescence system.

### Binding assays

Binding assays were essentially performed as previously reported (25). Cells grown in Ham's/F12 (without phenol red) supplemented with 10% stripped FBS were harvested and washed with cold TEDMo [10 mmol/liter Tris-HCl (pH 7.4), containing 1.5 mmol/liter EDTA, 1 mmol/liter dithiothreitol, and 10 mmol/liter sodium molybdate]. Cells were resuspended in TEDGMO (TEDMo plus 10% glycerol) and homogenized in an ultraturax. The homogenate was appropriately diluted, and 100  $\mu$ l containing 0.70 mg protein for androgen binding or 0.35 mg for estrogen binding were incubated overnight at 4 C in a final volume of 250  $\mu$ l in TEDGMO with increasing concentrations of [2,4,6,7,16,17-<sup>3</sup>H]-estradiol (specific activity, 150 Ci/mmol), in the absence (0.015, 0.03, 0.06, 0.12 nmol/liter) or presence (0.12 nmol/liter) of increasing concentrations of nonradioactive 17 $\beta$ -estradiol ( $10^{-11}$  to  $10^{-5}$  mol/liter) for estrogen binding or with increasing concentrations of [<sup>3</sup>H]R1881 (specific activity, 68 Ci/mmol) in the absence (0.125, 0.25, 0.5, 1 nmol/liter) or presence (1 nM) of increasing concentrations of nonradioactive R1881 ( $10^{-10}$  to  $10^{-6}$  mol/liter) for androgen binding. Dilution of tracer was performed to optimally characterize the high-affinity portion of the displacement curves (26). To prevent R1881 binding to progesterone receptor (PR), 1  $\mu$ mol/liter triamcinolone acetonide was added to each tube. Separation of bound and free ligand was achieved by 15 min treatment with 500  $\mu$ l of a suspension of dextran-coated charcoal (0.5% dextran, 0.5% charcoal) in 10 mmol/liter Tris-HCl (pH 7.4), 1.5 mmol/liter EDTA, 1 mmol/liter dithiothreitol at 4 C. The charcoal was pelleted by centrifugation for 10 min at  $1500 \times g$ , and 600  $\mu$ l supernatant was counted in Instagel plus (Packard, Meriden, CT) in a  $\beta$ -counter. Protein content was determined by the method of Bradford (26a) with BSA as standard. Data were analyzed by the computer program LIGAND (27).

### Estradiol stimulation of PR expression

PR expression in hfPSMC after 7-h stimulation with 17 $\beta$ -estradiol and tamoxifen was evaluated by Western blot analysis of total protein extracts separated by 8% reducing SDS-PAGE and transfer on nitrocellulose membranes (28). Transferred nitrocellulose was blocked for 2 h at room temperature in 5% BSA-TTBS, washed in TTBS, and incubated for another 2 h with monoclonal c262 anti-PR antibody (1:400 in 2% BSA-TTBS) followed by antimouse peroxidase-conjugated secondary IgG (1:4000). Finally, reacted proteins were revealed by BM enhanced-chemiluminescence system. Quantification of bands corresponding to PR in Western blots was made directly on the films by image scanning analysis using Photoshop 5.5 software. Data have been reported as mean  $\pm$  SEM of percentage increase, with the control taken as 100%.

### Cell proliferation assay

For growth measurement,  $2 \times 10^4$  cells were seeded onto 12-well plates in growth medium. After 24 h, the growth medium was removed, and the cells were washed twice in PBS and then incubated in phenol red- and serum-free medium containing 0.1% BSA. After 24 h, increasing concentrations of 17 $\beta$ -estradiol, T, or the synthetic androgen R1881 were added. A fixed concentration (100 nmol/liter) of tamoxifen was simultaneously added to cultures stimulated with 17 $\beta$ -estradiol, whereas a fixed concentration (300 nmol/liter) of letrozole was added to cells stimulated with T. Cells in phenol red- and serum-free medium containing 0.1% BSA and vehicle were used as basal controls. After 24 h, cells were trypsinized, and each experimental point was derived from hemocytometer counting averaging at least six different fields for each well. In single experiments, each experimental point was repeated in triplicate; experiments were performed at least three times.

### Aromatase activity in hfPSMC culture

Aromatase activity in hfPSMC was evaluated by in-cell <sup>3</sup>H-water release assay (29). hfPSMC cultures at 80% confluence were added to

a solution of 1 $\beta$ -<sup>3</sup>H androstenedione and nonradioactive steroid (to obtain the required final concentration of 200 nmol/liter) together with 1  $\mu$ mol/liter PR to inhibit 5 $\alpha$ -reductase activity. To assess the specificity of the reaction, one dish for each experiment was incubated in the presence of 1  $\mu$ mol/liter letrozole. After 48 h of incubation, conditioned medium was collected and added to an equal volume of dextran-coated charcoal suspension (1% charcoal, 0.5% dextran). Samples were centrifuged at  $2000 \times g$  for 15 min, and the supernatant was extracted by shaking 30 min at room temperature with chloroform (1:2.5). The extracted aqueous phase was counted in a  $\beta$ -counter. Although proliferation experiments were performed mostly at 24-h incubation, for evaluation of aromatase activity 48-h incubation time was chosen to enhance the enzymatic activity and improve detection.

### Statistical analysis

Statistical analysis was performed by one-way ANOVA and unpaired or paired Student's *t* tests, when appropriate, taking *P* values less than 0.05 as significant. The computer program ALLFIT (30) was used for the analysis of sigmoidal dose-response curves to obtain estimates of half-maximal inhibition (IC<sub>50</sub>) and stimulation (EC<sub>50</sub>) values. The binding data were evaluated quantitatively with nonlinear least-squares curve fitting using the computer program LIGAND (27). Data were expressed as mean  $\pm$  SEM.

## Results

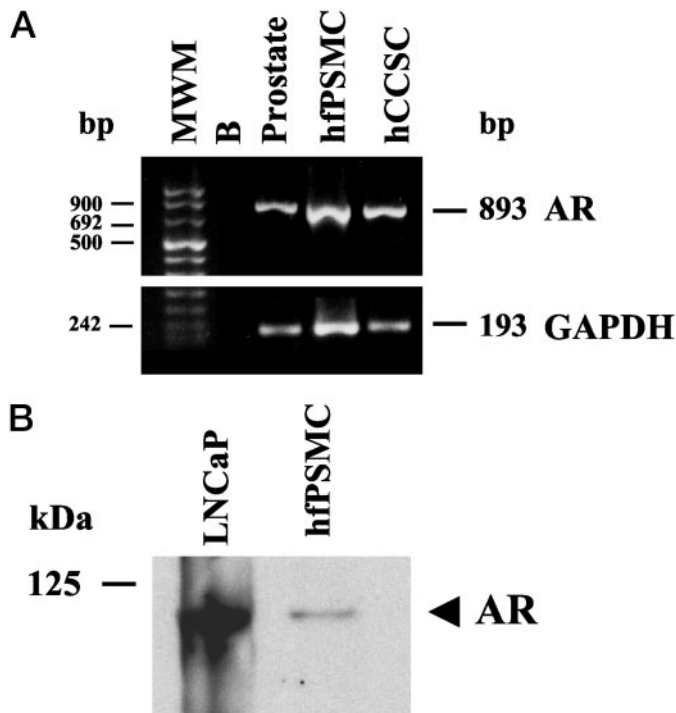
### ER and AR are present in the developing human fetal penile tissue

Transverse sections of the developing human male external genitalia (12 wk gestation) are shown in Fig. 1. In these sections, condensing connective tissue to form the corpus cavernosum (CC) and the remodelling urethra are clearly shown. Positivity for both ER (Fig. 1A) and AR (Fig. 1B) was present throughout the entire developing organ, including the skin, the urethra, and the glans stromal cells of CC. Positivity for both sex steroid receptors occurred apparently in the same structures, as can be appreciated at higher magnification (Fig. 1C, ER; Fig. 1D, AR). Among cellular structures, numerous wide and convoluted blood vessels surrounding and invading the CC appeared to be positive for ER and AR in both endothelial and smooth muscle cells (Fig. 1C, ER; Fig. 1D, AR). Stromal cells in the urethra were also stained. Smooth muscle cells expressed ER as shown at high magnification of the section at the level of the inner prepuce (Fig. 1E). The specificity of the immunohistochemical staining was demonstrated by the absence of labeling in sections incubated with normal serum avoiding the primary antibodies (Fig. 1F).

### Presence of AR and ER in hfPSMC

To investigate the presence and activity of both AR and ER in human fetal penile tissue, we established cultures of hfPSMC from four samples of fetal penile tissues. We first characterized the cultured cells obtained as smooth muscle cells by immunohistochemical positivity for  $\alpha$  smooth muscle cell antigen (data not shown). Both karyotype analysis and PCR on genomic DNA extracted from the cells using primers for the male specific *sry* gene on Y chromosome (data not shown) confirmed the male sex of fetuses from which cell cultures were obtained.

RT-PCR analysis of total RNA using primers designed for the AR revealed the presence of a single cDNA band of the expected length (893 bp) in both hfPSMC and adult



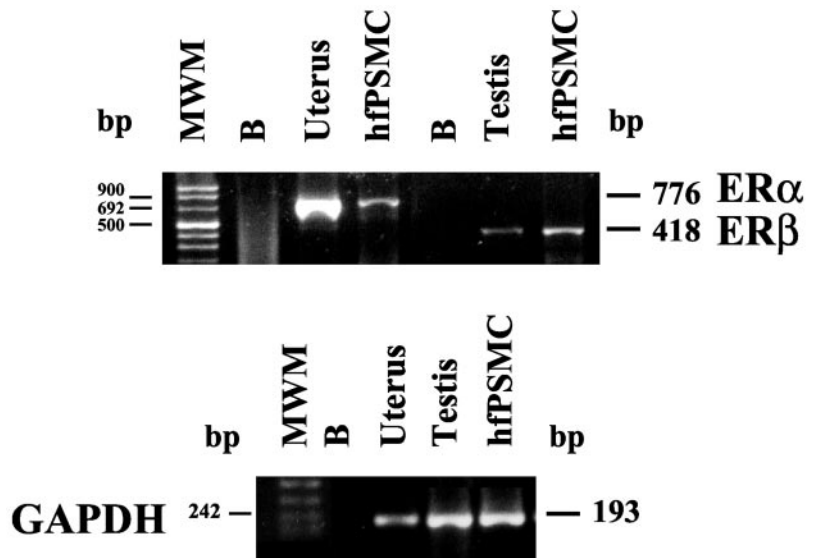
**FIG. 2.** AR expression in hfPSMC. **A**, Ethidium bromide-stained agarose gels, showing RT-PCR products for AR (*top*) and GAPDH (*bottom*) primers on total RNA from hfPSMC. Adult CC stromal cells (hCCSC) and prostate RNA were used as positive control for AR expression. A single band at the expected molecular weight (893 bp) was present in hfPSMC compared with positive controls. GAPDH amplification (193 bp) was used as housekeeping gene. MWM, Molecular weight marker; B, negative control without RNA. **B**, Western blot detection of AR protein in hfPSMC. All lysates were obtained as described in *Materials and Methods*, and 30  $\mu$ g of proteins from hfPSMC and LNCaP cells were separated by 8% SDS-PAGE, transferred onto nitrocellulose membrane, and probed for AR expression with anti-AR polyclonal antibody (1:100). A single band of about 110 kDa molecular weight (*arrowhead*) was present in both hfPSMC compared with LNCaP cells, used as positive control. Molecular weight marker (kilodaltons) is indicated to the *left* of the blot.

stromal CC cells, as well as in the prostate used as positive control (Fig. 2A). Western blot analysis of hfPSMC protein lysates using a polyclonal antibody raised against human AR showed a single band of about 110 kDa corresponding to AR, as confirmed by the presence of a band migrating at the same molecular weight in lymph node carcinoma of prostate (LNCaP) lysates used as positive controls (Fig 2B). AR expression in hfPSMC detected by both RT-PCR and Western blot analysis was also confirmed by immunocytochemistry on fixed cells (see Fig. 8A, *inset*).

Expression of both forms of ER (ER $\alpha$  and ER $\beta$ ) in hfPSMC has been demonstrated by RT-PCR on total RNA extracted from hfPSMC, testis, and uterus, used as positive controls (Fig. 3). RT-PCR has been conducted using couples of primers in which the sense and antisense primers map on two noncontiguous exons. This strategy avoids the possibility that the PCR product of the expected length may be due to genomic DNA contamination during RNA extraction, because it would generate a distinct higher PCR fragment. Expected transcripts for ER $\alpha$  (776 bp) and ER $\beta$  (418 bp) were both expressed in hfPSMC, compared with uterus and testis used as positive control (Fig. 3).

Western blot analysis of hfPSMC protein lysates with a monoclonal antibody directed against the conserved ligand binding domain of ER $\alpha$  and ER $\beta$  clearly demonstrated the presence of both a 65-kDa band corresponding to ER $\alpha$  and a 54-kDa band corresponding to ER $\beta$  compared with MCF7 cells, used as positive control (Fig. 4). ER expression in hfPSMC detected by both RT-PCR and Western blot analysis was also confirmed by immunocytochemistry on fixed cells (see Fig. 7A, *inset*). Moreover, we found that hfPSMC not only express both AR and ER but also specifically bind the respective labeled androgen and estrogen ligands. In binding studies, we found the presence of low capacity ( $B_{\max} = 8.1 \pm 1.5 \times 10^{-12}$  mol/liter for R1881 and  $B_{\max} = 2.6 \pm 0.5 \times 10^{-12}$  mol/liter for 17 $\beta$ -estradiol) and high affinity ( $K_d = 1.9 \pm 0.5 \times 10^{-10}$  mol/liter for R1881 and  $0.5 \pm 0.1 \times 10^{-10}$  mol/liter for 17 $\beta$ -estradiol) binding sites for both ligands (Fig. 5, A for R1881, and B for 17 $\beta$ -estradiol).

**FIG. 3.** Expression of ER $\alpha$  and ER $\beta$  in hfPSMC. *Top*, RT-PCR for ER $\alpha$  (*upper*) and ER $\beta$  (*lower*) were performed on total RNA extracted from hfPSMC, human uterus, and testis. Human uterus was used as positive control for ER $\alpha$  expression (*left*), whereas testis was used as positive control for ER $\beta$  expression (*right*). A single band at the expected molecular weight was found for both ER $\alpha$  (776 bp, *upper*) and ER $\beta$  (418, *lower*) in hfPSMC compared with positive controls. *Bottom*, GAPDH amplification (193 bp) was used as housekeeping gene. MWM, Molecular weight marker; B, negative control without RNA.



### Functional activity of ER in hfPSMC

To demonstrate functional activity of ER present in hfPSMC, we evaluated the ability of 17 $\beta$ -estradiol to regulate PR levels, because estrogens have been widely reported to up-regulate PR expression (31). After cell treatment with 17 $\beta$ -estradiol (1 nmol/liter) with or without tamoxifen (100 nmol/liter), levels of PR protein were measured by Western blot analysis of hfPSMC protein extracts using a monoclonal antibody directed against the ligand binding domain of PR. MCF7 lysates were used as positive control. Seven-hour treatment with 1 nmol/liter 17 $\beta$ -estradiol induced a clear increase of expression of the 120-kDa PR<sub>B</sub> isoform compared with untreated sample (Fig. 6, A and B). Conversely, the concomitant presence of tamoxifen 100 nmol/liter signifi-

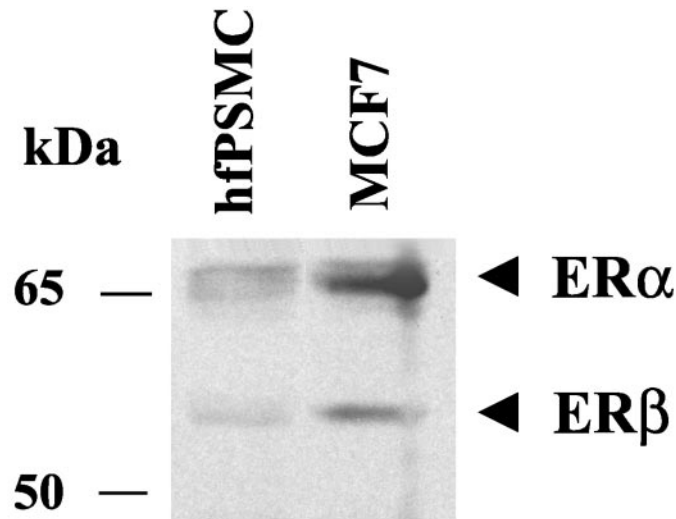


FIG. 4. Western blot detection of ER $\alpha$  and ER $\beta$  proteins in hfPSMC. Thirty micrograms of total proteins extracted from hfPSMC and MCF7 cells were separated by 10% SDS-PAGE, transferred onto nitrocellulose membrane, and probed for both ER $\alpha$  and ER $\beta$  using H226 monoclonal antibody (1:400). Two bands at the expected molecular weight (65 kDa for ER $\alpha$ , and 55 kDa for ER $\beta$ ) were revealed in hfPSMC compared with the positive control, MCF7. Molecular weight markers (kilodaltons) are indicated to the left of the blot.

cantly reduced the stimulatory effects of estrogens (Fig. 6, A and B). The average percentage of PR<sub>B</sub> positivity, compared with control taken as 100%, as evaluated in five different experiments is reported in Fig. 6B.

### Effects of androgens and estrogens on hfPSMC proliferation

To investigate the possible role of ER in fetal penile tissues, we incubated hfPSMC with increasing concentrations of 17 $\beta$ -estradiol ( $10^{-13}$  to  $10^{-6}$  mol/liter). As shown in Fig. 7A, 24-h incubation of hfPSMC with 17 $\beta$ -estradiol resulted in a significant and dose-dependent decrease in cell proliferation rate with an IC<sub>50</sub> =  $0.2 \pm 0.1 \times 10^{-10}$  mol/liter. This value is in good agreement with the affinity constant of the ER derived from binding studies (Fig. 5B). The inhibitory effect exerted by 17 $\beta$ -estradiol was counteracted by the simultaneous treatment with tamoxifen (100 nmol/liter; Fig. 7B), confirming the antiestrogenic effect of tamoxifen on PR<sub>B</sub> level (Fig. 6, A and B). Conversely, androgens stimulated a time-dependent (Fig. 8A) and dose-dependent (Fig. 8, B and C) increase in hfPSMC proliferation. In particular, 24-h treatment with increasing concentrations of the synthetic androgen R1881 resulted in a significant increase in cell growth with an EC<sub>50</sub> =  $4.0 \pm 0.9 \times 10^{-10}$  mol/liter (Fig. 8B, squares). Similarly to R1881, the aromatizable androgen T significantly stimulated cell proliferation in a dose-dependent manner ( $10^{-10}$  to  $10^{-7}$  mol/liter) with EC<sub>50</sub> =  $4.4 \pm 1.4 \times 10^{-10}$  mol/liter (Fig. 8B, triangles). Mathematical analysis using the ALLFIT program (30) of sigmoidal growth curves indicated significant differences between maximal effects ( $E_{\max}$  for T,  $138 \pm 2$ ;  $E_{\max}$  for R1881,  $153 \pm 2$ ;  $P < 0.05$ ). At high doses ( $10^{-8}$  and  $10^{-7}$  mol/liter), R1881 and T stimulation of cell growth was significantly different as evaluated by ANOVA (Fig. 8B). However, the simultaneous incubation of the hfPSMC cultures with the specific aromatase inhibitor letrozole (300 nmol/liter) completely rescued T-induced cell proliferation (Fig. 8C) to the R1881 level (Fig. 8B), consistent with the presence of endogenous aromatase activity. We therefore evaluated aromatase activity by in-cell <sup>3</sup>H-water release assay on both 48-h letrozole-treated and untreated cells. Preliminary experiments showed a  $123 \pm 3\%$  increase of aro-

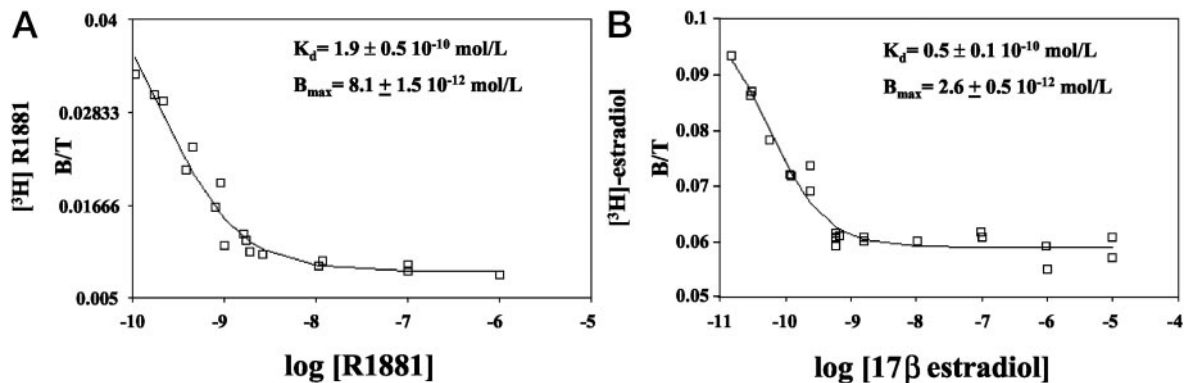


FIG. 5. Binding of sex steroids to hfPSMC. A, Homologous competition curve for [<sup>3</sup>H]R1881 with unlabeled R1881 obtained pooling data from two experiments performed in triplicate. Ordinate, Bound to total (B/T) ratio for [<sup>3</sup>H]R1881. The concentrations of the tracer were progressively reduced to optimally characterize the high-affinity region of the curve. Abscissa, Total concentration of labeled and unlabeled R1881. B, Homologous competition curve for [<sup>3</sup>H]estradiol with unlabeled 17 $\beta$ -estradiol obtained pooling data from two experiments performed in triplicate. Ordinate, B/T ratio for [<sup>3</sup>H]estradiol. The concentrations of the tracer were progressively reduced to optimally characterize the high-affinity region of the curve. Abscissa, Total concentration of labeled and unlabeled estradiol.



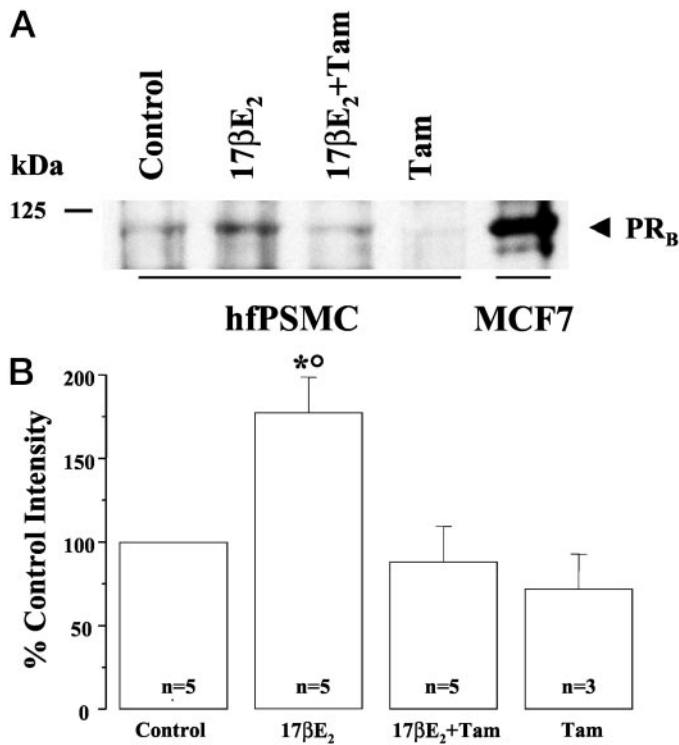


FIG. 6. PR expression in hfPSMC is induced by  $17\beta$ -estradiol ( $17\beta E_2$ ) and inhibited by tamoxifen (Tam). A, ER functional activity was evaluated in hfPSMC as the ability to stimulate  $PR_B$  expression.  $PR_B$  protein was detected by Western blot analysis of cells stimulated for 8 h with  $17\beta$ -estradiol (1 nmol/liter) and Tam (100 nmol/liter) alone or in combination. Thirty micrograms of total proteins were separated by 10% SDS-PAGE, transferred onto nitrocellulose membrane, and probed for  $PR_B$  using c262 monoclonal antibody (1:400). A single band at the expected molecular weight (120 kDa) was revealed in hfPSMC compared with the positive control, MCF7 cells. Molecular weight marker (kDa) is indicated to the left of the blot. B, Means  $\pm$  SEM of  $PR_B$  band intensity were evaluated by image scanning in hfPSMC and expressed as percentage of control intensity taken as 100% in the indicated number of different experiments. \*,  $P < 0.05$  vs. control; °,  $P < 0.005$  vs.  $17\beta E_2 + Tam$ , Student's  $t$  test for paired data. A significant increase in  $PR_B$  expression was stimulated by  $17\beta$ -estradiol, whereas Tam completely blocked  $17\beta$ -estradiol stimulation.

matase activity over control ( $100 \pm 1\%$ ), which was totally blunted by the concomitant presence of  $1 \mu\text{mol/liter}$  letrozole ( $108 \pm 18\%$ ;  $P < 0.01$  vs. untreated cells at 48 h).

### Discussion

The present study demonstrates for the first time the presence of functional ER in human male external genitalia. In particular, this finding is relevant not only for understanding the physiology of the development of human male external genitalia, but also because it can explain the possible mechanism of action by which exposure to environmental toxicants with estrogenic activity interferes with this process in the male fetus. In fact, prenatal exposure to these factors has been demonstrated to alter the normal development of fetal sexual structures, and for these reasons these toxicants have been classified as endocrine disruptors (14). In particular, reduced production of androgens and exposure to molecules acting as antiandrogens are implicated in sex reversal of male sexual structures. However, in the last few years, the role of

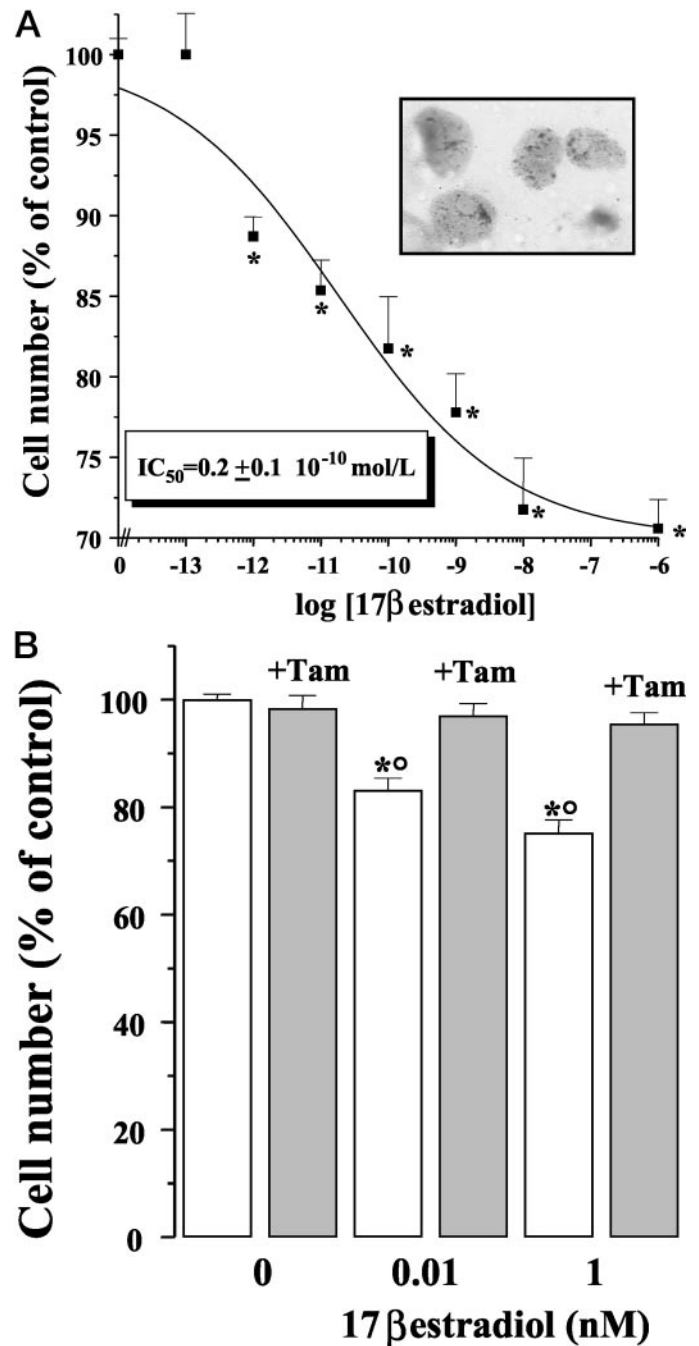
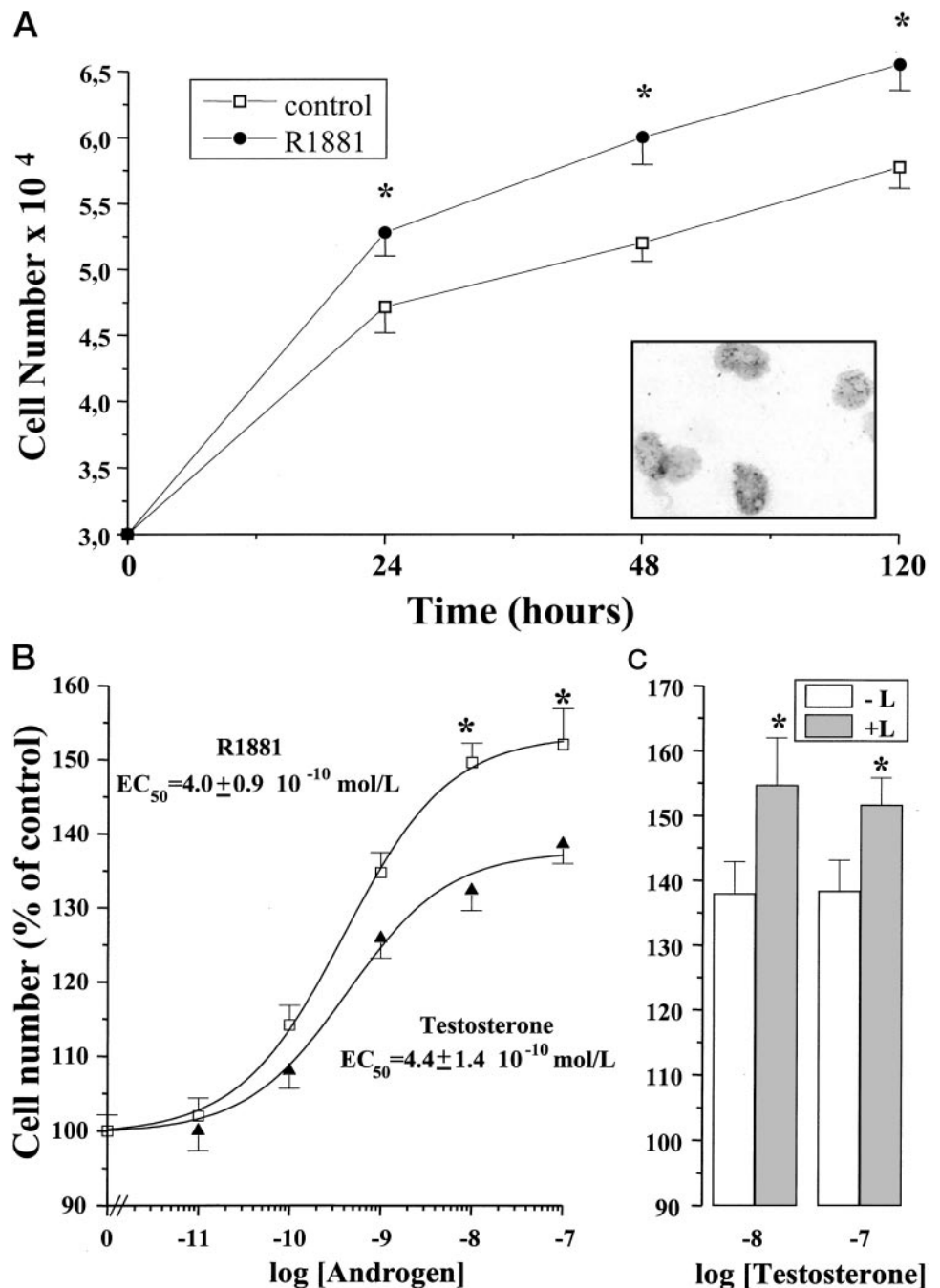


FIG. 7. Estrogens inhibit proliferation in hfPSMC. A, Twenty-four-hour incubation with increasing concentrations of  $17\beta$ -estradiol ( $10^{-13}$  to  $10^{-6}$  mol/liter) resulted in a statistically significant decrease in hfPSMC proliferation rate with  $IC_{50} = 0.2 \pm 0.1 \times 10^{-10}$  mol/liter. Ordinate, Cell number expressed as percentage of the control; abscissa, logarithmic scale of  $17\beta$ -estradiol molar concentrations. Data were expressed as the means  $\pm$  SEM values obtained in six different experiments. \*,  $P < 0.001$  vs. control (0 nM), Student's  $t$  test for paired data. Inset, Immunocytochemistry in hfPSMC shows positivity for ER in cell nuclei (magnification,  $\times 300$ ). B, The inhibitory effect of  $17\beta$ -estradiol on hfPSMC was counteracted by a fixed dose of the antiestrogen tamoxifen (+ Tam, 100 nmol/liter). Data were expressed as the means  $\pm$  SEM values obtained in three different experiments. \*,  $P < 0.01$  vs. control (0 nM); °,  $P < 0.005$  vs. Tam-treated. Student's  $t$  test for unpaired data.

FIG. 8. Androgens stimulate cell proliferation in hfPSMC: effects of letrozole. A, Time course of R1881-induced cell growth. The stimulatory effect of 1 nmol/liter R1881 on hfPSMC proliferation was statistically significant over different times of incubation (24, 48, and 120 h). Data were expressed as the means  $\pm$  SEM values obtained in three different experiments. \*,  $P < 0.05$  vs. respective time points on control curve (squares), Student's *t* test for paired data. *Inset*, Immunocytochemistry in hfPSMC shows positivity for AR in cell nuclei (magnification,  $\times 300$ ). B, Twenty-four-hour incubation with increasing concentrations of the synthetic androgen R1881 ( $10^{-11}$  to  $10^{-7}$  mol/liter; squares) and T ( $10^{-10}$  to  $10^{-7}$  mol/liter; triangles) resulted in an increase of hfPSMC proliferation rate with  $EC_{50} = 4.0 \pm 0.9 \times 10^{-10}$  mol/liter and  $4.4 \pm 1.4 \times 10^{-10}$  mol/liter, respectively. *Ordinate*, Cell number expressed as percentage of the control; *abscissa*, logarithmic scale of androgen molar concentrations. Data were expressed as the means  $\pm$  SEM values obtained in five different experiments. All points were statistically significant vs. respective controls (0), except for  $10^{-11}$  mol/liter, Student's *t* test for paired data. At high doses ( $10^{-8}$  and  $10^{-7}$  mol/liter), the effects between R1881 and T were statistically different as evaluated by ANOVA test. \*,  $P < 0.05$ . C, The simultaneous incubation of the hfPSMC cultures with the aromatase inhibitor letrozole (L; 300 nmol/liter) further increased T-induced cell proliferation to values comparable to the those obtained with the same doses of R1881 (panel B). Data were expressed as the means  $\pm$  SEM values obtained in two different experiments. Student's *t* test for paired data. \*,  $P < 0.05$  vs. respective controls without L.



estrogens in determining feminization of the developing male genital tract has been emerging, suggesting a model called Developmental Estrogenization Syndrome as the result of disruption of embryonal programming and gonadal development during fetal life (32), leading to the so-called Testicular Dysgenesis Syndrome characterized by morphological and functional abnormalities in both internal and external urogenital structures (33).

In the present study, we show the expression of ER and AR in fetal penile tissue and cells during the first trimester of pregnancy. These data, obtained by different experimental techniques, including immunohistochemistry, RT-PCR,

Western blot, and binding analysis are in contrast to those of Kallo *et al.* (34), who excluded ER positivity in male external genitalia on the basis of immunohistochemistry. Because that study (34) was conducted on fetuses older than 11–12 wk, a decrease of ER expression with age might account for the differences in ER positivity in male fetuses observed in the two studies. In contrast, distribution of AR in fetal penis and urethra, analogous to what we found, has been demonstrated recently in humans (6). Our immunohistochemical results clearly show that expression of both ER and AR is localized in the same structures of the developing male external genitalia, suggesting putative interactions between effects ex-



erted by the two steroid hormones. In particular, the positivity at the level of the forming male urethra suggests sensitivity of this structure to both hormones, thus supporting a possible explanation for the occurrence of hypospadias and related external genital defects when androgen-estrogen balance is disrupted during male fetal development. We also demonstrate that even ER are functionally active, because hfPSMC bind estrogen with high affinity and low capacity. Moreover, estrogens induce an increase in PR receptor level, a well known transcriptional target of estrogen (31).

More importantly, we demonstrate for the first time a direct effect of estrogen on the growth of fetal penile smooth muscle cells *in vitro*. Estrogens inhibit cell proliferation in a dose-dependent manner from  $10^{-12}$  mol/liter, whereas tamoxifen acts as an antiestrogen to reverse the estrogen effect. In contrast, androgens such as T and the nonaromatizable androgen R1881 stimulate cell proliferation, suggesting that the development of male urogenital structures results from a complex balance between estrogens and androgens. The intracellular signaling involved in mediating estrogen effects still has to be elucidated, although the involvement of a cAMP-adenosine pathway in estradiol inhibition of smooth muscle cell growth has been suggested (35). In the fetal circulation, estrogens may be derived from different sources, *i.e.* maternal, placental, and fetal tissues. However, only free estrogens can interact with ER. Normal buffering mechanisms consisting of high-affinity estrogen-binding proteins such as circulating  $\alpha$ -fetoprotein (36) and a Leydig-specific estrogen sulfotransferase (37, 38) control the concentration of free estradiol in male fetus, preventing estrogens from affecting fetal tissues that contain ER. However, many synthetic estrogens, such as DES, are effective at lower concentrations than endogenous estradiol, because they do not bind to these proteins (39). Studies on animal models defective for these binding proteins could help us in understanding the detrimental effects exerted by an increase in free estrogen and estrogenic compounds on the development of male genital structures. Moreover, a maternal vegetarian diet in pregnancy has been demonstrated to be associated with increased hypospadias in male sons, suggesting that phytoestrogens present in high levels in vegetarian diet could interfere with normal male genitalia development (40), probably through flavonoid inhibition of estrogen binding to  $\alpha$ -fetoprotein as demonstrated in a rat model (41). All of these findings suggest that endocrine disruptors with estrogenic activity interfere with normal development of external genitalia.

Recently, in the effort to elucidate the molecular mechanisms underlying cellular androgen-estrogen balance, a model based on the direct interaction between these two steroid hormones through formation of a complex between their activated receptors at the plasma membrane has been proposed (42). The effects exerted by these steroids have been called nongenomic, because they are atypically mediated by nuclear receptors associated at plasma membrane level (43).

The present study suggests that human penile cells are not only a target of estrogens, because they possess ER, but seem also to be a potential fetal source of estrogens. In fact, a specific aromatase inhibitor such as letrozole potentiates the stimulatory effects of T on cell proliferation, increasing the stimulated cell growth to the extent of the same doses of

the nonaromatizable androgen, R1881, suggesting that these cells might aromatize T to estrogen.

In conclusion, this study demonstrates that ER are present and functional in external genitalia of human male fetuses. Moreover, estrogen inhibition of cell growth, in contrast to the stimulatory effect of androgens, suggests that feminization of the external male genitalia due to prenatal exposure to abnormal levels of external estrogens may result from a direct effect of estrogens on cell growth. In fact, although estrogens have been demonstrated to act indirectly, reducing fetal androgen production by limiting growth of Leydig cells and inhibiting activity of steroidogenic enzymes involved in T synthesis (44), this is the first time that a direct effect on inhibition of growth of hfPSMC has been described.

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