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Estradiol Acts as a Germ Cell Survival Factor in the Human Testis *in Vitro**

VIRVE PENTIKÄINEN, KRISTA ERKKILÄ, LAURA SUOMALAINEN, MARTTI PARVINEN, AND LEO DUNKEL

Hospital for Children and Adolescents, University of Helsinki (V.P., K.E., L.S. L.D.), FIN-00029 Helsinki, Finland; and Department of Anatomy, University of Turku (M.P.), FIN-20520 Turku, Finland

ABSTRACT

The necessity of estrogens for male fertility was recently discovered in studies on both estrogen receptor α knockout and aromatase (*cyp 19* gene) knockout mice. However, direct testicular effects of estrogens in male reproduction have remained unclear. Here we studied the protein expression of ERα and the recently described estrogen receptor β in the human seminiferous epithelium and evaluated the role of 17β-estradiol, the main physiological estrogen, in male germ cell survival. Interestingly, both estrogen receptors α and β were found in early meiotic spermatocytes and elongating spermatids of the human testis. Furthermore, low concentrations of 17β-estradiol (10^{-9} and 10^{-10} mol/L) effectively inhibited male germ cell apoptosis, which was induced *in vitro* by incubating segments of human seminiferous tubules without survival factors (*i.e.* serum and hormones). Dihydrotestosterone, which, in addition to estradiol, is an end metabolite of testosterone, was also capable of inhibiting testicular apoptosis, but at a far higher concentration (10^{-7} mol/L) than estradiol. Thus, estradiol appears to be a potent germ cell survival factor in the human testis. The novel findings of the present study together with the previously reported indirect effects of estrogens on male germ cells indicate the importance of estrogens for the normal function of the testis. (*J Clin Endocrinol Metab* **85:** 2057–2067, 2000)

RECENT CONCERN over the potentially harmful effects of environmental estrogen-like chemicals on male fertility (1, 2) has aroused growing interest in understanding the physiological effects of estrogens in the male. Although estrogens have been regarded as female steroid hormones, they are now known to have profound effects on both female and male reproductive systems. In males, estrogens are synthesized mainly in the testis, where they are formed from testosterone by the enzyme P450 aromatase. In the rat, there is an age-related change in the aromatization site from Sertoli cells in the immature testis to Leydig cells in the adult testis (3, 4). In various other species, including humans, P450 aromatase has been found to be present in the Leydig cells of the adult testis (5–9). In addition, in the mouse (6), rat (7), brown bear (9), and rooster (10), aromatase has been found in meiotic and postmeiotic germ cells of the testis, predominantly in spermatids.

Estrogens exert their cellular effects through estrogen receptors (ER) that exist in at least two subtypes, ERα (11, 12) and the recently described ERβ (13, 14), which differ in the C-terminal ligand-binding domain and in the N-terminal *trans*-activation domain. These two subtypes of ER have similar high affinities for 17β-estradiol, but some synthetic and naturally occurring ligands have different relative affinities for ERα and ERβ (15). In the male reproductive tract, ERα has been shown to be strongly expressed in the epididymis and efferent ductules (16–20). It has also been found in the Leydig cells of the rat testis (16), whereas the seminiferous epithelium has been thought to be negative for ERα. ERβ messenger ribonucleic acid (mRNA), in turn, has been described in germ cells, particularly in primary spermatocytes and round spermatids, of the human testis (21). In addition, ERβ mRNA and protein have been shown to be expressed by Leydig cells and elongated spermatids of the mouse testis (22) as well as by Sertoli cells and type A spermatogonia of the developing rat testis and by Sertoli cells, pachytene spermatocytes and round spermatids of the adult rat testis (23, 24). Thus, the expression patterns of ERs seem to be rather complicated and to show at least some species variation. Moreover, a recent study revealed a novel functional estrogen receptor on the human sperm membrane that is clearly smaller than the ERα and ERβ (25). The physiological significance of this ER remains to be elucidated.

Recent investigations of mice deficient in ERα (ERα knockout, ERαKO) (26, 27) or aromatase (*cyp 19* gene knockout, ArKO) (28) have provided direct evidence for a physiological role of estrogens in male reproductive organs. The ERαKO males were infertile (26). Their testes appeared normal until puberty, but then began to degenerate, with disruption of spermatogenesis (26). The infertility was suggested to be caused by impaired fluid reabsorption in the efferent ductules, resulting in diluted sperm, increased back-pressure in the seminiferous tubules, and related atrophy of the seminiferous epithelium (27). Thus, estrogens have been thought to have only an indirect effect on the developing germ cells of the seminiferous epithelium. However, ArKO mice were also shown to possess progressive disruption of spermatogenesis and infertility, but there was no evidence of abnormal fluid reabsorption by the efferent ductules (28). These results

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Address all correspondence and requests for reprints to: Virve Pentikäinen, M.D., Hospital for Children and Adolescents, University of Helsinki, P.O. Box 281, FIN-00029 Helsinki, Finland. E-mail: virve.pentikainen@hus.fi.

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from ArKO mice and the recently reported expression of ERβ in the human (21), mouse (22), and rat (23, 24) seminiferous epithelium suggest additional direct effects of estrogens on spermatogenesis.

Spermatogenesis is a unique process of germ cell proliferation and maturation from diploid spermatogonia through meiosis to mature haploid spermatozoa. Before reaching maturity, a number of germ cells undergo physiological apoptotic death, which has been shown to be controlled by FSH and testosterone (reviewed in Ref. 29). In this context, we have recently shown that testosterone inhibits apoptosis of human testicular germ cells *in vitro* (30). The role of estrogens, however, has been completely unknown. The aim of the present study was to evaluate the direct effects of the main physiological estrogen, 17β-estradiol, on germ cell apoptosis in the human testis. As no reports concerning the protein expression of ERα and ERβ in the human testis were available, we first used immunohistochemistry and Western blotting to study the expression of these receptors in the adult human testis. We then induced germ cell apoptosis in our recently described *in vitro* model (30) and studied the effects of 17β-estradiol and dihydrotestosterone (DHT), the two end metabolites of testosterone, on the process of cell death.

Subjects and Methods

Patients

Testis tissue was obtained from men, aged 60–80 yr, undergoing orchidectomy as treatment for prostate cancer. They had not received hormonal or chemotherapeutic medication or radiotherapeutic treatment for the cancer before the operation. They had no endocrinological disease, and none of them had suffered from cryptorchidism. The operations were performed between November 1996 and December 1999 at the Department of Urology, University of Helsinki, and at the Helsinki City Health Department, Surgical Unit (Helsinki, Finland). The ethics committees of the Hospital for Children and Adolescents and the Department of Urology, University of Helsinki, approved the study protocol.

Immunohistochemical staining of ERα and ERβ

Small segments of human seminiferous tubules (~1 mm in length) were squashed under coverslips and fixed as previously described (31). These squash preparations were rehydrated, washed twice for 5 min each time in phosphate-buffered saline (PBS), and blocked with blocking solution (PBS containing 5% normal serum, 3% BSA, and 0.1% Tween 20) for at least 1 h at room temperature. In our preliminary studies we found that the negative controls, in which the primary antibody was replaced with PBS, stayed negative regardless of whether the endogenous peroxidases were blocked in methanol containing 1% H_2O_2. Therefore, it appears that in the human testis, endogenous peroxidases are not present in amounts that would affect the immunohistochemical staining of the ERs. The ERα protein in the preparations was detected using a rabbit polyclonal antibody to human ERα (HC-20, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 0.2 μg/mL or mouse monoclonal antibodies to human ERα (NCL-ER-6F11, Novocastra Laboratories Ltd., Newcastle, UK; or ER-1D5, DAKO Corp. A/S, Glostrup, Denmark) at dilutions of 1:10 and 1:100, respectively. The ERβ protein was detected using rabbit polyclonal antibody to human ERβ (PAI-313, Affinity BioReagents, Inc., Golden, CO) at 10 μg/mL. The primary antibodies were added to the preparations in blocking solution, and incubation was performed overnight at 4 C. After incubation, the slides were washed three times for 5 min each time in PBS. The primary antibody was detected using biotin-conjugated goat antirabbit IgG or horse antimouse IgG secondary antibodies from the corresponding ABC-Elite Kits (Vector Laboratories, Inc., Burlingame, CA) followed by incubation with ABC solution. For location of the antibody, 0.05% diaminobenzidine substrate (Sigma, St. Louis, MO) was added. Light counterstaining was performed with hematoxylin. A blocking peptide was used to verify the specificity of the polyclonal antibody to ERα.

Western blot analysis

Small tissue sections were homogenized on ice in homogenization buffer [1% Triton X-100, 150 mmol/L NaCl, 10 mmol/L Tris (pH 7.4), 1 mmol/L ethylenediamine tetraacetate, 1 mmol/L ethyleneglycol-bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, 0.2 mmol/L phenylmethylsulfonylfluoride, and 1 μg/mL leupeptin]. After centrifugation at 17,000 × *g* for 30 min, the supernatants were collected, and their protein concentrations were determined by DC protein assay (Bio-Rad Laboratories, Inc., Hercules, CA). Proteins (25–50 μg) were loaded onto SDS-polyacrylamide gels, and electrophoresis was performed at 180 V. The proteins were transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore Corp., Bedford, MA) by electrophoresis for 2 h at 4 C in transfer buffer (26 mmol/L Tris, 192 mmol/L glycine, and 10% methanol) at 100 V. The transfer was checked by staining with 0.2% Ponceau S in 3% trichloroacetic acid. ERα and ERβ proteins on the membranes were detected using rabbit polyclonal antibodies to human ERs. The ERα antibodies HC-20 (Santa Cruz Biotechnology, Inc.) and H-184 (Santa Cruz Biotechnology, Inc.) recognize amino acids 576–595 at the carboxyl-terminus and 2–185 at the amino-terminus of human ERα, respectively, and they were used at 0.1 and 0.4 μg/mL. The ERβ antibody PAI-313 (Affinity BioReagents, Inc.) was used at 1 μg/mL. The primary antibodies were followed by horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). The bound secondary antibody was located with the ECL detection kit (Amersham Pharmacia Biotech, Arlington Heights, IL). The specificity of the HC-20 antibody to ERα was confirmed using the corresponding blocking peptide.

Tissue culture

Apoptosis of the human testicular germ cells was induced *in vitro* by incubating segments of seminiferous tubules under serum- and hormone-free culture conditions (*i.e.* without survival factors). Segments of seminiferous tubules were cultured, instead of isolated germ cells, to maintain an environment as physiological as possible for the germ cells. Testis tissue was microdissected under a transillumination stereomicroscope in a petri dish containing PBS supplemented with 0.1% BSA (Sigma). Segments of seminiferous tubules (~3 mm in length) were isolated and transferred to culture plates. Each plate contained tissue culture medium (nutrient mixture Ham's F-10, Life Technologies, Inc., Europe, Paisley, UK) supplemented with 0.1% human albumin (Sigma) and 10 μg/mL gentamicin (Life Technologies, Inc.). The samples were incubated for 2–24 h under serum- and hormone-free conditions (*i.e.* without survival factors) at 34 C in a humidified atmosphere containing 5% CO_2.

Inhibition of germ cell apoptosis in the testis tissue culture

To study the effects of estrogens on male germ cell apoptosis, 17β-estradiol (Sigma) was added to the tissue cultures at final concentrations of 10^{-7}, 10^{-9}, and 10^{-10} mol/L (in preliminary studies, estradiol concentrations from 10^{-6}–10^{-10} mol/L were tested). To test whether the effects of 17β-estradiol were mediated by the ERs, an ER antagonist, ICI 182,780 (Tocris Cookson Ltd., Bristol, UK), was added at 10^{-7} mol/L simultaneously with estradiol. The effects of DHT on germ cell death were also studied by adding DHT (5α-androstan-17β-ol-3-one, Fluka Chemie Ag, Buchs, Switzerland) to the culture medium at final concentrations of 10^{-7}, 10^{-8}, and 10^{-9} mol/L. Apoptosis was detected by Southern blot analysis of low molecular weight DNA fragmentation, by *in situ* end labeling (ISEL) analysis of apoptotic cells in the squash preparations of seminiferous tubules, and by electron microscopy using the established morphological criteria of apoptosis.

Southern blot analysis of apoptotic DNA fragmentation

Tissue samples were snap-frozen in liquid nitrogen and stored at −70 C until DNA isolation. Genomic DNA was extracted using the Apoptotic DNA Ladder Kit (Roche Molecular Biochemicals, Mannheim, Germany)

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according to the manufacturer's instructions, with some modifications. Briefly, the testis tissue samples were homogenized and incubated for 10 min at room temperature in binding/lysis buffer (6 mol/L guanidine-HCl, 10 mmol/L urea, 10 mmol/L Tris-HCl, and 20% TritonX-100, pH 4.4). The samples were then mixed with isopropanol (final proportion of isopropanol, 25%), loaded into polypropylene tubes, and centrifuged for 1 min at 8000 rpm. The tubes were washed twice with washing buffer (20 mmol/L NaCl and 2 mmol/L Tris-HCl, pH 7.5), and the bound DNA was eluted from the tubes with 10 mmol/L Tris, pH 8.5. Finally, the samples were incubated with ribonuclease (2.5 μg/mL; deoxyribonuclease-free ribonuclease, Roche Molecular Biochemicals) for 20 min at room temperature. After quantification, the DNA samples were 3′-end labeled with digoxigenin-dideoxy-UTP (Dig-dd-UTP; Roche Molecular Biochemicals) by the terminal transferase (Roche Molecular Biochemicals) reaction, subjected to electrophoresis on 2% agarose gels, and blotted onto nylon membranes overnight. The next day the DNA was cross-linked to the membranes by UV irradiation. The membranes were then washed and blocked for 30 min at room temperature. Apoptotic, 3′-end labeled, DNA on the membranes was detected by the antibody reaction (anti-Digoxigenin-AP, alkaline phosphatase-conjugated; Roche Molecular Biochemicals), as recently described (30). For the luminescence reaction, the membranes were incubated in disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl)phenyl phosphate solution (Roche Molecular Biochemicals) for 5 min at room temperature. The membranes were then enclosed in hybridization bags, incubated for 15 min at 37 C, and exposed to x-ray films. The films were scanned with a tabletop scanner (Microtek ScanMaker, Microtec International, Inc., Taiwan), and the digital image was analyzed with NIH-Image (1.61) analysis software. The digitized quantification of the low molecular weight DNA fragments (185-bp multiples) of the sample cultured for 4 h without survival factors was set at 1.0 (100% apoptosis), and the amounts of low molecular weight DNA fragments in the other samples were expressed in relation to it.

ISEL of apoptotic DNA

Squash preparations from human seminiferous tubules were rehydrated and washed twice for 5 min each time in distilled water. After incubation for 10 min with terminal transferase reaction buffer (1 mol/L potassium cacodylate, 125 mmol/L Tris-HCl, and 1.25 mg/mL BSA, pH 6.6), the apoptotic DNA was 3′-end labeled with Dig-dd-UTP (Roche Molecular Biochemicals) by the terminal transferase reaction for 1 h at 37 C. For the negative controls, the terminal transferase enzyme was replaced with the same volume of distilled water. Dig-dd-UTP was detected with the antidigoxigenin antibody conjugated to horseradish peroxidase (Anti-Digoxigenin-POD, Roche Molecular Biochemicals). For location of the antibody, 0.05% diaminobenzidine substrate (Sigma) was added. Light counterstaining was performed with hematoxylin, and the samples were dehydrated and mounted.

Electron microscopy

Segments of seminiferous tubules were cultured as described above. They were then fixed in 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer, dehydrated, and embedded in epoxy resin. The samples were sectioned at 50 nm with an E Ultramicrotome (Reichert Jung, Vienna, Austria) and stained with uranyl acetate and lead citrate. Observations were made with a JEM 1200 EX transmission electron microscope (JEOL USA, Inc., Tokyo, Japan). Germ cells were identified according to their characteristic morphology (32). Apoptosis was recognized by typical ultrastructural changes, including condensation of nuclear chromatin and degeneration of cytoplasmic organelles.

Statistical analysis

The experiments for Southern blot analysis of DNA fragmentation were repeated on at least three independent occasions. Quantitative data represent low mol wt DNA (optical density from x-ray films). The data obtained from the samples incubated for 4 h without survival factors were set at 1.0 (100% apoptosis), and the data from the samples treated with 17β-estradiol or DHT were compared to it. Data obtained from three to seven replicate experiments (mean ± SEM) were analyzed by one-sample t test. $P < 0.05$ was considered significant.

Results

Expression of ERα and ERβ in the adult human seminiferous epithelium

Localization of the ERα and ERβ proteins in the adult human testis was studied immunohistochemically using a rabbit polyclonal antibody (HC-20) and mouse monoclonal antibodies (ER-6F11 and ER-1D5) to human ERα and a rabbit polyclonal antibody to human ERβ. Representative samples of cells were obtained from human seminiferous epithelium by squashing segments of the seminiferous tubules under coverslips. With this technique, cells from the seminiferous epithelium migrate under the coverslip to produce a monolayer. The cells maintain their morphological characteristics, allowing identification of individual cell types.

Strong positive staining for the ERα protein was observed in early meiotic germ cells (zygotene and early pachytene primary spermatocytes) and in early elongating spermatids (Fig. 1A). Whether we used the polyclonal (HC-20) or the monoclonal (ER-6F11 or ER-1D5) antibodies to ERα, the result was the same. The specificity of the staining with the HC-20 antibody was confirmed by incubating the primary antibody with an excess of the corresponding peptide before use. When this blocking peptide was used, there was only faint background staining, which was not located in the cells. There was no staining when the primary antibodies were replaced with PBS (negative controls).

The ERβ protein was strongly expressed by the same types of germ cells (Fig. 1B) as the ERα. The strong positive staining of the early primary spermatocytes and the early elongating spermatids for the ERβ protein accords with the previously reported expression of ERβ mRNA in primary spermatocytes and spermatids of the human testis. No staining was observed in the negative controls, *i.e.* when the primary antibody was replaced with an equal volume of PBS.

Western blot analysis of human seminiferous tubules showed strong expression of both ERα and ERβ (Fig. 2). Both polyclonal ERα antibodies HC-20 and H-184 recognized a band corresponding to a molecular mass of about 80 kDa, which is clearly higher than the usually reported molecular mass of human ERα (66–70 kDa). The 80-kDa band was also detected in the samples of human ovary and endo/myometrium with both antibodies, although, with H-184, extended ECL exposure time was needed to detect the band in endo/myometrium. With HC-20, we observed two additional faint bands of about 50–55 kDa in both the male and female tissue samples. The 80-kDa band and the upper of the additional bands disappeared when the HC-20 antibody was incubated with the corresponding blocking peptide before use, indicating specificity of these bands. With H-184, in turn, an additional faint band of approximately 60 kDa was detected in all of the male and female tissue samples studied. To further confirm the specificity of the polyclonal antibodies to ERα, we performed the Blast sequence similarity search with the peptide sequences corresponding to HC-20 and H-184. The only proteins for which similarity with these sequences was found were ERα with its different splice variants. Thus, in Western blot analysis of human seminiferous tubules, ovary, and endo/myometrium, we observed a band repre-

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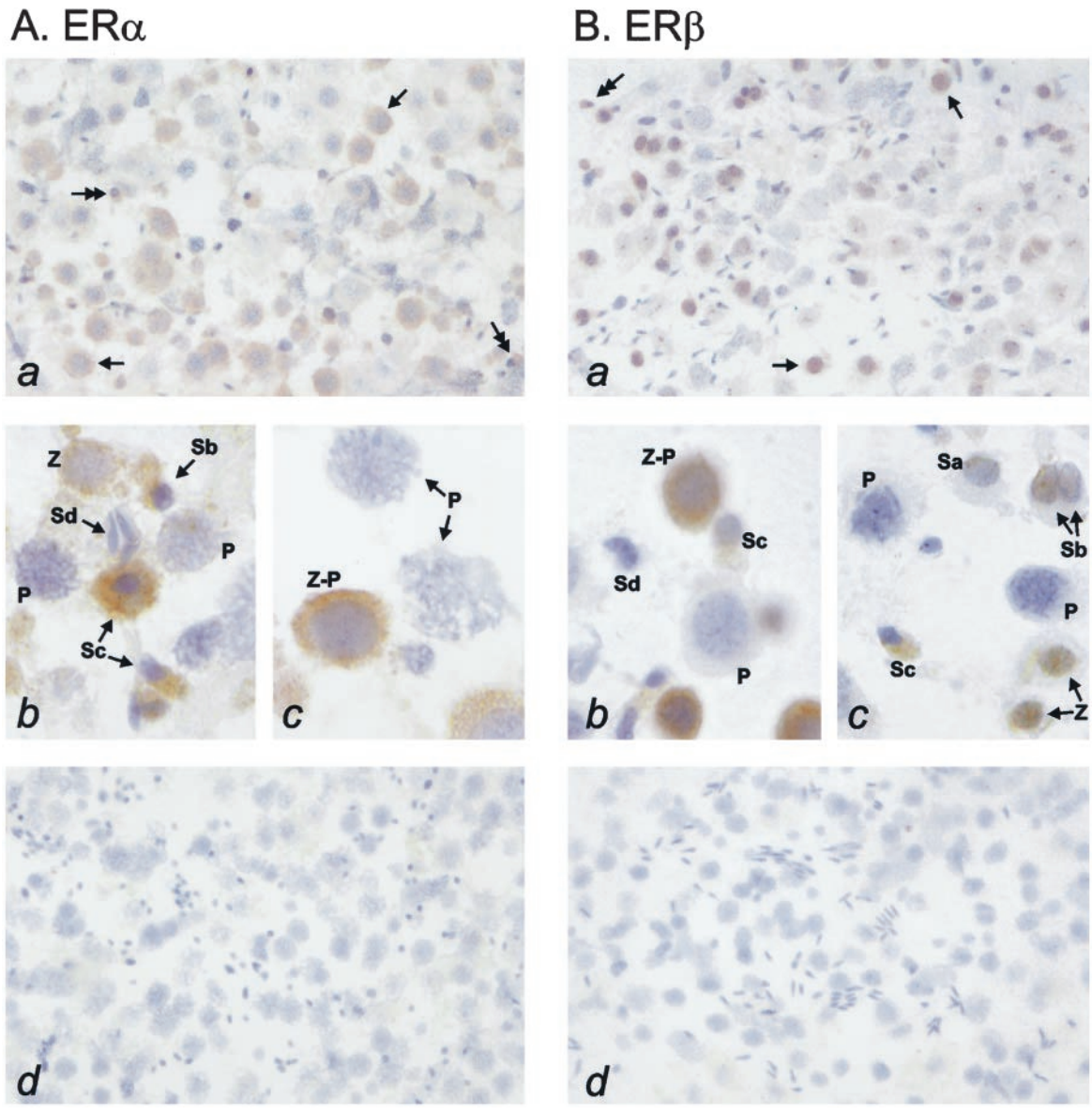

FIG. 1. Localization of ERα (A) and ERβ (B) in the seminiferous epithelium of the adult human testis. Segments of seminiferous tubules were squashed and analyzed immunohistochemically as described in *Subjects and Methods*. A(a), Strong positive staining for ERα was observed with the HC-20 antibody in early meiotic germ cells, namely zygotene and early pachytene primary spermatocytes (*arrow*), and in early elongating spermatids (*double arrow*); original magnification, ×20. A(b and c), Higher magnification (×100) in which the structure of the nuclear chromatin can be seen and the individual germ cells reliably identified. A(d), No specific staining was observed when the HC-20 antibody was incubated with the corresponding blocking peptide before use; original magnification, ×20. B(a), The ERβ protein was also found to be strongly expressed in the early meiotic germ cells (*arrow*) and in the elongating spermatids (*double arrow*); original magnification, ×20. B(b and c), ×100 magnification showing individual germ cells. B(d), Negative control, in which the primary antibody was replaced with PBS; ×20 magnification. Z, Zygotene spermatocyte; Z-P, zygotene/very early pachytene spermatocyte; P, pachytene spermatocyte; Sa, round spermatid; Sb and Sc, early elongating spermatids; Sd, late elongating spermatid.

senting the ERα protein and migrating at a mobility of 80 kDa. The other specific bands observed may represent different splice variants of the ERα. Western blot analysis of the same tissues using the polyclonal antibody to ERβ revealed a protein of approximately 60 kDa, corresponding to the size of human ERβ. The expression of both receptors remained constant during the culture of the seminiferous tubules in serum- and hormone-free conditions for various lengths of time.

In vitro induction of human testicular apoptosis

In the present *in vitro* model, human germ cells were cultured in their natural surroundings, *i.e.* in the seminiferous tubules, to maintain as physiological an environment as possible. Germ cell apoptosis was induced in this model by incubating segments of seminiferous tubules under serum- and hormone-free conditions (*i.e.* without survival factors). Apoptotic cells were identified by electron microscopy, using the characteristic morphology of the different germ cell types

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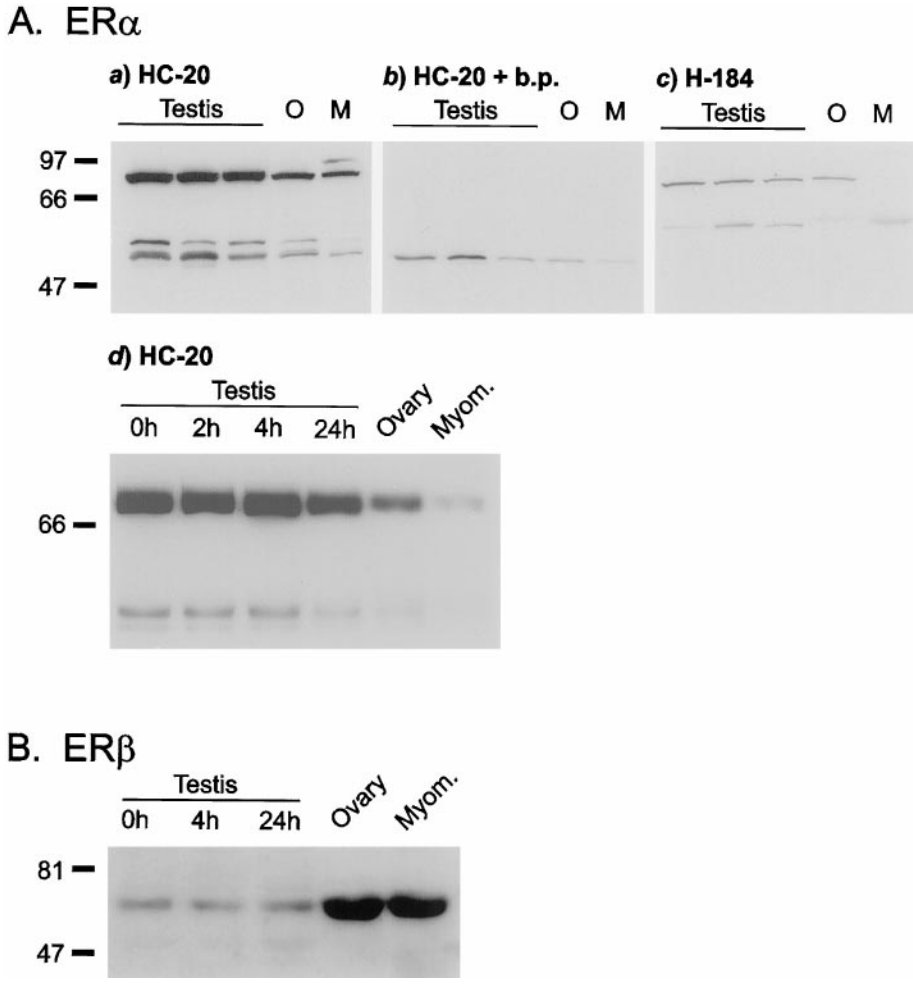

FIG. 2. Western blotting of the ER expressed in the human seminiferous tubules. Extracts of seminiferous tubules cultured for 0, 2, 4, and 24 h under serum- and hormone-free conditions were subjected to electrophoresis on SDS-PAGE gels, blotted on polyvinylidene difluoride membranes, and analyzed with rabbit polyclonal antibodies to human ERα (HC-20 or H-184, Santa Cruz Biotechnology, Inc.) or human ERβ (PAI-313, Affinity BioReagents, Inc.) as described in *Subjects and Methods*. A(a), Expression of the ERα protein was observed in human seminiferous tubules (samples from three men), ovary, and myometrium. The HC-20 antibody (specific for the carboxyl-terminus of the ERα) recognized a specific band corresponding to a molecular mass of about 80 kDa. In addition, two bands approximately 50–55 kDa were detected. A(b), The specificity of the 80-kDa band and the upper of the additional bands was confirmed using a blocking peptide (b.p.) to HC-20. A(c), With the H-184 antibody (specific for the amino-terminus of the ERα), the same 80-kDa band and an additional band approximately 60 kDa were detected. A(d), The expression of ERα remained constant in the samples of seminiferous tubules cultured for increasing lengths of time. B, In the Western blot analysis of ERβ, a clear band approximately 60 kDa was observed. The expression remained constant when the samples were cultured for increasing lengths of time. Strong expression of the ERβ was also seen in the human ovary and myometrium used as controls. Numbers on the *left* correspond to molecular masses (kilodaltons) of marker proteins. The experiments were repeated at least three times.

(32) and the established morphological criteria of apoptosis. Typical signs of the apoptotic cells were, for example, condensation of nuclear chromatin, degeneration of cytoplasmic organelles, and, in the later stages of apoptosis, dispersion of the nuclear envelope. The morphological signs of apoptosis were seen most often in spermatocytes and spermatids. Some of the apoptotic spermatids showed a ring-like condensation of chromatin around the nuclear periphery, which is characteristic for apoptosis of this type of germ cell. Late apoptotic cells were impossible to identify.

Inhibition of human testicular apoptosis by 17β-estradiol

To evaluate the direct effects of estrogens on male germ cells, we studied the role of the natural estrogen, 17β-estradiol, in germ cell apoptosis in the present *in vitro* model. Interestingly, 17β-estradiol effectively inhibited the germ cell apoptosis that was induced *in vitro* by withdrawal of serum and hormones (Fig. 3). The most effective concentrations were 10^{-10} and 10^{-9} mol/L, which are in range of the previously reported physiological estradiol concentrations in human spermatic vein and testis tissue. In Southern blot analysis, the total amount of apoptotic low molecular mass DNA fragmentation was suppressed by 47% ($P < 0.001$) and 41% ($P = 0.003$) at estradiol concentrations of 10^{-10} and 10^{-9} mol/L, respectively. Estradiol concentration of 10^{-7} mol/L did not significantly inhibit germ cell death. The suppressive effect of estradiol on germ cell apoptosis was blocked by the ER antagonist ICI 182,780 at 10^{-7} mol/L. This result was obtained from three independent experiments.

To confirm the results of Southern blot analysis and to

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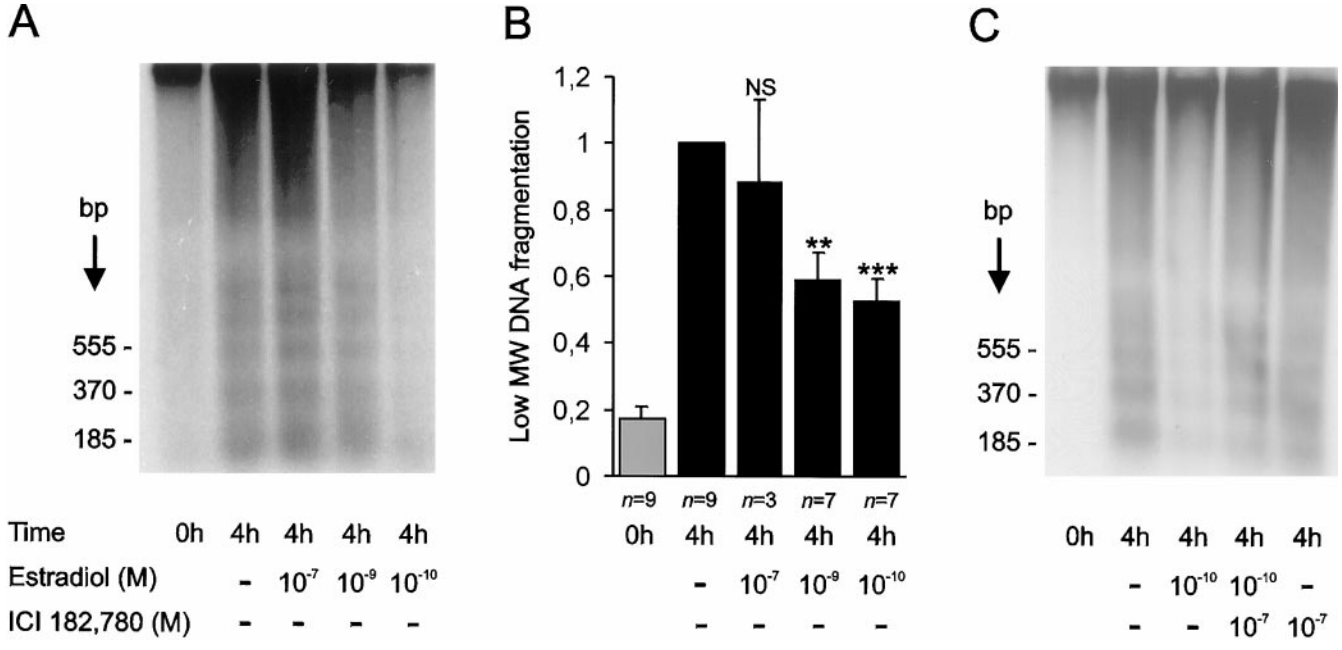

FIG. 3. Inhibition of *in vitro* induced human testicular apoptosis by 17β-estradiol. Segments of seminiferous tubules were incubated for 4 h under serum- and hormone-free conditions in the presence or absence of 17β-estradiol. DNA from the seminiferous tubules was extracted, 3′-end labeled with Dig-dd-UTP, and subjected to electrophoresis on 2% agarose gels. The labeled DNA was detected with disodium 3-(4-methoxy-spiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.$1^{3,7}$]decan}-4-yl)phenyl phosphate as described in *Subjects and Methods*. Immediately after the operation (0 h), the DNA samples showed no apoptotic ladder pattern in Southern blot analysis. In contrast, clear apoptotic DNA fragmentation was observed in samples cultured for 4 h without survival factors in the absence of estradiol. This apoptosis was effectively inhibited by 17β-estradiol. A, X-Ray of a gel from a representative experiment in which 17β-estradiol was added to the culture medium at concentrations of 10^{-7}, 10^{-9}, and 10^{-10} mol/L. B, Low molecular weight (MW) DNA (<1.3 kb) quantification showing the effective, concentration-dependent inhibition of testicular apoptosis by 17β-estradiol. The physiological (10^{-10} mol/L) concentration of estradiol appeared to be the most effective in suppressing apoptotic DNA fragmentation. Each value represents the mean of independent experiments (number of experiments indicated as *n*) ± SEM. ***, $P < 0.001$; **, $P < 0.01$. C, The ER antagonist ICI 182,780 blocked the antiapoptotic effect of 17β-estradiol. The experiment was repeated three independent times.

obtain information on the morphology of the germ cells treated with estradiol, we performed ISEL analysis of squash preparations from human seminiferous tubules taken immediately after the operation (0 h) or cultured in serum-free conditions in the presence or absence of 17β-estradiol. The inhibitory effect of estradiol on germ cell apoptosis was evident. In the samples taken immediately after the operation, only a few apoptotic cells were observed (Fig. 4A), whereas in the samples cultured for 4 h in the absence of serum and hormones, their amount was greatly increased (Fig. 4B). As in the electron microscopy, the apoptotic cells were most often identified as spermatocytes and spermatids. When 17β-estradiol was added to the culture medium, the number of positively staining cells was effectively reduced (Fig. 4C). When the terminal transferase enzyme was replaced with distilled water, there was no staining (negative control, data not shown).

Effect of DHT on in vitro-induced apoptosis of human testicular germ cells

In addition to 17β-estradiol, testosterone can be metabolized *in vivo* to DHT. Therefore, we further studied the effects of this other end metabolite of testosterone on the *in vitro* induced apoptotic death of testicular germ cells. In the present *in vitro* model, DHT was also capable of inhibiting testicular apoptosis (Fig. 5). However, the lowest concentrations of DHT needed for effective inhibition of germ cell death were 100-1000 times the effective concentrations of 17β-estradiol, indicating the relatively high potency of estrogens as germ cell survival factors compared with that of androgens. Apoptotic low molecular mass DNA fragmentation was suppressed by 38% at DHT concentration of 10^{-7} mol/L ($P < 0.05$). Lower concentrations of DHT did not significantly inhibit germ cell death.

Discussion

Estrogens have recently been shown to be essential for male reproduction (26, 28), but the mechanisms of estrogen action in the male have remained enigmatic. In the present study we demonstrate that in addition to the previously suggested indirect effects of estrogens on the seminiferous epithelium, estrogens may contribute directly to the survival of germ cells in the testis.

We observed strong expression of both the ERα and the ERβ proteins in the developing germ cells of the human testis. Expression of ERα in the testicular germ cells is a novel finding. Previous studies have shown ERα expression in the epididymis and the efferent ductules of various species (16–20) as well as in the Leydig cells of the rat testis (16). Testicular germ cells, however, have not been shown to express ERα in any species, and therefore, direct ERα-mediated effects of estrogens on spermatogenesis have not been sug-

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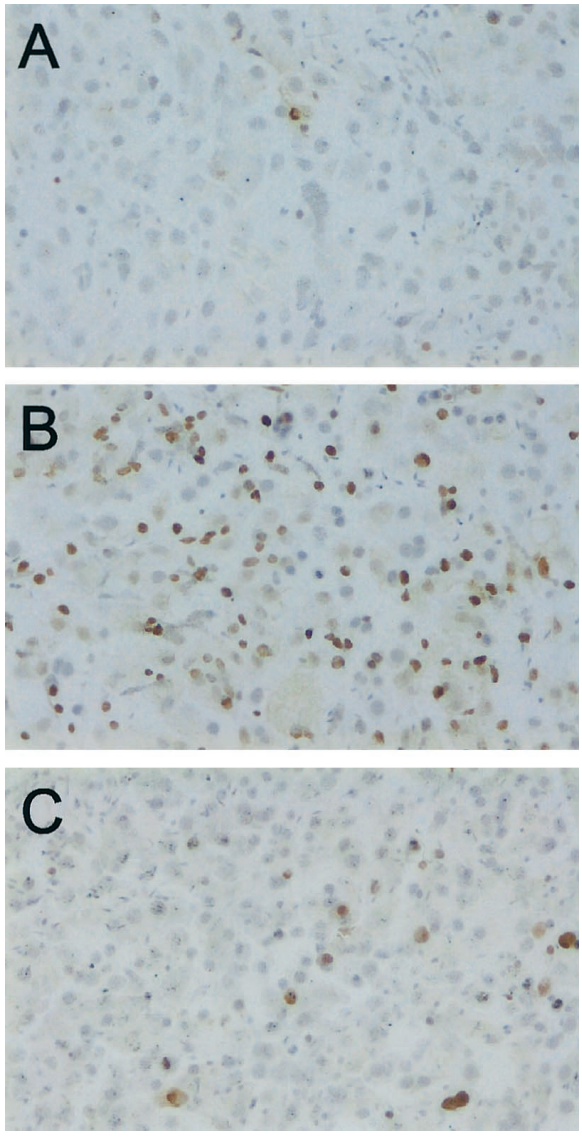

FIG. 4. ISEL analysis of the estradiol-mediated inhibition of *in vitro* induced apoptosis of testicular germ cells. Segments of seminiferous tubules were incubated under serum- and hormone-free culture conditions in the absence or presence of 17β-estradiol. After incubation, the tubules were squashed and fixed, and apoptotic cells were detected by *in situ* 3′-end labeling of apoptotic DNA, as described in *Subjects and Methods*. A, Only a few apoptotic cells were observed in the samples squashed immediately after the operation (0 h). B, The number of apoptotic cells was greatly increased in the samples cultured for 4 h in the absence of serum and hormones. C, Germ cell apoptosis was effectively inhibited when 17β-estradiol at 10^{-10} mol/L was added to the culture medium.

gested. In the present study the cells staining positively for ERα by immunohistochemistry were identified mainly among early meiotic germ cells in stages VI and I to II of the human seminiferous epithelium cycle (zygotene and early pachytene primary spermatocytes) and as elongating spermatids in stages IV to V of the cycle (Fig. 6). When the seminiferous tubules were cultured in serum-free conditions for increasing lengths of time, the expression remained constant regardless of the amount of germ cell apoptosis. The expression pattern of the ERβ protein in the human seminiferous epithelium was similar to that of the ERα protein (Fig. 6). Although the ERβ mRNA has recently been found in the germ cells of the human testis (21), this is the first report confirming the expression of ERβ in the human testis at the protein level.

The functional role of estrogens in the human testis was then studied in our recently established *in vitro* model (30). In this model, germ cells of the human testis were induced to undergo apoptosis by incubating segments of seminiferous tubules under serum- and hormone-free culture conditions (*i.e.* without survival factors). The apoptotic cells were identified as spermatocytes and spermatids. Interestingly, this *in vitro* induced apoptosis of male germ cells was effectively inhibited when the natural estrogen, 17β-estradiol, was added to the culture medium. As only seminiferous tubules and some occasional interstitial Leydig cells, but not other parts of the testis, are present in our culture model, this effect of estradiol must be a direct effect on either the cells of the seminiferous tubules or the Leydig cells. Although estrogens have a well documented inhibitory effect on Leydig cell androgen secretion and RNA synthesis (33–39), the concentrations of estradiol needed for *in vitro* inhibition of Leydig cell function in the previous studies (36–39) were at least 1000-fold the concentrations capable of effectively inhibiting germ cell apoptosis in the present study. Therefore, the possible inhibitory effect of these low apoptosis-inhibiting concentrations of estradiol on the occasional Leydig cells present in our culture model and consequent indirect effects of estradiol on germ cell survival seem very unlikely. Moreover, as both subtypes of ER are expressed in germ cells of the types that can be induced to undergo apoptosis, and as this apoptosis can be blocked by 17β-estradiol, we infer that estrogens are directly involved in the regulation of male germ cell survival.

The suppressive effect of 17β-estradiol on germ cell apoptosis was blocked by an ER antagonist, ICI 182,780, indicating that estradiol was functioning through its receptors. The intracellular mechanisms of ER action in the present study and the possible involvement of both ERα and ERβ in male germ cell survival are not known. The classic ER signaling pathway involves binding of the ligand-bound ER (either α or β) to the estrogen-responsive element that regulates transcription of target genes. However, ERs may also mediate gene transcription by binding to an activating protein-1 (AP1) element together with the transcription factors Fos and Jun. ERα and ERβ have been shown to signal in opposite ways from the AP1 site; when bound to 17β-estradiol, ERα activates and ERβ inhibits transcription (40). In contrast to the natural estrogen, antiestrogens, including tamoxifen, raloxifene, and ICI 164,384, have been shown to effectively activate transcription from an AP1 site when bound to ERβ (40). The effects of AP1-mediated regulation of transcription on germ cell death are not known. Thus, the possible involvement of AP1 activation in estrogen prevention of germ cell apoptosis remains unknown. Moreover, recent studies have revealed a novel nongenomic signaling pathway through ERs located on the plasma membrane (reviewed in Ref. 41). Binding of 17β-estradiol to the membrane receptors results in rapid (within minutes) activation of the mitogen-activated protein kinase signaling cascade. In en-

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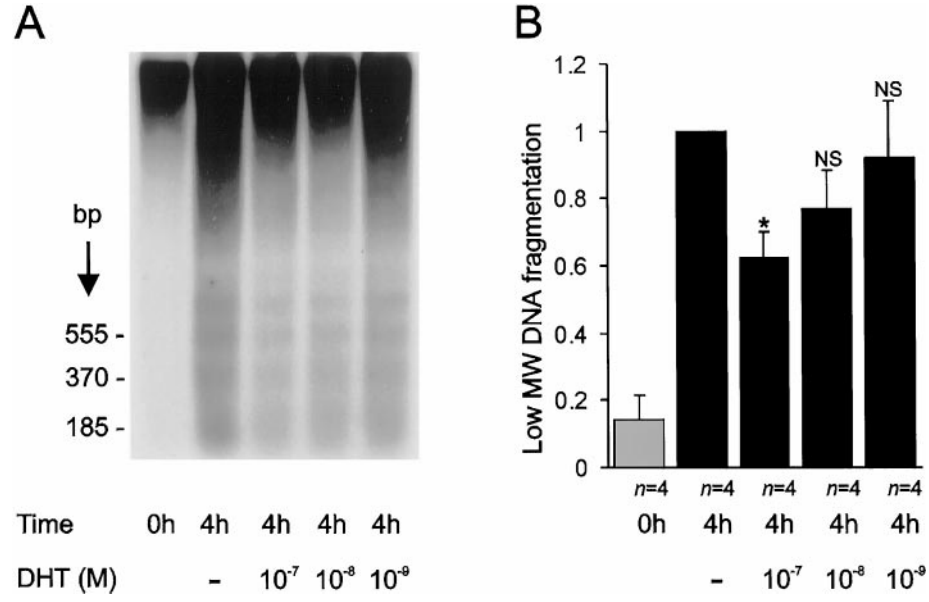

FIG. 5. Effect of DHT on *in vitro* induced apoptosis of human testicular germ cells. Segments of seminiferous tubules were incubated under serum- and hormone-free conditions in the presence or absence of DHT. Southern blot analysis of apoptotic DNA was performed as described in Fig. 4. A, Inhibition of apoptotic DNA laddering by DHT. B, Quantification of DHT-mediated inhibition of low molecular weight DNA fragmentation was performed as described in *Subjects and Methods*. Each value represents the mean of four independent experiments ± SEM. *, $P < 0.05$.

dothelial cells this has been associated with activation of endothelial nitric oxide synthase and production of nitric oxide, which, at physiologically relevant levels, has been shown to suppress apoptotic pathways in a variety of cell types (reviewed in Ref. 42). As in the present study the antiapoptotic effect of 17β-estradiol on germ cells was seen after 4-h incubation, it is possible that the survival of germ cells is at least partly mediated by this rapid nongenomic ER signaling pathway.

In our previous studies, testosterone has also been shown to be an effective inhibitor of germ cell apoptosis in the human testis *in vitro* (30). However, the concentrations of testosterone (10^{-7} mol/L) required for this apoptosis inhibiting effect were 100-1000 times the effective concentrations of estradiol (10^{-9}–10^{-10} mol/L). Of note, the relative potencies of testosterone and estradiol are in the range of their relative physiological concentrations in the spermatic vein (43–47) and testis tissue (48, 49). *In vivo,* testosterone can be metabolized to either estrogens or DHT. We found that DHT was also capable of inhibiting germ cell death in our *in vitro* model, but, as with testosterone, the lowest effective concentrations of DHT were strikingly higher than the effective concentrations of estradiol. Thus, *in vitro* estradiol appears to be a more potent inhibitor of male germ cell death than the androgens testosterone and DHT. The essential role of androgens in completing normal spermatogenesis is well established. However, the mechanism by which androgens regulate spermatogenesis has not been resolved, and even the site of androgen action within the testis has remained unclear. Some reports have shown immunoreactive androgen receptor in developing germ cells (50–53), and others have suggested that only testicular somatic cells, namely Leydig, Sertoli, peritubular myoid, and smooth muscle cells surrounding the walls of the blood vessels, express AR (54–58). If the germ cells lack functional AR, the effects of androgens on spermatogenesis may be forwarded to germ cells through paracrine regulation of germ cells by the neighboring somatic cells, especially by the Sertoli cells providing structural and nutritional support to the developing germ cells. The results of the present study suggest another interesting mechanism for testosterone-mediated survival of germ cells. Taken the present finding that estradiol is much more potent than androgens in inhibiting germ cell apoptosis and the previously reported expression of P450 aromatase by adult Leydig and germ cells in several species (5–10), it is possible that testosterone should at least partly be metabolized to estrogen to mediate its protective effects on germ cells.

The importance of local conversion of testosterone to estrogen in the testis was recently demonstrated in ArKO mice lacking the functional aromatase (*cyp 19*) gene (28). These mice were initially fertile, but developed progressive infertility with disruptions to spermatogenesis between 4.5 months and 1 yr despite any decrease in the levels of circulating gonadotropins or androgens. Spermatogenesis was arrested at early spermiogenic stages and was characterized by increased germ cell apoptosis and reduction in the number of round and elongated spermatids. Interestingly, in the ArKO mice there was no evidence of abnormal fluid reabsorption by the efferent ductules, which was reported to be the primary defect leading to infertility in ERαKO mice (27). Thus, the mechanism of defective spermatogenesis in the ArKO animals appears to be a direct effect of estrogen withdrawal on germ cell development, rather than an indirect effect as in ERαKO mice. Another ArKO mouse with a different mutation site in the aromatase gene was recently described (59). These animals at the age of 10–18 weeks had sperm present in their epididymis, but they were infertile. Further studies of older animals are needed to show whether disruption of spermatogenesis also develops in these animals.

The relevance of estrogen formation for human male reproduction was shown by case reports of two men with homozygous inactivating mutation in the ERα gene (60) or in the P-450 aromatase gene (61). The patient with mutation of the ERα gene had normal male genitalia and sperm den-

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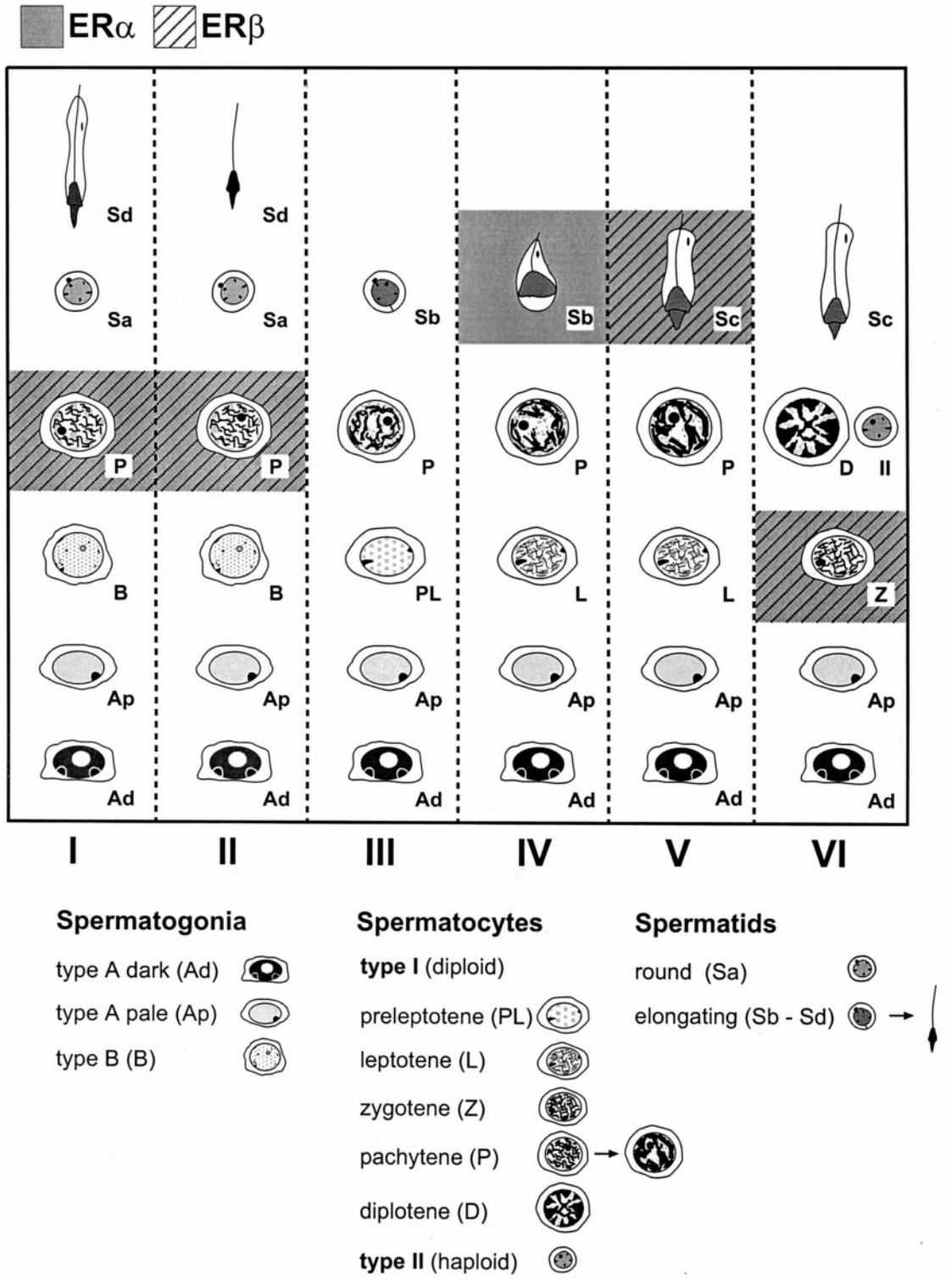

FIG. 6. Cycle of the human seminiferous epithelium [adapted from Clermont (64)]. In the present study the ERα protein was expressed in the early meiotic germ cells (zygotene and early pachytene primary spermatocytes) of stages VI and I to II of the cycle and in the early elongating spermatids of stages IV to V of the cycle. The ERβ protein possessed an expression pattern similar to that of ERα. Both subtypes of ER were expressed in germ cells that can be induced to undergo apoptosis (*i.e.* primary spermatocytes and spermatids), which could then be blocked by 17β-estradiol.

sity, but sperm viability was severely decreased. The mutation in the aromatase gene resulted in infertility with a decreased sperm count and 100% immotile spermatozoa. Thus, studies of both human and mouse have shown that male infertility results from blocking either the ERα or aromatase gene. Somewhat surprisingly, the recently described male

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ERβKO mice lacking ERβ were fertile (62). Fertility was, however, assessed between 6 and 12 weeks of age, at which age no change in testicular morphology in ArKO mice was observed (28, 63). Therefore, the evaluation of fertility in older ERβKO animals will be very interesting.

In the present study the effects of estrogens in the human testis have been evaluated in an *in vitro* culture model that may naturally have some limitations as to approximation of germ cell physiology *in vivo*. However, we believe that in the present *in vitro* model, conditions were sufficiently close to the situation *in vivo*, firstly because in this model the germ cells were allowed to stay in their natural environment (*i.e.* in the seminiferous tubules), and secondly because germ cell death can be blocked by physiological concentrations of testicular hormones, such as testosterone and estradiol. *In vivo*, the situation may, of course, be complicated by the effects of locally produced compounds on interstitial cells. In the case of estrogens, the locally produced hormones could theoretically inhibit Leydig cell androgen production, leading to unpredictable effects on spermatogenesis. However, the *in vivo* significance of our results indicating direct protective effects of estrogens on male germ cells expressing ERs is strongly supported by the previously shown production of estrogens by testicular germ cells in several species (6, 7, 9, 10) and by the recently reported infertility and germ cell apoptosis without accompanying alterations of efferent ductules in ArKO mice (28).

In conclusion, our results describe a novel function of estradiol in the human testis. As receptors for estrogens (both α and β) are strongly expressed by the differentiating and proliferating germ cells of the human testis, and as the *in vitro* induced apoptosis of these cells can be blocked by the natural estrogen 17β-estradiol, it appears very likely that estrogens act as germ cell survival factors in the human testis. The present results together with the results of recent studies of ERαKO (26, 27) and ArKO (28) mice indicate the importance of estrogens for the normal function of the adult testis. Therefore, the effects of physiological estrogens on the male reproductive system should be carefully studied when considering the potential effects of environmental estrogens on male reproductive health.

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