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Androgens Inhibit the Stimulatory Action of 17 β -Estradiol on Normal Human Breast Tissue in Explant Cultures

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Background: The data concerning the effects and safety of androgen in human breast tissue are conflicting.

Objective: Our aim was to analyze the effects of androgens on normal human breast tissue (HBT).

Approach: We cultured explants of HBT (obtained from reduction mammoplasty operations of postmenopausal women) with or without testosterone (T) and 5 α -dihydrotestosterone (DHT) or in combination with 17 β -estradiol (E₂) for 7 and 14 d to study the effects of androgens on proliferation, apoptosis, target gene expression, and steroid receptors. The androgen receptor (AR) and estrogen receptor (ER) dependences of the effects were studied with the antihormones bicalutamide and fulvestrant, respectively.

Results: The hormone responsiveness of cultured breast tissue was assessed by assaying apolipoprotein-D and prostate-specific antigen expression increased by androgens and amphiregulin and trefoil factor-1 expression induced by E₂ treatment. T and DHT reduced proliferation and increased apoptosis in breast epithelium, the effects of which were reversed by bicalutamide. In combination with E₂, they suppressed E₂-stimulated proliferation and cell survival. DHT also inhibited basal ($P < 0.05$) and E₂-induced expression of cyclin-D1 mRNA ($P < 0.05$). Immunohistochemistry showed that T ($P < 0.05$) and DHT ($P < 0.05$) increased the relative number of AR-positive cells, whereas ER α -positive ($P < 0.001$) cell numbers were strongly decreased. The percentage of ER β -positive cells remained unchanged. E₂ treatment increased ER α -positive ($P < 0.01$) cells, whereas AR- ($P < 0.05$) and ER β -expressing ($P < 0.001$) cells diminished. These effects were repressed in combination cultures of E₂ with T and DHT.

Conclusion: T and DHT inhibited proliferation and increased apoptosis in the epithelium of cultured normal HBT and opposed E₂-stimulated proliferation and cell survival in an AR-dependent manner. These effects were associated with changes in the proportions of ER α - and AR-positive epithelial cells. (*J Clin Endocrinol Metab* 97: 0000–0000, 2012)

Androgens perform physiologically important functions in females, but their effects on mammary tissue and their role in the development of breast cancer remain unclear (1, 2). There are conflicting reports on the inhib-

itory effects of androgens on the proliferation of breast epithelial cells, which may play a protective role in the pathogenesis of breast cancer (3). Epidemiological studies demonstrate that high levels of endogenous androgens in

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Abbreviations: Apo-D, Apolipoprotein-D; AR, androgen receptor; AREG, amphiregulin; Bclt, bicalutamide; CPP32, cleaved caspase-3; C_t, cycle threshold; DHT, 5 α -dihydrotestosterone; E₂, 17 β -estradiol; ER, estrogen receptor; Fulv, fulvestrant; HBT, human breast tissue; PHH3, phosphohistone-3; PSA, prostate-specific antigen; qRT-PCR, quantitative RT-PCR; T, testosterone; TFF1, trefoil factor-1.

postmenopausal women are associated with an increased risk of breast cancer, independent of circulating estrogen levels (4). Experimental data from animal studies and cell cultures with human breast cancer cell lines have indicated both proliferative (5) and antiproliferative (6) effects on cell growth. Recently the presence of an androgen receptor (AR) in tumor tissue was found to be correlated with favorable outcomes in patients (7), which suggests a protective role for AR signaling in breast tissue.

Most *in vitro* studies using breast cancer cell lines show that androgens inhibit the proliferative effects of estrogen (8, 9). However, the results diverge, depending on the specific cell line, the status and type of steroid receptor, and the dose of androgen (10). Rodent models have limitations because of the marked biological differences between the species, which limits the validity of the data. Studies on nonhuman primates with similarities to humans in terms of the mammary gland, reproductive physiology, and peripheral steroid hormone metabolism showed that the administration of testosterone markedly reduced estrogen-induced proliferation and estrogen receptor (ER)- α gene expression (6, 11). Female-to-male transsexuals treated with androgens exhibited involutionary changes similar to those observed in the breasts of menopausal women, including marked reduction of glandular tissue, involution of the lobuloalveolar structures, prominence of fibrous connective tissue, and the presence of only small amounts of adipose tissue (12). However, the doses of androgens used in those treatments were notably high. Thus, further clarification of the effects of androgens on human breast tissue (HBT) is required.

In this study, we aimed to clarify the action of exogenous androgens on normal HBT. The effects of testosterone (T) and 5 α -dihydrotestosterone (DHT) and in combination with 17 β -estradiol (E₂) on HBT were studied using a modified explant culture system that allows the maintenance of epithelial-stromal interactions and the monitoring of the responses of HBT to steroid hormones (13).

Subjects and Methods

HBT sampling

HBT samples were collected from postmenopausal women (n = 19) aged 46–64 yr undergoing reduction mammoplasties at Turku University Central Hospital (Turku, Finland). None of the women had used hormonal therapy within the previous 6 months. Informed written consent was obtained from all of the patients before surgery. The study protocol was approved by the Joint Ethics Committee of Turku University and Turku University Central Hospital.

Organ culture

HBT were processed for explant culture as described previously (13) (Supplemental Methods, published on The Endocrine Society's Journals Online web site at <http://jcem.endojournals.org>). 17 β -Hydroxy-3-oxo-4-androstene (T; Fluka Chemie AG, Buchs, Switzerland), 5 α -androstane-17 β -ol-3-one (DHT; Fluka-Chemie), and E₂ (Sigma Aldrich GmbH, Steinheim, Germany), or a combination thereof was added to the basal medium. T and DHT were used at a concentration of 1 nM. E₂ was used at a final concentration of 10 nM. The steroids were dissolved in dimethyl sulfoxide (Sigma). The HBT samples were collected on d 7 and 14 of the culture for further analyses [histology, whole mounts, immunohistochemistry, Western blots, and quantitative RT-PCR (qRT-PCR)]. To study the AR- and ER-mediated effects of androgens and E₂, the antiandrogen bicalutamide (Bclt) and the antiestrogen fulvestrant (Fulv) were used (both kindly provided by Hormos Medical, Turku, Finland) at concentrations of 1 μ M.

Whole mounts

HBT samples were spread onto a glass slide, fixed in a 1:3 mixture of glacial acetic acid/absolute ethanol, hydrated, stained overnight in 0.2% carmine and 0.5% AlK(SO₄)₂, dehydrated in graded solutions of ethanol, and cleared in xylene.

Morphology and immunohistochemistry

For histology and immunohistochemistry, the samples were fixed in 4% paraformaldehyde, and paraffin sections (4 μ m) were mounted on SuperFrost *Plus slides (Menzel GmbH & Co. KG, Braunschweig, Germany). The sections were deparaffinized in xylene and rehydrated through graded ethanol solutions. Endogenous peroxidase activity was blocked using 0.3% H₂O₂ for 30 min. The sections were then incubated with primary antibodies (Supplemental Table 1) overnight at 4 C followed by incubation with biotin-labeled secondary antibody diluted 1:200 for 1 h at room temperature. Immunoreaction was detected using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) and peroxidase substrate 3,3'-diaminobenzidine (Vector Laboratories) following the manufacturer's instructions. The samples were subsequently counterstained with Mayer's hematoxylin and mounted in Mountex (Histolab Products AB, Göteborg, Sweden).

Image analysis and histomorphometry

The sections were interpreted under light microscopy in high-power fields. An Olympus BX-60 (Olympus Deutschland GmbH, Hamburg, Germany) microscope with Cell \hat{D} imaging system connected to a Leica DC300F camera (Leica Microsystems GmbH, Heidelberg, Germany) was used for the image analysis. For each sample, more than 1000 common epithelial cells were scored for the various markers, and the data were expressed as the percentage of cells that were positive.

A histomorphometric evaluation was performed to estimate the changes of epithelial morphology (acinar and ductal wall thickness) during different hormonal treatments using OsteoMeasure system (OsteoMetrics, Atlanta, GA) as described previously (13) (Supplemental Methods).

Western blot analyses

Cultured frozen HBT was homogenized in lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0). The protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad Labora-

ories, Hercules, CA). Next, 50 μg of protein was heated to 108 C for 5 min in Laemmli sample buffer with 2 β -mercaptoethanol and loaded onto a 12% SDS-PAGE gel. After gel electrophoresis, the proteins were transferred to a polyvinylidene fluoride membrane (Bio-Rad Laboratories). The primary antibodies used for the Western blots are presented in Supplemental Table 1. The secondary goat antimouse IgG-horseradish peroxidase (Bio-Rad Laboratories) was used at a dilution of 1:10,000. Detection was performed using ECL-Plus (Amersham, Piscataway, NJ). The band intensities were quantified using Analysis MCID version 5.0 software (Imaging Research, Inc., Ontario, Canada), and the results are expressed as relative density of target to tubulin (loading control) ratios.

Quantitative RT-PCR

Total RNA from the frozen HBT was isolated using the TRIsure reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. One microgram of total RNA was reverse transcribed with AMW reverse transcriptase (Finnzymes, Espoo, Finland). The cDNA was diluted 1:10 in nuclease-free water, and 8 μl was analyzed in duplicate by RT-PCR in an CFX384 RT-PCR detection system (Bio-Rad Laboratories) using the DyNamo SYBR Green qPCR kit (Finnzymes) with 0.4 $\mu\text{mol/liter}$ of each primer (Oligomer, Helsinki, Finland) (Supplemental Table 2) in a reaction mixture with a total volume of 20 μl . The negative controls contained water in place of first-strand cDNA. The relative gene expression levels were normalized to a calibrator that was chosen to be the control group. The final results were expressed as n-fold differences in gene expression relative to keratin-18 and the control. The results were calculated using the cycle threshold (C_t) method as follows: $n\text{-fold} = 2^{-(\Delta C_t\text{sample} - \Delta C_t\text{calibrator})}$, where the ΔC_t values of the sample and the controls were determined by subtracting the average C_t value of the housekeeping gene from the average C_t value of the different genes analyzed.

Statistical analysis

Statistical analysis was performed using general linear mixed models. The effect of the treatment type and time were also tested. The Tukey-Kramer method was used to adjust the P values of the pairwise comparisons, and the residuals were checked for justification of the analysis. The data are expressed as mean \pm SD. $P < 0.05$ were considered to be statistically significant. Statistical analyses were carried out using the IBM SPSS Statistics 19 system (IBM Corporation, White Plains, NY).

Results

Effects of androgens on HBT morphology

For the control HBT cultured for 7 or 14 d, the explants maintained their structural integrity and glandular morphology. The epithelial cells were cuboidal and were arranged in two regular layers. Pilot testing was done to determine the optimal concentration for studying the biological effects of androgens on HBT. The concentration of 1 nM T and 1 nM DHT were used in further experiments alone or in combination with E_2 .

The 10-nM concentration of E_2 was used based on previous studies, which determined effective concentrations

of these steroid hormones *in vitro* (11). As previously shown (13), HBT was strongly stimulated with E_2 . The acinar epithelium became stratified, and a portion of luminae was obliterated. The epithelial cells varied in size and shape, and they appeared to lose their polarized orientation in relation to the basement membrane, especially in the explants grown with E_2 for 14 d (Fig. 1A, *upper panel*). A combination of E_2 /T or E_2 /DHT prevented the E_2 -induced changes. Similar to the controls, the epithelium remained cuboidal, regular and organized, and the epithelial cells retained their polarity and height. The histomorphometry showed that the thickness of acinar and ductal wall was increased by treatment with E_2 and decreased by T and DHT significantly (Fig. 1A, *lower panel*). Whole mounts confirmed the involutive effects of DHT and the stimulatory effects of E_2 on the lobule structure of explants cultured for 14 d (Fig. 1B).

Hormone responsiveness of androgen and E_2 target gene expression in cultured HBT

Hormone responsiveness was examined by studying the expression of androgen-regulated prostate-specific antigen (PSA) and apolipoprotein D (Apo-D) (14) *vs.* E_2 -regulated trefoil factor-1 (TFF1) (14) and amphiregulin (AREG) (15, 16) in HBT grown with or without T or DHT *vs.* E_2 , respectively. Immunohistochemistry showed that PSA stained faintly in the myoepithelial cells of the control samples, whereas it was strongly expressed in the epithelial cells of HBT cultured with androgens (Fig. 2A, *left panel*). In the presence of Bclt, T/Bclt, or DHT/Bclt, the PSA immunoreaction was low. The quantification of PSA-positive cells showed that androgens increased the percentage of PSA-positive cells compared with the controls (Fig. 2A, *right panel*). Bclt decreased the PSA-positive cell proportion to approximately 25% and significantly attenuated the effects of T and DHT. Treatment with E_2 and Fulv had a negative effect on the percentage of PSA-positive cells. Fulv decreased the PSA-positive cell count to approximately 30%. The addition of E_2 to Fulv significantly increased the percentage of PSA-positive cells, but their level remained lower than in the control groups. Western blot analyses showed that T and DHT increased the expression of Apo-D and their effects were blocked by Bclt (Fig. 2B).

TFF1 expression was strong in the epithelial cells of explants cultured with E_2 (Fig. 2C, *left panel*). In the Fulv-treated explants, TFF1 was observed only in clumps of detached epithelial cells located in the luminae and not in the epithelium. The addition of Fulv with E_2 diminished TFF1 staining in the epithelial cells, but there were TFF1 cell clumps in some luminae. Treatment with E_2 increased the level of TFF1 mRNA up to 6-fold compared with the

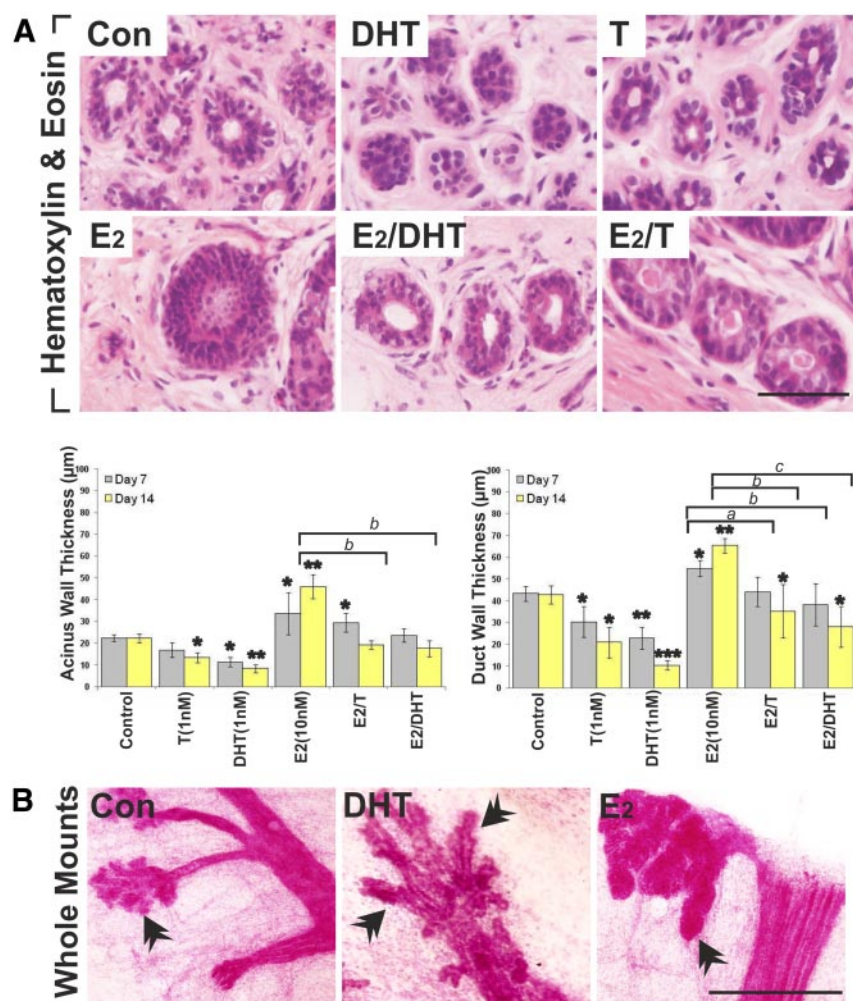


FIG. 1. Representative images of histological staining, whole-mount micrographs of cultured HBT in different treatment groups, and histomorphometric analyses. A, Histological analysis revealed differences in the morphology of HBT explants after hormonal treatment *in vitro* for 14 d (upper panel). The histology of the explants grown in the presence of T (1 nM) and DHT (1 nM) was not different from that of the control explants grown in the presence of vehicle (Con). E₂ (10 nM) treatment caused the overgrowth of epithelium and hyperplasia in some lobules. In the explants treated with E₂/T (10 nM/1 nM) and E₂/DHT (10 nM/1 nM), the luminae were larger and filled with secretions in the E₂/T explants. H&E staining was done. Scale bar, 200 μm. Histomorphometrical method used to determine the acinar and ductal wall thickness in cultured human breast explants (lower panel). The asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) indicate the statistical significance between the treatment and the control groups. The letters (a, $P < 0.05$; b, $P < 0.01$; c, $P < 0.001$) correspond to the statistical significance between E₂ (10 nM) vs. E₂/T (10 nM/1 nM) and E₂ (10 nM) vs. E₂/DHT (10 nM/1 nM). B, Whole-mount micrographs of HBT explants cultured with DHT (1 nM) and E₂ (10 nM) (lower panel). The arrows indicate the lobules, which differ in size and shape between the treatments. Alumine carmine staining is shown. Scale bar, 200 μm.

controls ($P < 0.001$) (Fig. 2C, right panel). Fulv decreased the basal and E₂-induced TFF1 mRNA. Both androgens inhibited TFF1 mRNA expression, and Bclt alone increased it. Interestingly, the addition of T to Bclt further increased TFF1 mRNA compared with the control, whereas the addition of DHT to Bclt reversed TFF1 mRNA to the control level (Fig. 2C). The Western blotting experiments showed that E₂ increased the level of AREG in HBT (Fig. 2B), an effect opposed by Fulv.

Androgens decrease epithelial cell proliferation in HBT

The proliferation was determined by immunohistochemistry with phosphohistone-3 (PHH3) (Fig. 3A) and Ki67 antigen (Fig. 3B). The relative numbers of PHH3-positive cells were lower compared with those of the Ki67-positive cells, reflecting the expression pattern of the markers during the cell cycle (17). The number of PHH3 cells was lower in T- ($P < 0.05$) and DHT-treated ($P < 0.001$) HBT compared with the controls (Fig. 3A). Interestingly, the numbers of PHH3-positive cells increased in Bclt-treated HBT ($P < 0.01$) compared with the controls. The addition of Bclt to T or DHT opposed these androgen effects (Fig. 3A). The highest percentage of PHH3-positive cells was observed in the E₂ group when compared with the controls and other treatments (Fig. 3A, right panel). Similar results were obtained with Ki67 (Fig. 3B). The effects of E₂/T and E₂/DHT on proliferation were assessed only with Ki67. As previously shown (13), the proliferation increased in the E₂-treated HBT ($P < 0.01$) and decreased in a time-dependent manner in HBT treated with T and DHT for 7 and 14 d (Fig. 3C, right panel). The combination of T and DHT with E₂ opposed the E₂-alone effects as compared with the HBT cultured with E₂ alone at both 7 and 14 d.

Expression of cyclin-D1 in HBT cultured with androgens and E₂

To study the mechanisms of androgen inhibition of HBT proliferation in the presence and absence of E₂, the expression of cyclin-D1 was studied by immunohistochemistry and qRT-PCR (Fig. 4). Approximately 10% of the epithelial cells were immunopositive for

cyclin-D1 in the controls, whereas a larger proportion was observed in the E₂-treated explants (Fig. 4A, left panel). Surprisingly, there seemed to be more cyclin-D1-positive cells in the T-treated HBT than in the control HBT, and few cyclin-D1-expressing cells were found in the DHT-treated explants. The qRT-PCR results showed that DHT decreased cyclin-D1 mRNA (Fig. 4B), whereas the level of cyclin-D1 mRNA was increased in T-treated HBT. How-

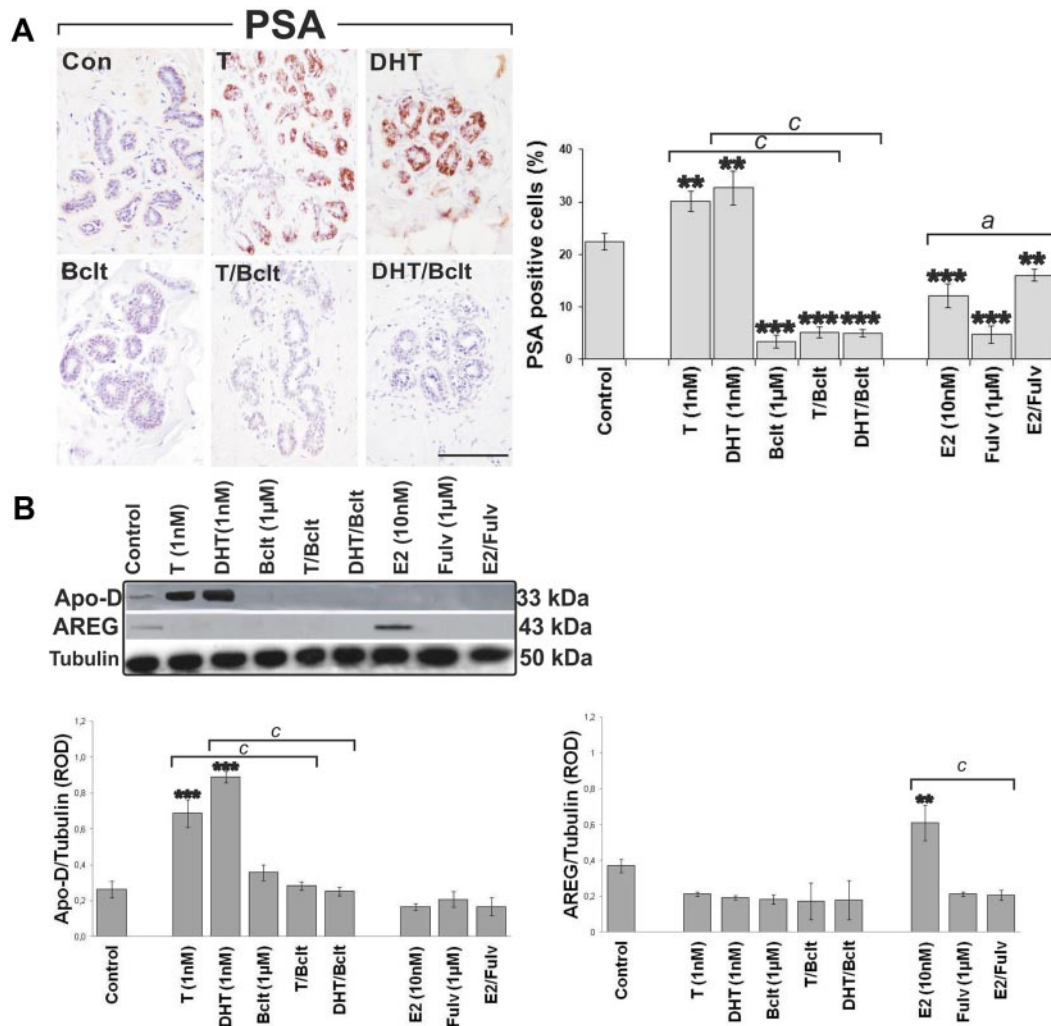


FIG. 2. Androgen and estrogen responsiveness of HBT in culture. HBT was cultured with T (1 nM) or DHT (1 nM) and E₂ (10 nM) in the presence or absence of Bclt (1 μM) or Fulv (1 μM). A, Immunostaining for PSA in HBT cultured without hormones (Con) was faint and mainly located in myoepithelial cells (*left panel*). The epithelial and myoepithelial cells of HBT cultured with T (1 nM) and DHT (1 nM) showed strong immunoreactivity for PSA. Scale bar, 100 μm. The results of the PSA-positive cell counts in different treatment groups are shown as columns (mean ± SD; n = 7) (*right panel*). The asterisks (*, P < 0.05; **, P < 0.01; ***, P < 0.001) indicate the statistical significance between the treatment and the control groups. The letters (a, P < 0.05; b, P < 0.01; c, P < 0.001) correspond to the statistical significance between T (1 nM) vs. T/Bclt (1 nM/1 μM), DHT (1 nM) vs. DHT/Bclt (1 nM/1 μM), and E₂ (10 nM) vs. E₂/Fulv (10 nM/1 μM). B, The expression of apolipoprotein (Apo-D) and AREG in cultured HBT (n = 4) was analyzed by Western blots. Tubulin was blotted as the protein loading control. Densitometry analysis was performed to evaluate the Apo-D to tubulin and AREG to tubulin ratios. Data are expressed as relative OD (ROD). The columns report the mean ± SD values (n = 4). The asterisks (*, P < 0.05; **, P < 0.01; ***, P < 0.001) indicate the statistical significance between the treatment and the control groups. The letters (a, P < 0.05; b, P < 0.01; c, P < 0.001) correspond to the statistical significance between T (1 nM) vs. T/Bclt (1 nM/1 μM) and DHT (1 nM) vs. DHT/Bclt (1 nM/1 μM) and E₂ (10 nM) vs. E₂/Fulv (10 nM/1 μM). C, Representative images of HBT explants immunostained for TFF1 are shown for different study groups (*left panel*). The strongest immunopositivity for TFF1 was found in the epithelial cells of explants cultured with E₂ (10 nM). The addition of Fulv (1 μM) with E₂ (10 nM) caused decreased expression of TFF1. In the control and Fulv (1 μM)-treated explants, a degree of immunoreactivity for TFF1 was observed only in the cell clumps that were in the luminae. Scale bar, 100 μm. The highest level of TFF1 mRNA was detected by qRT-PCR in HBT treated with E₂ (*right panel*). The columns report the mean ± SD values (n = 4). The asterisks (*, P < 0.05; **, P < 0.01; ***, P < 0.001) indicate the statistical significance between the treatment and the control groups. The letters (a, P < 0.05; b, P < 0.01; c, P < 0.001) correspond to the statistical significance between T (1 nM) vs. T/Bclt (1 nM/1 μM), DHT (1 nM) vs. DHT/Bclt (1 nM/1 μM), and E₂ (10 nM) vs. E₂/Fulv (10 nM/1 μM).

ever, the level of cyclin-D1 mRNA in the T-treated HBT was much lower than that in the E₂-treated HBT. In the combination cultures, both androgens clearly decreased E₂-induced cyclin-D1 mRNA (Fig. 4C).

Androgens increase apoptosis in cultured HBT

To investigate how androgen signaling affects cell survival in HBT, cleaved caspase-3 (CPP32), a marker

for apoptosis, was assessed (Fig. 5). The proportion of CPP32-positive cells was increased in the HBT cultured with T (P < 0.001) and DHT (P < 0.001) for 7 and 14 d compared with the controls (Fig. 5). E₂/T and E₂/DHT reduced the percentage of apoptotic cells significantly when compared with the control and E₂-alone groups.

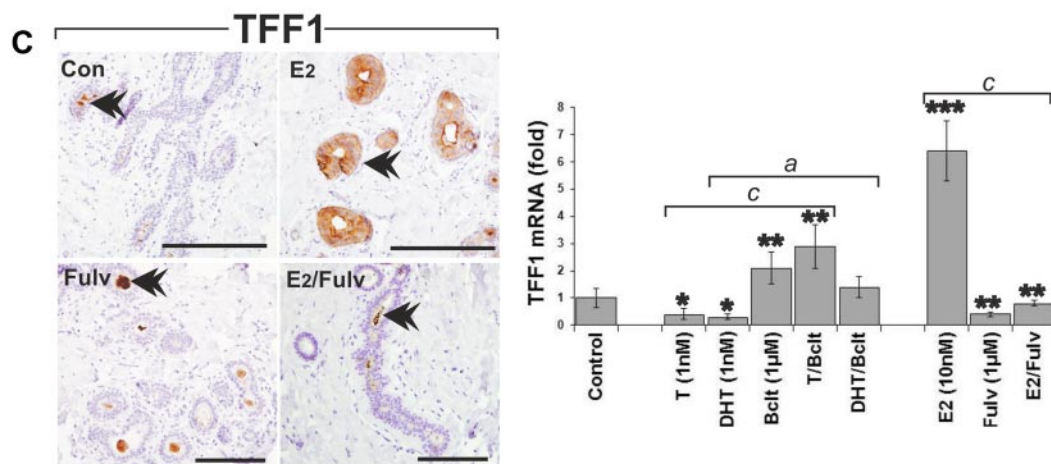


FIG. 2. Continued.

Expression of AR, ER α , and ER β in androgen- and E $_2$ -treated HBT

Immunohistochemical stainings showed that in noncultured HBT, 34% of the cells were positive for the AR, 26% for ER α , and 80% for ER β . The AR were mainly located in the nuclei of acinar and ductal epithelial cells and, to a minor extent, in stromal cells. ER α was observed only in epithelial cells, and ER β was observed in the nuclei of epithelial cells and in scattered stromal cells. The percentage of AR cells was significantly increased in the T and DHT groups compared with the controls, whereas E $_2$ treatment reduced the proportion of AR cells (Fig. 6A). Addition of E $_2$ to T and DHT raised AR-positive cell numbers compared with E $_2$ -alone treatment. The count of ER α -positive cells was lowered by T and DHT in comparison with the control ($P < 0.001$), whereas E $_2$ increased them significantly (Fig. 6B). The E $_2$ effect was opposed by T and DHT in the E $_2$ /T and E $_2$ /DHT cultures (Fig. 6B). The proportion of ER β -positive cells was not affected by T and DHT, but E $_2$ significantly decreased their expression (Fig. 6C). The combination of T and DHT with E $_2$ opposed the E $_2$ effects, but the number of ER β -positive cells remained lower than in the controls.

Discussion

In this study, we investigated how androgens influence HBT and how they modulate E $_2$ stimulation of the breast. We used organ cultures of HBT obtained from reduction mammoplasty surgeries of postmenopausal women in which HBT may differ from breast tissues in other populations. As we reported previously, the HBT explants retain their structural integrity and appropriate hormonal responses in this model (13).

The maintenance of hormone responsiveness of cultured HBT was demonstrated by studying the expression

of known androgen and E $_2$ target genes (14, 18). PSA is widely used as an androgen-regulated marker in prostate tissue, but it is also expressed in the breast (19) and other tissues (20). Our cultures showed that T and DHT strongly induced PSA expression in HBT. Bclt inhibition of androgen-induced PSA expression demonstrated that expression was mediated by the AR. The percentage of PSA-positive cells in the E $_2$, Fulv, and E $_2$ /Fulv cultures decreased, the decrease of which was probably caused by indirect mechanisms such as repression of AR expression (21) and because the PSA gene is not known to contain estrogen response elements (14). Extranuclear ER actions (22) provide another possible mechanism for E $_2$ suppression of PSA. The expression of Apo-D, another androgen target gene (14), was also induced in an AR-dependent manner. These results suggest that T and DHT can directly influence target gene expression in HBT.

The strong up-regulation of TFF1 and AREG expression in the presence of E $_2$ showed the appropriate response to E $_2$ and Fulv inhibition of stimulation indicated an ER-mediated effect. Fulv alone decreased TFF1 mRNA, suggesting that TFF1 expression in basal conditions may have been slightly increased by ER activation by endogenous ligands (a possibility discussed later) or by ligand-independent mechanisms. Both androgens inhibited TFF1 expression. Interestingly, Bclt alone and T/Bclt increased TFF1 mRNA compared with the control. This finding suggests that the AR is involved in the repression of TFF1 expression or in decreasing the proportion of cells expressing the protein. It is also possible that T aromatization to E $_2$ may contribute to increased TFF1 mRNA level in T/Bclt group.

The E $_2$ stimulation of epithelial cell proliferation is generally considered to increase the risk for the development of breast cancer (23). However, conflicting studies have been reported on the effects of exogenous androgens on the proliferation of mammary epithelial cells and the as-

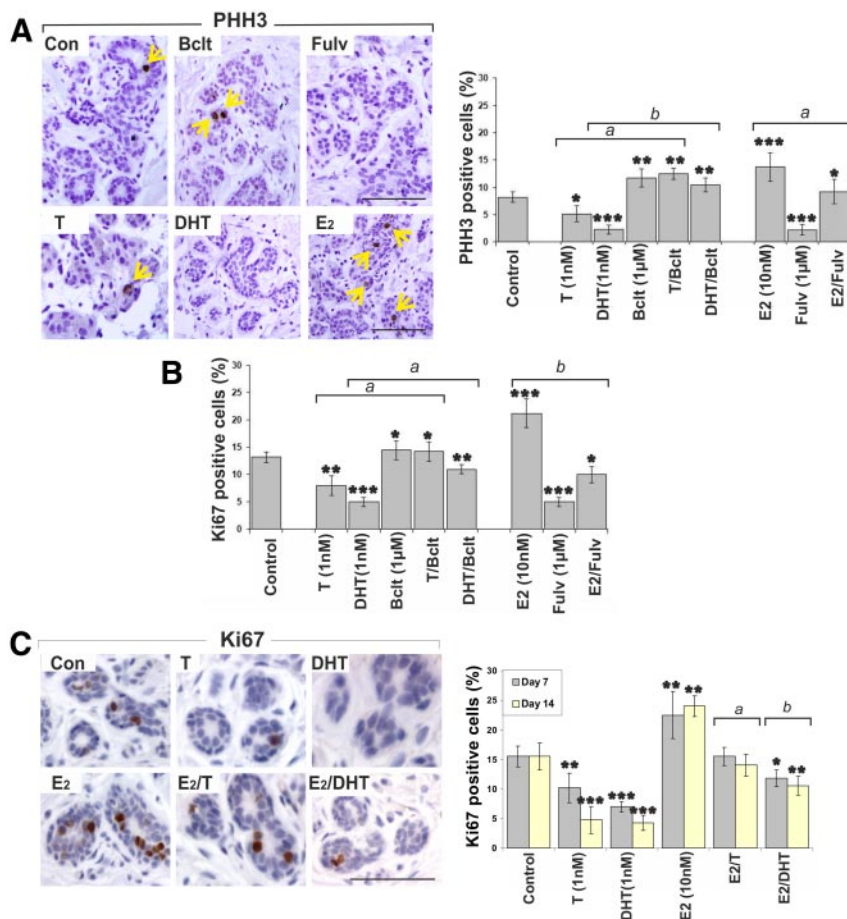


FIG. 3. The effects of androgens and E₂ on epithelial cell proliferation in HBT. **A**, The effects of T, DHT, Bclt, E₂, and Fulv on the expression of PHH3 in cultured HBT for 7 d (left panel). The results of PHH3 immunostaining are shown for the different treatment groups. The arrows indicate mitotic cells in the cultured HBT. Scale bar, 100 μ m. PHH3-positive cells in different treatment groups after 7 d (right panel) are shown. The columns show the mean \pm SD values (n = 7). The asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) indicate the statistical significance between the treatment and the control groups. The letters (a, $P < 0.05$; b, $P < 0.01$; c, $P < 0.001$) correspond to the statistical significance between T (1 nM) vs. T/Bclt (1 nM/1 μ M), DHT (1 nM) vs. DHT/Bclt (1 nM/1 μ M), and E₂ (10 nM) vs. E₂/Fulv (10 nM/1 μ M). **B**, Effects of T, DHT, Bclt, E₂, and Fulv on the expression of Ki67 in cultured HBT for 7 d. The columns show the mean \pm SD values (n = 7). The asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) indicate the statistical significance between the treatment and the control groups. The letters (a, $P < 0.05$; b, $P < 0.01$; c, $P < 0.001$) correspond to the statistical significance between T (1 nM) vs. T/Bclt (1 nM/1 μ M), DHT (1 nM) vs. DHT/Bclt (1 nM/1 μ M), and E₂ (10 nM) vs. E₂/Fulv (10 nM/1 μ M). **C**, The proliferation in HBT cultured with T, DHT, E₂, E₂/T, and E₂/DHT for 14 d was determined by Ki67 immunoreactivity. Representative images are shown for the different treatment groups (left panel). Scale bar, 200 μ m. Ki67-positive cells in the different treatment groups after 7 and 14 d are shown. The columns show the mean \pm SD values (n = 12) of the percentage of the Ki67-positive cell in the different treatment groups (right panel). The asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) indicate the statistical significance between the treatment and the control groups. The letters (a, $P < 0.05$; b, $P < 0.01$; c, $P < 0.001$) correspond to the statistical significance between E₂ (10 nM) vs. E₂/T (10 nM/1 nM) and E₂ (10 nM) vs. E₂/DHT (10 nM/1 nM).

sociated risk of breast cancer (5, 6, 24–28). Our study demonstrates that androgens decreased proliferation in a time-dependent manner and effectively opposed the stimulatory effects of E₂. Interestingly, Bclt increased the proportion of proliferating cells, which suggests that AR exerts an inhibitory effect on the basal levels of proliferation. Our results agree with a previous study on human breast

tissue by Hofling *et al.* (29), who found decreased Ki67 cells in women treated with androgen-containing hormone therapy. Corresponding results have also been obtained in primate model studies in which androgen treatment decreased mammary proliferation (11).

The mechanisms of androgen inhibition of HBT proliferation were further evaluated by studying the expression of cyclin-D1, which has a crucial role in triggering cell cycle progression from the G₁ to the S phase. In our experiments, DHT decreased cyclin-D1 mRNA and immunostaining. This result agrees with earlier observations with breast cancer cells (30). Comstock *et al.* (31) recently reported that ligand-bound AR interacts with specific androgen response element sequences on the cyclin-D1 promoter, which leads to the inhibition of cyclin-D1 transcription (30). In contrast, T increased cyclin-D1 mRNA levels. This effect may partly have been caused by androgen conversion to E₂ (10), as discussed later. Unexpectedly, Bclt alone and together with T or DHT decreased cyclin-D1 mRNA, which suggests promoter-specific Bclt effects and generally reflects the complex actions of ligand-bound AR on the cyclin-D1 promoter (30). The overall effects of T were antiproliferative, opposed by Bclt, which suggests that the regulation of cyclin-D1 was not critical among the mechanisms leading to the T-induced inhibition of proliferation. In combination cultures with E₂, both T and DHT clearly opposed the strong up-regulation of cyclin-D1 expression by E₂.

In addition to inhibiting proliferation, androgens contributed to epithelial regression by increasing apoptosis, an effect counteracted by E₂ in the combination culture. DHT has been reported to induce cell death (32) and inhibit the expression of antiapoptotic bcl-2 in breast cancer cells (8, 33), which could be a mechanism for increased cell death in androgen-treated HBT. However, *in vivo* treatment with DHT arrested growth but did not induce cell death in developing mouse mammary glands (34).

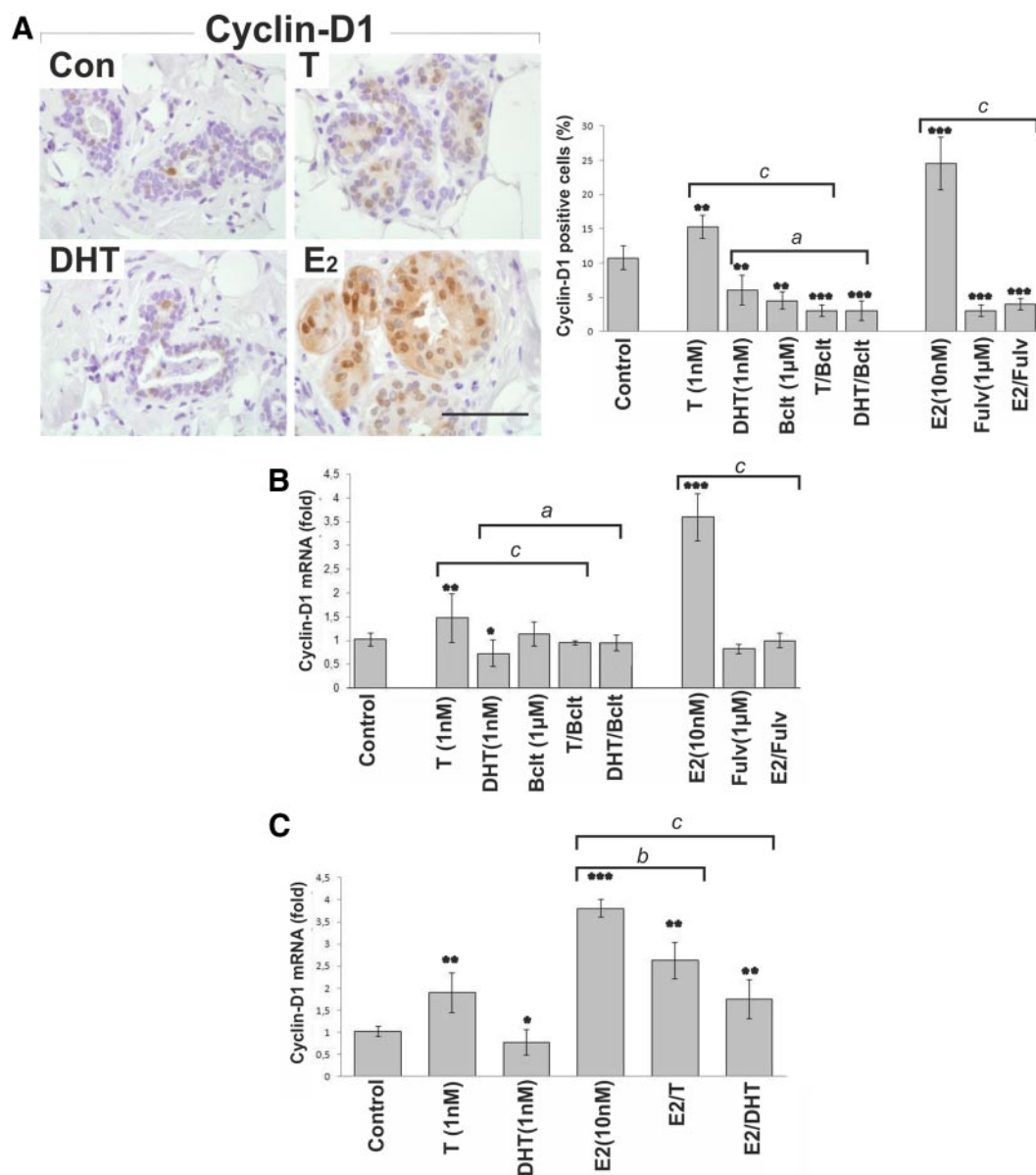


FIG. 4. Expression of cyclin-D1 in cultured HBT. A, The immunostaining of different treatment groups for cyclin-D1 was dependent on the treatment (*left panel*). Scale bar, 200 μ m. Cyclin-D1-positive cells in the different treatment groups after 7 d (*right panel*) are shown. The columns show the mean \pm SD values ($n = 4$) of the percentage of cyclin-D1-positive cells in the different treatment groups (*right panel*). The asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) indicate the statistical significance between the treatment and the control (Con) groups. The letters (a, $P < 0.05$; b, $P < 0.01$; c, $P < 0.001$) correspond to the statistical significance T (1 nM) vs. T/Bclt (1 nM/1 μ M), DHT (1 nM) vs. DHT/Bclt (1 nM/1 μ M), and E₂ (10 nM) vs. E₂/Fulv (10 nM/1 μ M). B, The highest level of cyclin-D1 expression was induced by E₂ alone. The columns show the mean \pm SD values ($n = 4$) for cyclin-D1 mRNA in the different treatment groups. The asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) indicate the statistical significance between the treatment and the control groups. The letters (a, $P < 0.05$; b, $P < 0.01$; c, $P < 0.001$) correspond to the statistical significance between T (1 nM) vs. T/Bclt (1 nM/1 μ M), DHT (1 nM) vs. DHT/Bclt (1 nM/1 μ M), and E₂ (10 nM) vs. E₂/Fulv (10 nM/1 μ M). C, T and DHT attenuated the E₂ effects on cyclin-D1 mRNA in HBT. The columns show the mean \pm SD values ($n = 4$) for cyclin-D1 mRNA for the different treatment groups. The asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) indicate the statistical significance between the treatment and the control groups. The letters (a, $P < 0.05$; b, $P < 0.01$; c, $P < 0.001$) correspond to the statistical significance between E₂ (10 nM) vs. E₂/T (10 nM/1 nM) and E₂ (10 nM) vs. E₂/DHT (10 nM/1 nM).

Androgens, T, and nonaromatizable DHT exerted similar effects on the proliferation, apoptosis, and expression of the marker proteins. As expected, DHT was generally more potent than T. In premenopausal women, circulating androgens include both T and DHT. In postmenopausal women, androgens are primarily formed from adrenal

precursors in peripheral tissues, such as the breast (35). The enzymatic activities for steroid sulfatases and 5 α -reductase have been demonstrated in normal HBT (1), which allow the synthesis of T and the conversion of T to DHT in breast tissue. In our HBT cultures, the T effects could be partly mediated by the more bioactive DHT. As

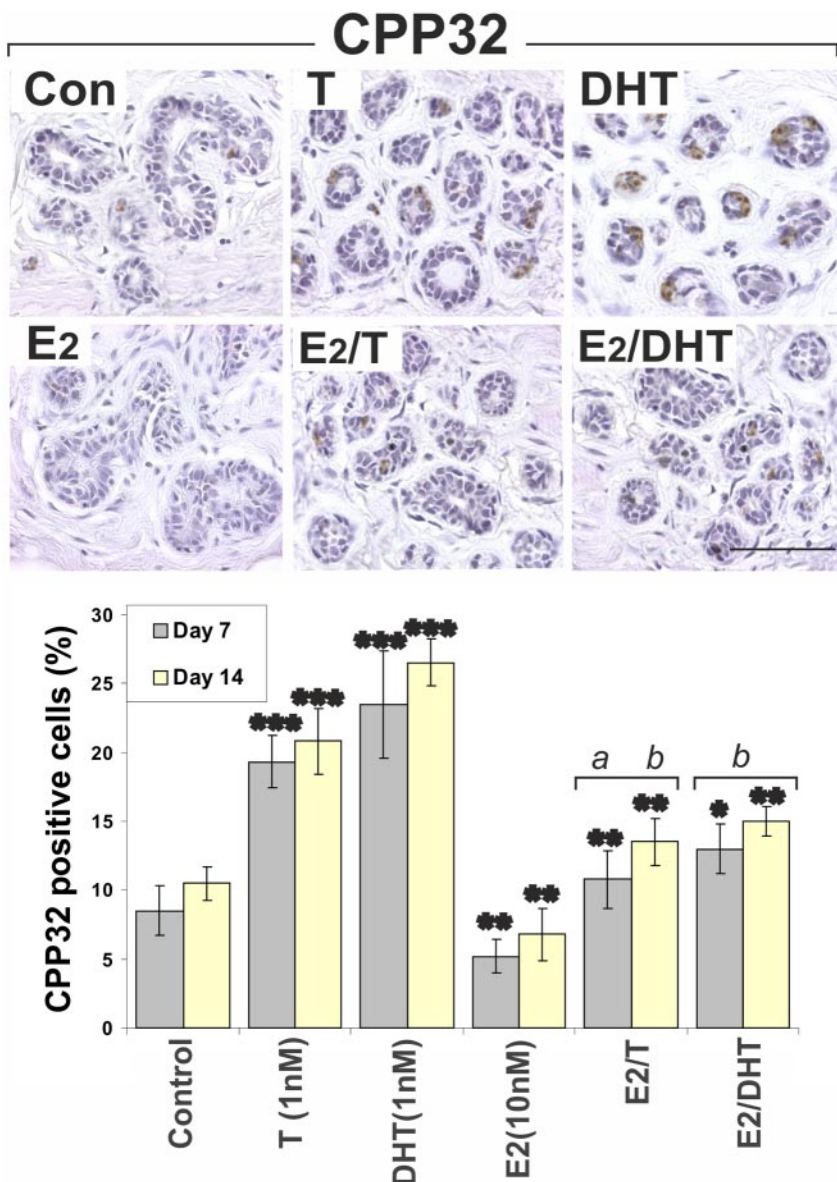


FIG. 5. The effects of T and DHT alone and in combination with E₂ (E₂/T and E₂/DHT) on apoptosis in HBT cultures. Apoptosis in HBT cultured with T (1 nM), DHT (1 nM), E₂ (10 nM), E₂/T (10 nM/1 nM), and E₂/DHT (10 nM/1 nM) for 7 and 14 d was determined by immunohistochemical staining of CPP32 (upper panel). Representative images are shown for different study groups after 14 d *in vitro*. Scale bar, 100 μ m. The scores of CPP32-positive cells were dependent on the treatment (lower panel). The columns show the mean \pm SD values (n = 12) for the percentage of CPP32-positive cells in the different treatment groups. The asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) indicate the statistical significance between the treatment and the control (Con) groups. The letters (a, $P < 0.05$; b, $P < 0.01$; c, $P < 0.001$) correspond to the statistical significance between E₂ (10 nM) vs. E₂/T (10 nM/1 nM) and E₂ (10 nM) vs. E₂/DHT (10 nM/1 nM).

previously reported (36), HBT was found to express low levels of aromatase catalyzing the conversion of T to E₂ (Supplemental Fig. 1). As discussed earlier, the conversion of T to E₂ may have contributed to T-increased cyclin-D1 mRNA expression, which was inhibited by DHT. Owing to lack of feasible methods, concentrations of steroids and their metabolites in small explants could not be measured, but according to previous reports, their levels in normal

postmenopausal HBT are very low (37). The overall similarity of other T and DHT responses and the experiments with Bclt suggest that the majority of the effects of T and DHT on proliferation and apoptosis in breast epithelial cells are mediated by the AR. Thus, the local production of androgens and the metabolism of T and E₂ may not have played a major role in culture conditions.

Immunohistochemistry showed that E₂ and androgen treatment markedly altered the percentage of ER α - and AR-positive cells in mammary epithelium. The androgen treatment increased the proportion of AR-positive cells, whereas ER α -expressing cells were strongly decreased. The ER β -expressing cells remained unaltered. Treatment with E₂ increased the number of ER α -positive cells, whereas those of ER β - and AR-positive cells decreased. In the combination cultures, E₂ and the androgens opposed each other. The altered proportions of steroid receptor-positive cells in the E₂ and androgen treatments could be explained by changes in receptor gene expression and/or the enrichment or reduction of receptor-positive cells, due to changes in proliferation, and/or apoptosis of selected cell populations. DHT has been found to increase AR expression (30) and decrease ER α expression in primate mammary glands *in vivo* (11) and in human breast cancer cells (8, 30). Our present experiments do not reveal the extent to which these mechanisms are involved in the changes in AR- and ER-positive cell percentages. The altered proportions of AR- and ER-expressing cells have an important role in E₂ stimulation and androgen repression of proliferation in mammary epithelial cells and in mammary gland response to hormonal therapies. Accordingly, one mechanism that could explain the androgen inhibition of proliferation is a strong decrease in ER α -positive cells. Whether AR-positive cells are able to divide in the mammary epithelium remains unknown, but recent reports demonstrated that androgens inhibit cyclin-D1 expression in MCF-7 cells in an AR-

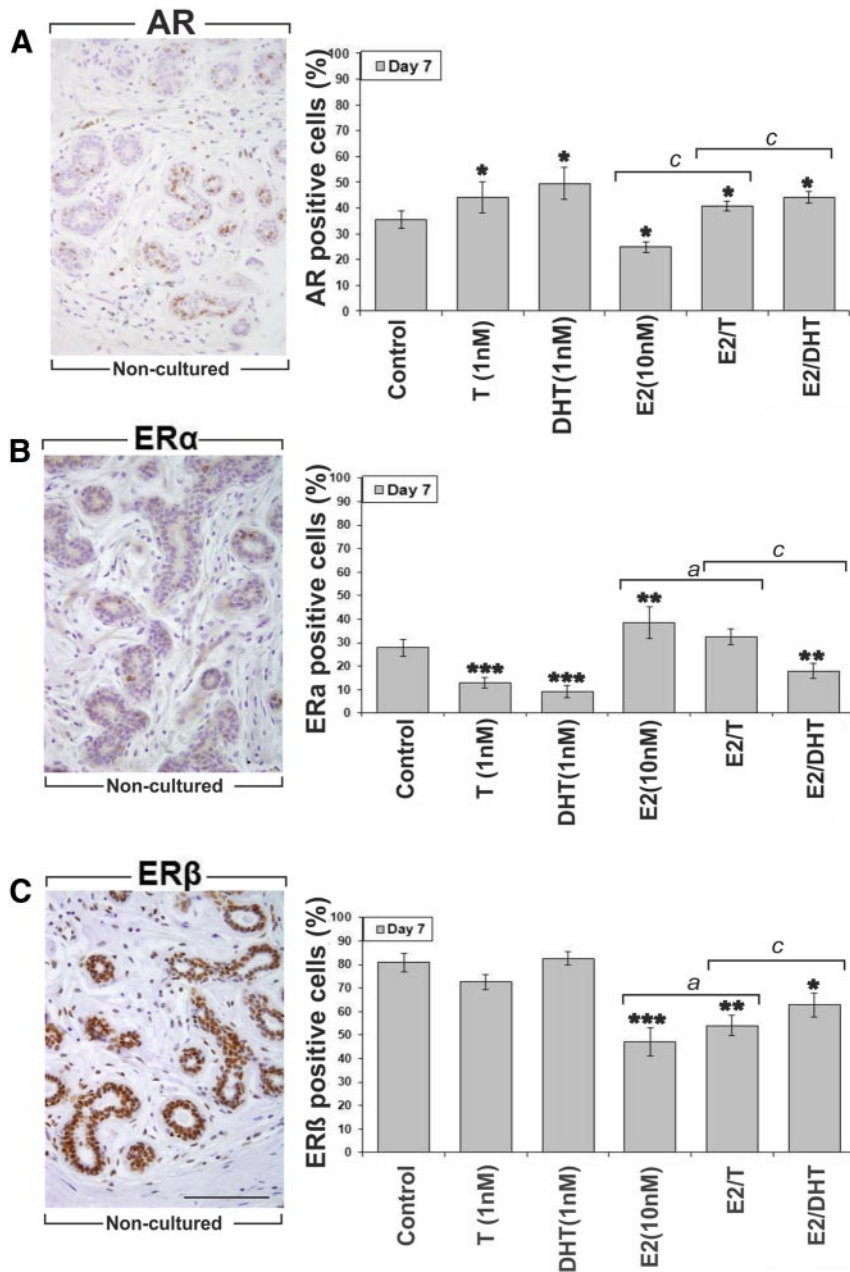


FIG. 6. The expression of AR, ER α , and ER β in noncultured and cultured HBT. Photomicrographs show the immunoreactivity for AR (A), ER α (B), and ER β (C) in noncultured HBT. Scale bar, 100 μ m. The scores of AR-, ER α -, and ER β -positive cells were calculated as percentages of positive cells. The columns show the mean \pm SD values ($n = 12$) for the AR-, ER α -, and ER β -immunopositive cells in the different treatment groups. The asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) indicate the statistical significance of differences between the treatment and control group.

dependent manner, which has been considered an explanation for androgen inhibition of proliferation in these cells (30).

An increase in ER α -positive cells was associated with stimulated proliferation and decreased apoptosis in E₂-treated explants. Conversely, decreased ER α positivity was linked to decreased proliferation and increased apoptosis in androgen-treated cultures. Previous studies (38) have shown that ER α -positive mammary epithelial cells

do not proliferate, whereas E₂-induced growth is mediated by paracrine stimulation of division in adjacent ER α -negative cells (38). A critical mediator of ER action is AREG (16), which was induced by E₂ in our HBT cultures. Because ER α -positive cells increased in the presence of E₂, it is likely that ER-negative cells rapidly and increasingly differentiate to ER α -positive cells after cell division.

Interestingly, the response of ER β -positive cell numbers to the T, DHT, and E₂ treatments seemed to resemble those of AR-positive cells, whereas the responses of ER α - and ER β -expressing cells were mostly the opposite. The function of ER β in breast tissue is largely unknown, but it is thought to have protective functions, similar to AR (39). Correspondingly, the expression of AR and ER β in tumor specimens has been associated with favorable outcomes in breast cancer patients (39).

Our results demonstrate that in addition to their direct inhibitory effects, T and DHT oppose the effects of E₂ on proliferation, apoptosis, and target gene expression in HBT, as previously reported in various experimental *in vivo* and *in vitro* models (6, 11). The mechanisms by which androgens inhibit E₂ in breast tissue may include T- and DHT-induced decreases in ER α -positive cells and/or in ER α protein expression (11, 30) or increased apoptosis of ER α -positive cells. Peters *et al.* (40) recently suggested that AR strongly inhibits ER α activity by binding to estrogen response elements in a subset of ER target genes, thereby preventing ER activation of these genes. Another mechanism that was recently suggested for maintaining the balance between ER/AR actions is the suppression of AR target gene expression by cyclin-D1 (31).

In addition to controlling the cell cycle, cyclin-D1 has other functions including the modulation of nuclear receptor actions (31). Comstock *et al.* (31) recently reported that cyclin-D1 can block the binding of AR to a subset of target genes, thereby inhibiting androgen action. If corresponding mechanisms work in the mammary epithelium, then E₂-induced cyclin-D1

could effectively inhibit androgen regulation of selected target genes and their functions.

Normal breast tissue is exposed to considerable levels of androgens (35). In premenopausal women, they are circulating androgens, such as T and DHT. In postmenopausal women, these androgens are primarily formed from adrenal precursors in peripheral tissues (10, 35). Thus, androgens belong to the physiological environment of normal breast tissue, and they may effectively balance the stimulatory effects of estrogens (1). E₂ and E₂/progesterone treatments in postmenopausal women lead to decreased serum androgen levels by feedback mechanisms and other systemic effects, such as changes in SHBG production. This disrupts the balance between hormones and may contribute to a risk of excessive E₂ stimulation of the breast (1). Therefore, adding androgen to postmenopausal hormone therapy has been suggested (6). In clinical use, androgen treatment for hypoactive sexual desire is associated with several beneficial effects in postmenopausal women, such as improved sexual function, general well-being and positive effects on bone mineral density (10, 41). However, androgen use causes side effects, including virilizing effects (acne and hirsutism), adverse lipid changes, reduced insulin sensitivity, and an altered pattern of fat deposition (10, 41). High levels of serum androgens have also been suggested to provide increased precursor supplies for E₂ synthesis in aromatase reactions, leading to the stimulation of proliferation in breast and even increased risk of breast cancer (9). Considering the strong inhibition of E₂ effects by androgens, this mechanism may not markedly affect the safety of androgen therapy at the tissue level. The development of novel selective androgen receptor modulators, which retain antiproliferative effects on mammary epithelium but lack adverse effects, could provide an important component to breast protective hormone therapy in the future.

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