# Overexpression of 17β-Hydroxysteroid Dehydrogenase Type 1 Increases the Exposure of Endometrial Cancer to 17β-Estradiol

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**Context:** The local interconversions between estrone (low activity) and 17β-estradiol (potent compound) by 17β-hydroxysteroid dehydrogenases (17β-HSDs) can lead to high 17β-estradiol generation in endometrial cancer (EC).

**Objective:** Examine the balance between the 17β-HSDs reducing estrone to 17β-estradiol (types 1, 5, 12, and 7) and those oxidizing 17β-estradiol to estrone (2, 4, and 8), in EC.

**Patients and Methods:** Reducing and oxidizing 17β-HSD activities (HPLC) and mRNA level (RT-PCR) were assessed in normal post-menopausal (n = 16), peritumoral endometrium (normal tissue beside cancer, n = 13), and 58 EC (29 grade 1, 18 grade 2, 11 grade 3).

**Results:** Grade 1 EC displayed a shifted estrone reduction/17β-estradiol oxidation balance in favor of 17β-estradiol compared with controls. This was more pronounced among estrogen receptor-α (ER-α)-positive biopsies. Type 1 17β-HSD mRNA (*HSD17B1* gene expression, real time PCR) and protein levels (immunohistochemistry) were higher in ER-α-positive grade 1 EC than controls. The mRNA coding for types 4, 5, 7, 8, and 12 17β-HSD did not vary, whereas that coding for type 2 17β-HSD was increased in high-grade lesions compared with controls. Three-dimensional *ex vivo* EC explant cultures demonstrated that 17β-HSD type 1 generated 17β-estradiol from estrone and increased tumor cell proliferation. Additional *in vitro* studies using EC cells confirmed that in the presence of 17β-HSD type 1, estrone induced estrogen signaling activation similarly to 17β-estradiol. Therefore, estrone was reduced to 17β-estradiol.

**Conclusions:** Type 1 17β-HSD increases 17β-estradiol exposure in grade 1 EC, thus supporting tumor growth. This enzyme represents a potential therapeutic target. **(*J Clin Endocrinol Metab* 97: E591–E601, 2012)**



Endometrial cancer (EC) is the most common gynecological malignancy in the Western world. Type I ECs (referred as EC from now on) represent more than 80% of all cases, frequently have low-grade histology, good prognosis, are estrogen driven, and unopposed exposure to estrogens is a risk factor (1–3).

Because EC is usually diagnosed after menopause, when the ovaries have already ceased hormone produc-

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Abbreviations: CCN, Cyclin; 3D, three dimensional; EC, endometrial cancer; ER, estrogen receptor; ERE, estrogen response element; F, forward; 17β-HSD, 17β-hydroxysteroid dehydrogenase; R, reverse.

**TABLE 1.** Clinical features of patients and controls and overview of metabolic enzyme mRNA, protein (17β-HSD type 1 only), and activity data

| | | | | | | | | | | 17β-HSD type 1 expression | | | | | | mRNA levels | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Age (yr) | | FIGO stage | | | HPLC analysis | | | HSD17B1 mRNA | | | Protein | | | HSD17B2[a] | | AKR1C3[b] | HSD17B12[b] | CYP19A1[b] Positive |
| Tissue sample | n | Range | Mean ± SD | IA/IB | II | III[c] | n | Activity ratio | *P* | n | Mean | *P* | n | Mean | *P* | n | Mean | *P* | Mean | Mean | (%) |
| Postmenopausal controls | 16 | 50–85 | 63 ± 10 | — | — | — | 16 | 0.4 | — | 12 | 1.2 | ns | 7 | 1.5 | — | 12 | 2.8 | — | 7.3 | 1.1 | 23.0 |
| Peritumoral controls[d] | 13 | 50–75 | 64 ± 7 | — | — | — | 13 | 0.6 | ns | 12 | 3.2 | ns | 0 | nd | — | 12 | 1.8 | ns | 2.0 | 1.2 | 9.1 |
| Grade 1 EC | 29 | 50–91 | 68 ± 10 | 20 | 2 | 7 | 27 | 4.2 | <0.001 | 29 | 40.2 | 0.09 | 9 | 7.5 | <0.05 | 29 | 7.7 | 0.09 | 1.5 | 0.8 | 9.4 |
| Grade 1 EC ER-α | 23 | nd | nd | — | — | — | 21 | 5.0 | <0.001 | 23 | 48.9 | <0.05 | 8 | 8.5 | <0.01 | 23 | 7.0 | ns | 1.7 | 0.8 | 12.0 |
| Grade 2 EC | 18 | 50–84 | 64 ± 8 | 14 | 3 | 1 | 18 | 1.0 | ns | 17 | 2.2 | ns | 5 | 5.3 | ns | 17 | 7.8 | <0.05 | 6.2 | 0.9 | 22.2 |
| Grade 3 EC | 11 | 50–79 | 63 ± 10 | 8 | 3 | 0 | 10 | 0.6 | ns | 11 | 14.3 | ns | 3 | 2.9 | ns | 11 | 3.5 | ns | 31.1[e] | 0.7 | 18.2 |

*P* value for significant based on unpaired Wilcoxon test compared with postmenopausal controls. $P < 0.05$ is considered significant. Nonsignificant $P > 0.1$ (if $0.05 < P < 1.0$, value is shown). nd, Not determined; ns, not significant; FIGO, Fédération Internationale de Gynécologie et d'Obstétrique; —, nonapplicable.

[a] Due to technical problems, 10 matched controls (not 12) and nine grade 3 samples (not 11) could be assessed for type 2 17β-HSD.

[b] None were significantly different compared with postmenopausal controls.

[c] FIGO stages III, IVa, and IVb.

[d] Peritumoral controls consist of normal endometrium adjacent to tumors (10 grade 1, two grade 2, and one grade 3).

[e] The high mean value of AKR1C3 among grade 3 lesions is nonstatistically different from controls because it was determined by one outlier (after its removal, mean value is 11).

tion, and because patients do not always present symptoms of high systemic estrogen overexposure, steroids must be supplied to endometrial cells by the local metabolism. Local estrogen metabolism contributes maintaining tissue hormone levels irrespective of the ovarian activity, and tissue estrogen levels may differ from those in the serum (4–6).

Estrogens are produced at extraovarian sites via adrenal androgen aromatization (4, 7): androstenedione, one of the most abundant circulating steroids in postmenopausal women (4), and testosterone [generated from the reduction of androstenedione by 17β-hydroxysteroid dehydrogenase (17β-HSD) type 5] (8, 9) are converted by aromatase to estrone and 17β-estradiol, respectively. The balance between estrone and 17β-estradiol is controlled by (at least one of) four 17β-HSDs (types 1, 5, 7, and 12), reducing estrone (with little activity) to 17β-estradiol (potent estrogen). Conversely, type 2 17β-HSD (and possibly types 4 and 8) oxidizes 17β-estradiol to estrone (8–10).

Expression of these enzymes in the endometrium and endometrial disorders has been reported (7, 11–15). We have shown that reducing and oxidizing 17β-HSDs principally control estrone/17β-estradiol interconversions and determine the tissue estrogen levels in endometriosis (11). Nevertheless, to date, the contribution of the distinct enzymes to the generation of 17β-estradiol in EC is still controversial (7, 9, 11, 14, 16–18).

In the present study, we have examined the estrogen metabolism in EC. The net 17β-HSD activity is shifted toward the reducing metabolism in cancers. The key enzyme controlling this event is type 1 17β-HSD.

## Materials and Methods

### Patient biopsies

EC tissue (type I endometrioid endometrial adenocarcinoma) from 58 patients undergoing hysterectomy (29 grade 1, 18 grade 2, and 11 grade 3) was collected. None of the patients had used hormonal medication within the last 6 months before surgery. Additional patients' characteristics are given in Table 1. From 13 patients, a macroscopically normal endometrium sample was obtained (peritumoral tissue). Control endometrium was obtained from 16 postmenopausal women undergoing hysterectomy for endometrial nonmalignant indications (six cervical lesions, three myomas, six prolapses, one Lynch Syndrome mutation preventive hysterectomy). Biopsies were immediately frozen in liquid nitrogen.

Fifteen-micrometer-thick slices were cut from frozen biopsies and lysed for enzyme activity and mRNA isolation. To ensure the presence of endometrial material only in these lysates, histology was performed at the start and the end of the material cut for lysate preparation. A pathologist confirmed the presence and grade of EC or the presence of normal endometrium (healthy and peritumoural controls) in all materials used. Peritumoural normal endometrium was not hyperplastic.

Four additional tumor samples were used to establish *ex vivo* explant cultures [three dimensional (3D)] as described below. The protocols were approved by the local ethical committee in our medical center.

### *Ex vivo* 3D EC explant cultures

EC 3D explant cultures were prepared as described (19). After macroscopic evaluation of EC by a pathologist, a fragment of

tumor was immersed and rinsed in sterile PBS and chopped into 2-mm$^3$ cubes. Six to eight cubes (100 mg tissue) were cultured *ex vivo* in six-well plates using Millicell inserts (Millipore Corp., Bedford, MA) in RPMI 1640 medium without phenol-red containing sodium-pyruvate and L-glutamine (Invitrogen, Life Technologies, Inc., Carlsbad, CA) and supplemented with 5% hormone-stripped serum (c.c.pro GmbH, Neustadt, Germany). Culturing time and stimulations are indicated (please see *Results* and figures). Estrone and 17$\beta$-estradiol were purchased from Sigma-Aldrich Chemie BV (Zwijndrecht, The Netherlands).

## Enzyme activity

Frozen samples were homogenized with a blender in a radioimmunoprecipitation assay buffer (50 mM Tris-HCL, pH 7.4; 1% Nonidet P-40; 0.25% sodium deoxycholate; 150 mM NaCl; 1 mM EDTA), debris was removed by centrifugation, and protein concentration was determined (BC assay protein quantitation kit; Uptima-Interchim, Montlucon, France).

The activity of reducing and oxidizing 17$\beta$-HSDs was determined using a described HPLC-based method (11, 20). Reactions were carried out at 37 C for 24 h using 10–20 $\mu$g of protein lysates and 10 nmol estrone (reductive activities) or 17$\beta$-estradiol (oxidative metabolism). The used HPLC system (Shimadzu LC-10AD setup; Kyoto, Japan) consisted of a SIL-10ADvp autosampler, FCV-10ALvp gradient mixer, LC10Avp pump with high-pressure gradient mixing extension, DGU-14A degasser, and a RF-10Axl fluorescence detector. Labsolutions software (Shimadzu) was used for instrument control, data acquisition, and calculation of peak areas.

## Histology, immunohistochemistry, and immunofluorescence

Hematoxylin and eosin staining was used for histology evaluation of formalin-fixed and frozen sections. Immunohistochemistry for estrogen receptor (ER)-$\alpha$ on formalin-fixed or frozen material was performed as described (19) using antibody D-5 (1:100; Dako, Glostrup, Denmark). For 17$\beta$-HSD type 1 detection on formalin-fixed tissue, antigen was retrieved with 1 mg/ml pepsin (Sigma-Aldrich Chemie) and rabbit monoclonal antibody EP1682-Y (1:100; Epitomics, Burlingame, CA) was used. Placental tissue served as positive control. Heat-induced epitope retrieval in Tris-EDTA buffer and antibodies sc-751 (1:200 rabbit polyclonal; Santa Cruz Biotechnologies, Heidelberg, Germany) and AB3 (1:50; mouse monoclonal; Calbiochem, San Diego, CA) were used for cyclins (CCN) A2 and CCND1 on formalin-fixed sections. Heat-induced epitope retrieval in citrate buffer and mouse monoclonal antibody Ki-S5 (1:100; Santa Cruz Biotechnologies) were used for Ki67 staining. Chemate Envision and 3,3-diaminobenzidine solution (Dako) were used to visualize antibody binding. Stainings were scored by two independent observers (A.R. and B.D.), and staining indexes were calculated as described (21).

For immunofluorescence, cells were methanol fixed on microscope cover glasses. Antihuman 17$\beta$-HSD type 1 EP1682-Y (1:1000; rabbit monoclonal; Epitomics) and swine-antirabbit IgG-fluorescein isothiocyanate (1:100, Dako) were used.

For all immunostainings, the omission of the primary antibody was used as negative control.

## RNA isolation, cDNA synthesis, and real-time PCR

Molecular biological techniques were performed with standard, manufacturer's recommended, or previously described protocols (11, 19, 22, 23). In short, RNA was isolated with the TRIzol reagent (Invitrogen, Carlsbad, CA) and assessed spectrophotometrically for quantity and purity (260:280 nm and 260:230 nm ratios), and cDNA was synthesized with the iScript cDNA synthesis Kit (Bio-Rad, Hercules, CA). Primers for PCR amplification were (5′ to 3′): *HSD17B1*, forward (F) GTTTATTGCGCCAGCAAGTT, reverse (R) CCCAACACCTTCTCCATGA; *HSD17B2*, F, TCAAAACTCCGGCAAATACC, R, ACCTGTGGATCAGAAGGCAG; *AKR1C3* (*HSD17B5*), F, AATGAGCAGCGCATCAGAC, R, GGGTGGCTAGCAAAACTATCA; *HSD17B12*, F, GTCTGGGGAGTGGGGAAT, R, CCTTCATTCCATGCTTTGCT; *CYP19A1*, F, ATGCGAGTCTGGATCTCTGG, R, GCCTTTCTCATGCATACC; housekeeping gene *β-actin*, F, GCCAACCGCGAGAAGATGAC, R, GATGGGCACAGTGTGGGTGAC; housekeeping *cyclophilin A*, F, CCGTGTTCTTCGACATTGCCGT, R, AATCCTTTCTCTCCAGTGCTCAGA. Real-time PCR was performed using the Sybr-green ABGene system (ABGene Ltd., Epsom, UK) and the Bio-Rad MyIQ apparatus.

## Cell lines, plasmids, and cell transfection

Protocols were previously described (19, 22, 23). The ECC1 endometrial cancer cell line (American Type Culture Collection, Manassas, VA), being estrogen responsive and well characterized in our laboratory, was maintained as described (22, 23). Three days before and during hormone stimulations (conditions indicated in *Results* and figures), cells were cultured in RPMI 1640 without phenol-red containing sodium-pyruvate and L-glutamine (Invitrogen, Life Technologies) supplemented with 5% hormone-stripped serum (c.c.pro GmbH).

Transient and stable transfection of ECC1 cells were performed using jetPEI (Q-Biogene, Heidelberg, Germany). Stable transfected ECC-HSD clones were obtained by hygromycin-B (Invitrogen) selection. Clones originated from one single cell (monoclonal) were isolated using serial dilutions on 96-well plates and microscopy examination. This procedure was repeated twice.

Expression plasmids for 17$\beta$-HSD types 1 and 12 were described previously (24). The expression plasmid for 17$\beta$-HSD type 5 (25) was a gift from Professor Penning (University of Pennsylvania, Pittsburgh, PA), whereas the ERE-TK-luciferase plasmid (ERE-LUC, containing the luciferase reporter gene under and estrogen response element (ERE), which is directly activated by estrogens via ER-$\alpha$) (22, 23) was a gift from Professor Schuele (Freiburg University, Freiburg, Germany).

For the luciferase assay, cells were transfected in 96-well plates, stimulated (conditions are indicated in *Results* and figures) and assayed with the Steady-GLO substrate and the GloMax 69 microplate luminometer (Promega GmbH, Mannheim, Germany). Plasmid pSV-$\beta$-galactosidase was cotransfected and used for transfection efficiently normalization by $\beta$-galactosidase spectrophotometric assay (19, 22, 23).

## Western blot analyses

Western blotting was performed using standard protocols. Equal amounts of proteins (concentration determined by BC Assay protein quantitation kit; Uptima-Interchim) were loaded, and efficiency of blotting on the membrane was confirmed with

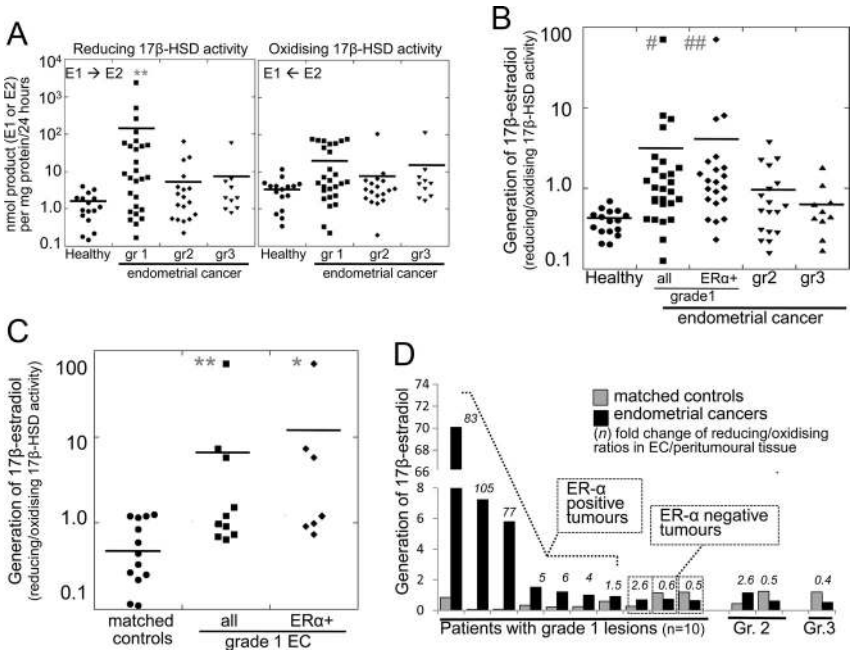


**FIG. 1.** Grade 1 EC generate more 17β-estradiol from estrone than controls. A, The activity of reducing and oxidizing 17β-HSDs was determined in healthy postmenopausal women (16), grade 1 (27), grade 2 (18), and grade 3 (10) EC. Mean values are indicated. Gr, Grade. B, The ratio of the 17β-HSD reducing *vs.* oxidizing enzyme activity, which represents the ability of each sample to generate 17β-estradiol from estrone, is shown on the chart for healthy postmenopausal women (16), grade 1 [all (27) or ER-α positive biopsies (21)], grade 2 (18), and grade 3 (10) EC (grade 2 and 3, irrespective of ER-α status). Mean values are indicated. C, The ability to generate 17β-estradiol (reducing *vs.* oxidizing enzyme activity ratio) is shown for 13 peritumoural endometrial biopsies (matched controls) and 10 corresponding grade 1 EC (seven ER-α positive). Mean values are indicated. Three tumor biopsies adjacent to the peritumoural tissues consist of high-grade lesions and their ability to generate 17β-estradiol does not differ from peritumoural controls (these data are shown in D). D, The ability to generate 17β-estradiol is shown for the 13 peritumoural (matched controls) and the corresponding adjacent EC for each patient. ER-α positive and -negative biopsies for grade 1 tumors are indicated. *Italicized numbers at the top* indicate the fold change of the reducing/oxidizing 17β-HSD activity (generation of 17β-estradiol) in the tumor *vs.* the adjacent peritumoural tissue. *P* values for statistical significance compared with healthy postmenopausal controls (A and B) or with matched controls (C) were calculated using the unpaired Wilcoxon-test. *, $P < 0.05$; **, $P < 001$; #, $P < 0.001$; ##, $P < 0.0001$.

Ponceau staining (Sigma-Aldrich Chemie). For CCNA2 and CCNE1, rabbit polyclonal sc-751 and mouse monoclonal sc-347 antibodies were used, respectively (both 1:500; Santa Cruz Biotechnologies). For 17β-HSD type 1, rabbit monoclonal antibody EP1682-Y (1:1000; Epitomics) was used. For 17β-HSD types 5 and 12, goat polyclonal antibody ab27491 (1:5000; Abcam, Cambridge, UK) and mouse polyclonal antibody A01 (1:5000; Abnova, Heidelberg, Germany) were used. As loading control, mouse monoclonal antihuman β-actin antibody AC-15 (Sigma-Aldrich Chemie) was used. Horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, Inc., Suffolk, UK) and the Western Lightning Ultra (PerkinElmer, Waltham, MA) were used for signal detection. Band intensities were quantified with the ImageJ program (http://rsb.info.nih.gov/ij/).

### Statistics

Statistical values were computed using the KaleidoGraph package (Synergy Software, Reading, PA). Patient data on enzyme activity and mRNA levels (continuous, nonparametric, and nonnormally distributed variables) were analyzed using the Wilcoxon statistic test. Studied groups were compared with controls as indicated in the figure legends. Replicates of *in vitro* experiments were analyzed with the Student *t* test by comparison with the control condition as indicated in the figure legends.

### National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) accession numbers

The genes and accession numbers include the following: type 1 17β-HSD, gene name *HSD17B1*, gene identification 3292; type 2 17β-HSD, gene name *HSD17B2*, gene identification 3294; type 4 17β-HSD, gene name *HSD17B4*, gene identification 3295; type 5 17β-HSD, gene name *AKR1C3*, gene identification 8644; type 7 17β-HSD, gene name *HSD17B7*, gene identification 51478; type 8 17β-HSD, gene name: *HSD17B8*, gene identification 7923; type 12 17β-HSD, gene name *HSD17B12*, gene ID 51144; and aromatase, gene name *CYP19A1*, gene identification 1588.

## Results

### EC have higher 17β-HSD reducing/oxidizing balance than controls

The enzyme activity of the reducing and the oxidizing 17β-HSDs was measured in EC biopsies (n = 58) and the control healthy postmenopausal tissues (n = 16). The reducing activity was 90-fold higher in the grade 1 EC than in controls, and it tended to return to normal levels (as in noncancerous tissue) in the high-grade EC. A similar trend, although nonsignificant, was found for the activity of the 17β-HSD-oxidizing (deactivating) 17β-estradiol (Fig. 1A).

Because the individual oxidizing and reducing activities can reflect differences in the prevalence of epithelial and stromal cells in the tissue and because the interconversions between 17β-estradiol and estrone determine the final tissue level of 17β-estradiol, the ratio between estrone reducing and 17β-estradiol oxidizing 17β-HSD enzyme activities was calculated. In healthy tissues, this balance favored estrone formation (Fig. 1B and Table 1). In contrast, 17β-HSD enzyme activity balance was shifted toward a preference for 17β-estradiol formation in grade 1 EC but not in grade 2 and grade 3 lesions. This shifted balance was more pronounced in ER-α-positive grade 1 biopsies.

For 13 patients, tumor and peritumoural (*i.e.* normal endometrium adjacent to cancer) tissues were obtained. The estrone-reducing to 17β-estradiol-oxidizing ratio in

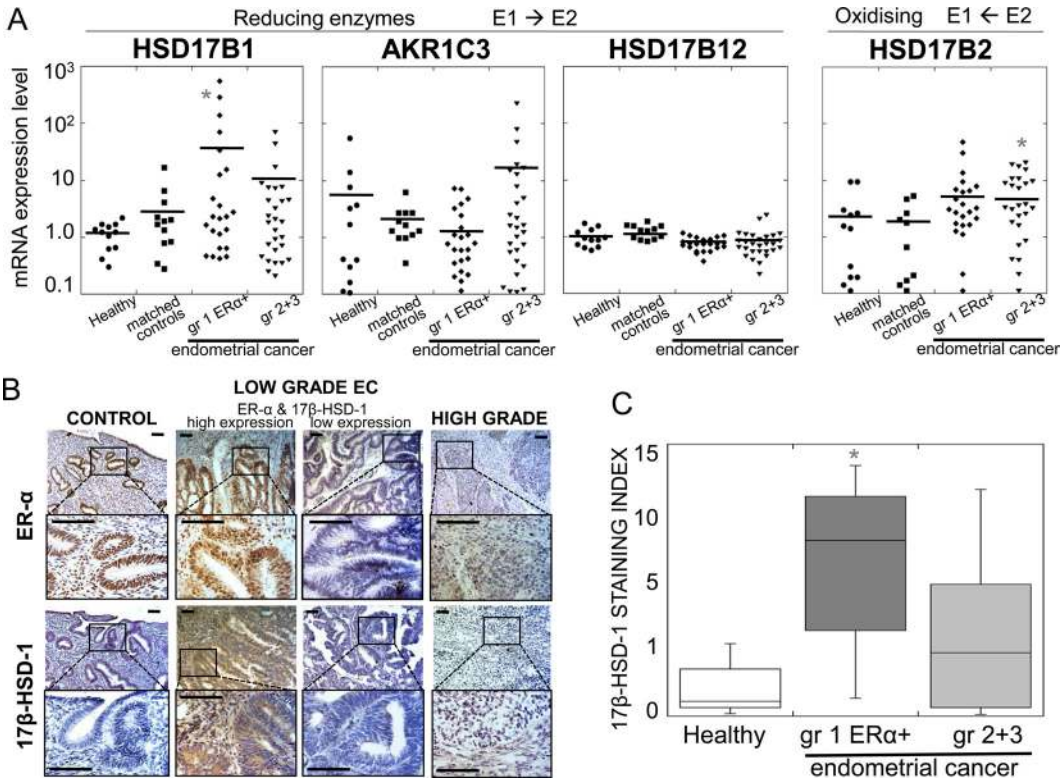


**FIG. 2.** *HSD17B1* mRNA is up-regulated in grade 1 EC expressing ER-$\alpha$. A, The *HSD17B1*, *AKR1C3*, and *HSD17B12* mRNA levels [coding for 17$\beta$-HSD types 1, 5, and 12, reducing estrone (E1) to 17$\beta$-estradiol (E2)], and the *HSD17B2* mRNA level (coding for 17$\beta$-HSD type 2, oxidizing 17$\beta$-estradiol to estrone) were determined by real-time PCR in endometrial biopsies from healthy postmenopausal women (12), peritumoural normal endometrium (12 matched controls; only 10 could be assessed for 17$\beta$-HSD type 2), grade 1 ER-$\alpha$-positive (23), and pooled grade 2 (17) and grade 3 (n = 11; nine could be assessed for 17$\beta$-HSD type 2) endometrial cancers. Mean values are indicated. gr, Grade. B, Representative immunohistochemistry for ER-$\alpha$ and 17$\beta$-HSD type 1 in normal postmenopausal control, low-grade EC with high (*left*) or low (*right*) expression of both ER-$\alpha$ and 17$\beta$-HSD type 1, and high-grade EC. *Bar*, 100 $\mu$m. C, Quantification of the 17$\beta$-HSD type 1 staining indexes in controls (n = 7), grade 1 ER-$\alpha$-positive (n = 8), and high-grade EC (five grade 2 and two grade 3). Mean values are indicated. *, $P < 0.05$. Statistic is based on the unpaired Wilcoxon test compared with the healthy postmenopausal controls. Results for grades 2 and 3 EC separately and for all grade 1 (ER-$\alpha$ positive and negative) are given in Table 1.

peritumoural tissues was similar to that of healthy controls (0.61 and 0.41, respectively; Table 1) but was significantly higher in the adjacent EC tissues (Fig. 1C). Next, the estrone-reducing to 17$\beta$-estradiol-oxidizing ratios calculated for the tumor and the corresponding peritumoural tissue in each patient were compared. In this way, the ability to generate 17$\beta$-estradiol of the normal tissue and the tumor, both located in the same uterus, could be assessed. Endometrial grade 1 lesions with ER-$\alpha$-positive cells showed the strongest preference for 17$\beta$-estradiol formation compared with the normal tissue (matched controls; Figure 1D). The estrone-reducing to 17$\beta$-estradiol-oxidizing ratios in tumor and peritumoural tissue were similar (or in favor of estrone formation) among grade 1 ER-$\alpha$-negative and high-grade EC.

## Type 1 17$\beta$-HSD is responsible for the estrone to 17$\beta$-estradiol conversion

Next, we wondered which specific 17$\beta$-HSD is involved in the observed shifted metabolism. Types 1, 5, and 12 are reducing 17$\beta$-HSDs reported to convert estrone to 17$\beta$-estradiol, whereas type 2 inactivates 17$\beta$-estradiol to estrone (8, 9). Real-time PCR was used the measure the gene expression of *HSD17B1*, *AKR1C3*, and *HSD17B12* (coding for types 1, 5, and 12 17$\beta$-HSD), and the expression of *HSD17B2*, coding for the oxidizing type 2 17$\beta$-HSD. *HSD17B1* mRNA was up-regulated in ER-$\alpha$-positive grade 1 EC (n = 23) compared with controls (n = 12; Fig. 2A), and significance was borderline when all grade 1 tumors (ER-$\alpha$ positive and negative; n = 29) were considered (Table 1). The level of *HSD17B1* did not vary among high-grade EC (n = 28), and those of *AKR1C3* and *HSD17B12* (reducing enzymes) were similar between cancers and controls (Fig. 2A and Table 1). The *HSD17B2* mRNA level was higher among grade 2 (and nonsignificantly among grade 1) tumors compared with controls (Fig. 2A and Table 1).

Because recent studies have excluded the involvement of types 4, 7, and 8 17$\beta$-HSD in estrogen metabolism (see *Discussion*), the transcript level of the corresponding genes (*HSD17B4*, *HSD17B7*, and *HSD17B8*) was as-

sessed among six controls and 18 EC only (10 grade 1 and eight grade 2 or 3 EC). Expression did not vary (results not shown).

Next, the protein expression of type 1 17β-HSD was assessed among randomly selected controls (n = 7), grade 1 (n = 9), and high-grade EC (n = 8). Type 1 17β-HSD was localized in the cytoplasm and staining was stronger in epithelial than stromal cells. In addition, its expression was strong when ER-α was expressed as well (Fig. 2B). Protein data confirmed mRNA results. Grade 1 EC showed increased type 1 17β-HSD immunoreactivity compared with controls, whereas high-grade lesions did not differ significantly from controls (Fig. 2, B and C, and Table 1).

Some authors have reported higher aromatase (*CYP19A1*) expression in EC than controls (7); therefore, we also assessed *CYP19A1* mRNA level by PCR. Expression was low and not quantifiable by RT-PCR (cycle threshold values above 36). Therefore, biopsies were classified simply as positive or negative. The percentage of biopsies expressing *CYP19A1* mRNA ranged between 9 and 23% and did not vary among EC and controls (Table 1). Additionally, *CYP19A1* and *AKR1C3* mRNA levels did not correlate.

### Type 1 17β-HSD generates 17β-estradiol from estrone *ex vivo*

To further prove the role of type 1 17β-HSD in the reduction of estrone to 17β-estradiol in EC, *ex vivo* tumor explant cultures were prepared (Fig. 3A). This 3D model is relevant to assess short-term hormonal responses because it maintains the *in vivo* tissue integrity. Cultures

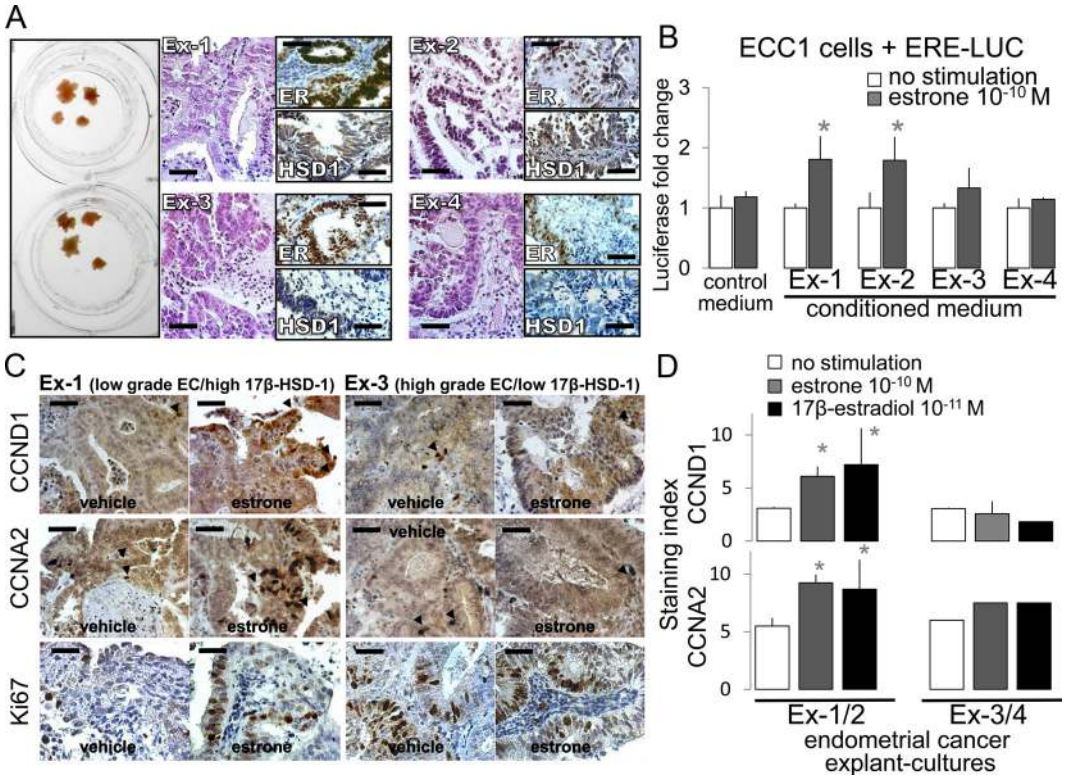


**FIG. 3.** Grade 1 ECs generate 17β-estradiol from estrone *ex vivo* A, 3D *ex vivo* explant cultures (*left panel*) were prepared from four EC biopsies, two derived from grade 1 EC (Ex-1 and Ex-2) and two derived from high-grade tumors (Ex-3 and Ex-4). The hematoxylin and eosin staining for histology as well as immunohistochemistry for ER-α and 17β-HSD-1 are shown at the *right*. *Bar*, 100 μm. B, ECC1 cells were transiently transfected with the ERE-LUC construct and (36 h later) stimulated overnight with either control medium supplemented with vehicle (no stimulation) or $10^{-10}$ M estrone or with media supplemented with vehicle or with $10^{-10}$ M estrone that were previously conditioned (*i.e.* used to culture for 2 h the *ex vivo* EC explants described in A). *Bars* represent the fold change in the luciferase activity. Values were normalized with β-galactosidase expression. Mean and SD are based on four replicates. *Asterisks* indicate $P < 0.05$ (*t* test) compared with no stimulation. Results were reproduced in two additional independent experiments. C, CCND1 and CCNA2 expression (nuclear staining; *arrowheads*) as well as Ki67 expression (proliferation control) was assessed by immunohistochemistry in the *ex vivo* explant cultures described in A after 24 h of stimulation with estrone $10^{-10}$ M or with vehicle alone. Representative pictures are shown. *Bar*, 100 μm. D, Quantification of the staining indexes (21) for CCND1 and CCNA2 (representative images shown in C) in the four explant cultures described in A. Staining indexes were first calculated by two observers (A.R. and B.D.) in four regions (a, b, c, d) of each sample. Three samples per CCND1 and three for CCNA2 were assessed for each explant culture: untreated (U-a, b, c, d), estrone treated (E1-a, b, c, d), or 17β-estradiol treated (E2-a, b, c, d); this last was used as positive control. Values of the two observers in each region were averaged (A.R./B.D.-Ua = average of A.R.-U-a and B.D.-U-a, *etc.*), and the mean values of the four regions per sample were computed: average A.R./B.D. (Ua, Ub, Uc, and Ud), *etc.*, which yield the staining index of Ex-1, Ex-2, Ex-3 and Ex-4, treated (E1), untreated (U), and positive control (E2). The *bars in the chart* show the average staining index of the two low-grade Ex-1/Ex2 (±SD) and the high-grade Ex-3/Ex-4 (±SD) tumors. *Asterisks* indicate $P < 0.05$ (*t* test) compared with no stimulation.

were prepared from four ECs: Ex-1 and Ex-2 were derived from low-grade tumors, Ex-3 and Ex-4 from high-grade lesions. All cultures were ER-$\alpha$ positive, but low-grade Ex-1 and Ex-2 had higher type 1 17$\beta$-HSD protein level than high-grade samples (Fig. 3A). To assess the ability of the tissues to reduce estrone, explant cultures were stimulated with vehicle or $10^{-10}$ M estrone for 2 h, and culture medium was harvested: preliminary tests had indicated 2 h as suitable to measure estrone to 17$\beta$-estradiol conversion. The media conditioned in the explant cultures were subsequently used to stimulate overnight ECC1 cells that were transiently transfected with the ERE-LUC construct. This construct contains the luciferase reporter gene under an ERE that is directly activated by estrogens via ER-$\alpha$. Because estrone, in contrast to 17$\beta$-estradiol, does not activate this promoter (Fig. 3B, control medium), the luciferase activity is a measurement of the conversion of estrone to 17$\beta$-estradiol that occurred in medium conditioned by each explant culture (note that ECC1 cells are estrogen responsive but do not convert estrone to 17$\beta$-estradiol; see below). Medium conditioned in Ex-1 and Ex-2 (grade 1 lesions and high type 1 17$\beta$-HSD level) activated the ERE-LUC (Fig. 3B), indicating conversion of estrone to 17$\beta$-estradiol. This was not observed for the high-grade lesions with a low level of type 1 17$\beta$-HSD (Ex-3 and Ex-4).

The physiological response of endometrial cells to estrogens is first the up-regulation of a number of cyclins [the transcription of CCNA2, CCND1, CCNB1, and CCNE1 is directly induced by ligand-activated ER-$\alpha$) ( 22, 26, 27)], followed by cell proliferation. To confirm that the 17$\beta$-estradiol generated from estrone will therefore activate the complete estrogen signaling and ultimately lead to cell proliferation, explant cultures were maintained (24 h) in the presence of vehicle or estrone ($10^{-10}$ M), and CCNA2 and CCND1 expression was determined by immunohistochemistry. In Ex-1 and Ex-2, estrone induced cyclin expression (Fig. 3, C and D), confirming its conversion to 17$\beta$-estradiol. In contrast, estrone did not affect cell growth in high-grade lesions (Ex-3 and Ex-4). Proliferation was confirmed by Ki67 staining. In Ex-1 and Ex2, approximately 10–15% on nuclei were Ki67 positive with no stimulation and clearly increased to about 40–50% upon estrone stimulation. The Ki67 positive nuclei (~40%) remained constant in Ex-3 and Ex-4 in the presence or absence of estrone (Fig. 3C).

### Type 1 17$\beta$-HSD converts estrone into a potent activator of estrogen signaling *in vitro*

We next confirmed that 17$\beta$-HSD type 1 can reduce estrone *in vitro* using the endometrial cancer cell line ECC1. This line is estrogen responsive (ER-$\alpha$ positive) but

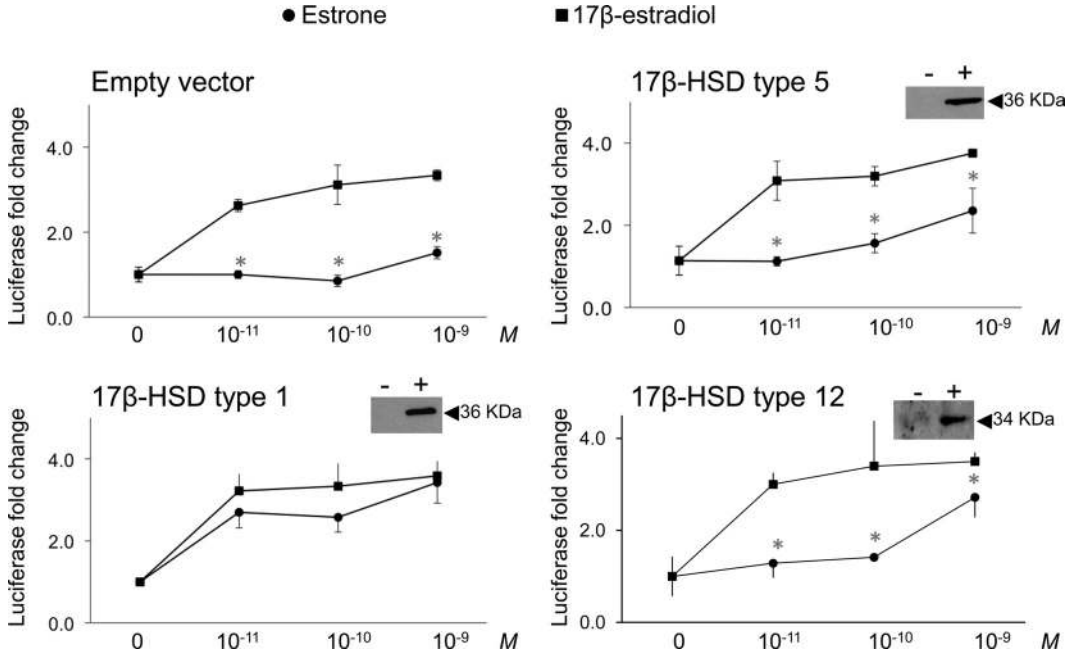


**FIG. 4.** Type 1 17$\beta$-HSD converts estrone into a potent activator of estrogen signaling *in vitro*. Endometrial cancer ECC1 cells were transiently transfected with the expression plasmids for 17$\beta$-HSD types 1, 5, and 12 or with empty vector along with the ERE-LUC construct. Forty-eight hours after transfection, cells were stimulated overnight with increasing concentrations of estrone or 17$\beta$-estradiol ($10^{-11}$ to $10^{-9}$ M). Luciferase activity was measured and values were normalized with $\beta$-galactosidase. Each point was measured in triplicate (mean ± SD is shown). *Asterisks* indicate $P < 0.05$ (*t* test) compared with the luciferase induction obtained using corresponding 17$\beta$-estradiol concentrations. The *panels at the top of each graph* indicate the expression of the transfected cDNA by Western blot. The same amounts of total protein were loaded in each lane, as determined by protein concentration and confirmed by Ponceau staining after blotting. +, Transfection with 17$\beta$-HSD cDNA (indicated type); −, empty vector-transfected cells. Results were reproduced in two additional independent experiments.

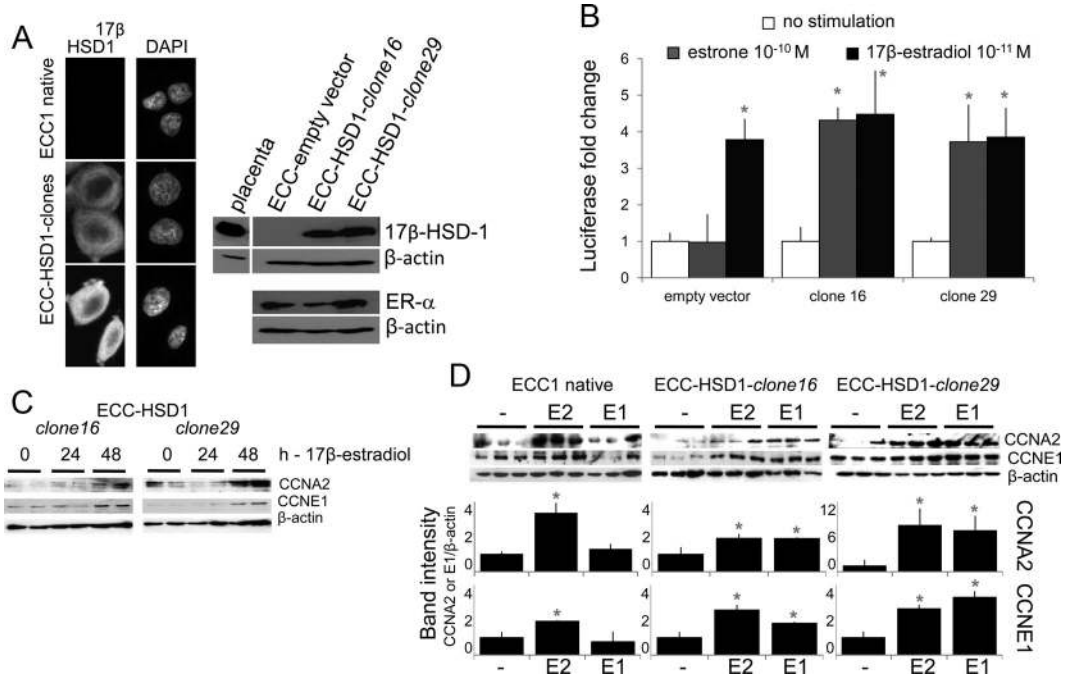


**FIG. 5.** Type 1 17β-HSD converts estrone into a potent inducer of cyclin expression in EC cells. A–C, Characterization of ECC1 cells stably transfected and expressing type 1 17β-HSD: ECC-HSD1 clones. A, *Left panels*, Immunofluorescence of native ECC1 cells (type 1 17β-HSD negative) and two ECC-HSD1 clones expressing type 1 17β-HSD. *Right panels*, Expression of type 1 17β-HSD and ER-α was determined by Western blot on empty vector-transfected ECC1 cells and ECC-HSD1-clone16 and -clone29, used for further analyses in B–D. Placenta tissue was used as type 1 17β-HSD positive control. B, ECC-HSD1-clone16 and -clone29 were transiently transfected with ERE-LUC and 36 h later were stimulated overnight with vehicle (no stimulation), $10^{-10}$ M estrone, or $10^{-11}$ M 17β-estradiol, and luciferase activity was measured. Values were normalized with β-galactosidase. Mean ± SD are based of triplicates, and *asterisks* indicate $P < 0.05$ (*t* test) compared with no stimulation. C, ECC-HSD1-clone16 and -clone29, like native cells (not shown), respond to 17β-estradiol ($10^{-11}$ M) with cell cycle progression (22, 26, 27), indicating that selected clones have a functioning estrogen signaling machinery. D, Western blot was used to determine the expression of CCNA2 and CCNE1 in ECC1 native cells, ECC-HSD1-clone16 and -clone29, after stimulation (48 h) with vehicle (−), $10^{-11}$ M 17β-estradiol (E2), or $10^{-10}$ M estrone (E1). Images of the blots are shown at the *top*. The charts at the *bottom* report the band intensities of the cyclins normalized with β-actin. Mean ± SD are based of three replicates per treatment, and *asterisks* indicate $P < 0.05$ (*t* test) compared with no stimulation. Results were reproduced in a second independent experiment.

unable to interconvert estrogens (HPLC data, not shown) because it does not express most estrogen metabolic enzymes like 17β-HSD types 1, 5, and 12 (Fig. 4) as well as types 2 and 7 17β-HSD and aromatase (RNA data, not shown).

Types 1, 5, and 12 17β-HSD were expressed in ECC1 cells by transient transfection along with the ERE-LUC construct. When ECC1 cells were transfected with the empty vector (used as control), stimulation with increasing concentrations of estrone ($10^{-11}$ to $10^{-9}$ M) did not result in any significant increase of the luciferase activity (Fig. 4), whereas similar concentrations of 17β-estradiol, as expected, induced strong activation of the luciferase. Under these experimental conditions, conversion of estrone to 17β-estradiol would result in luciferase activity. Expression of 17β-HSD type 1 resulted in conversion of estrone to 17β-estradiol (estrone and 17β-estradiol similarly activated the ERE-LUC), whereas 17β-HSD types 5 and 12 resulted in minor ERE-LUC activation at high estrone concentrations only (Fig. 4).

### In presence of type 1 17β-HSD, estrone induces cyclin expression *in vitro*

The role of 17β-HSD type 1 was further assessed using stably transfected ECC1 cells (ECC-HSD1). A number of ECC1-HSD1 clones with different 17β-HSD type 1 expression levels (immunofluorescence and Western blot; Fig. 5a) were characterized. ECC-HSD1-clone16 and -clone29 were selected for further analyses because 17β-HSD type 1 protein (Fig. 5A) and enzyme activity levels (HPLC; results not shown) were similar to those of placenta and other human tissues and because they still expressed ER-α (Fig. 5A). Type 1 17β-HSD expression and estrogen responsiveness were confirmed by the ability of estrone to activate the ERE-LUC similar to 17β-estradiol (Fig. 5B).

Next, the ability of estrone to fully activate estrogen signaling by inducing cyclin expression was assessed by measuring CCNA2 and CCNE1 level (22, 26, 27). Figure 5C confirmed that monoclonal selection/expansion of ECC-HSD1 clones did not affect their ability to up-regulate these cyclins upon estrogen stimulation.

In untransfected (or empty vector transfected; not shown) ECC1 cells, $10^{-11}$ M 17β-estradiol, but not estrone ($10^{-10}$ M), induced CCNA2 and CCNE1 protein expression (48 h stimulations; Fig. 5D). However, stimulation of ECC-HSD1-clone16 and ECC-HSD1-clone29 with estrone resulted in up-regulation of the cyclins comparably with that observed after stimulation with 17β-estradiol.

## Discussion

The conversion of estrone to 17β-estradiol was examined in EC. The activity ratio of the reducing to oxidizing 17β-HSD was higher in grade 1 EC than in healthy postmenopausal endometrium and peritumoural tissues, thus leading to higher local 17β-estradiol generation from estrone. This feature was more pronounced among ER-α-positive biopsies. This suggests that in estrogen-responsive EC cells, generally well differentiated and with low-grade histology, increased expression of 17β-HSD type 1 and 17β-estradiol synthesis provides a growth advantage. The fact that reducing to oxidizing 17β-HSD ratio and 17β-HSD type 1 level did not vary among high-grade lesions, which often do not express ER-α and are insensitive to estrogens, strengthen this conclusion. Interestingly, a recent publication has clearly indicated that local estrogen metabolism is altered in low-grade EC (3).

As an additional evidence of high 17β-estradiol exposure in the patients' tumor microenvironment, we have observed that the majority of the ECs (nine of 11 tested) had higher mRNA level of the estrogen-responsive gene trefoil factor 1 (*TFF1*) compared with the corresponding matched peritumoural tissue (data not shown).

### Type 1 17β-HSD determines elevated levels of 17β-estradiol

Literature is controversial regarding which 17β-HSD is responsible for the 17β-estradiol generation and may be a therapeutic target. Types 1, 5, 7, and 12 17β-HSD were reported to reduce estrone to 17β-estradiol (8, 9). Although initial investigations detected no/very low expression (mRNA and protein) of type 1 17β-HSD in normal and malignant endometrium (7), two recent studies described a down-regulation of *HSD17B1* mRNA in EC compared with peritumoural tissues (13, 28). Both studies also assessed the mRNA level of *HSD17B7* and *HSD17B12*. *HSD17B7* was down-regulated in cancers in the first study (13) and unchanged in the other (28), whereas *HSD17B12* did not vary in the first (13), but it was up-regulated in malignant tissue in the second study (28). Also regarding *AKR1C3* (type 5 17β-HSD), elevated (7, 15), equal (13), and lower mRNA (14) in EC than controls have been reported.

Similarly, regarding the oxidative metabolism, reduced (7), no variation (13), or increased *HSD17B2* mRNA in EC compared with controls (28) have all been described.

In these studies, no selection of patients occurred. However, large interindividual variations in estrogen metabolism as well as in the levels of circulating steroids exist (present study and Refs. 3, 11, and 28). Therefore, it is important to stratify the specimens with confirmed unbalanced enzyme conversion, like in our sample collection, to identify the relevant enzyme. Our data indicate that 17β-HSD type 1 (*HSD17B1* mRNA) was up-regulated only in the group of EC with high estrone to 17β-estradiol conversion and ER-α expression. RNA data were confirmed at the protein level. In line with a previous study (28), *HSD17B2* mRNA, coding for type 2 17β-HSD oxidizing 17β-estradiol, was increased in grade 2 lesions. The biological significance of this event has to be explored. *HSD17B12* and *AKR1C3* levels did not vary significantly between the EC and controls, although a nonsignificant increase of *AKR1C3* was observed among the grade 3 lesions (Table 1). Because types 4, 7, and 8 17β-HSD, initially reported to oxidize (types 4 and 8) or reduce (type 7) estrogens, have been more recently assigned to the fatty acid/cholesterol metabolism (8–10), their mRNA level was assessed in a limited group of samples and did not vary between EC and controls.

The 3D *ex vivo* EC model confirmed that ECs generate 17β-estradiol from estrone and enhance proliferation through 17β-HSD type 1. This was substantiated by *in vitro* data on ECC1 cells, which, however, do not completely exclude that types 5 and 12 17β-HSD can reduce estrone at high concentration (above $10^{-9}$ M; Fig. 4), as indicated previously (16, 29–31). Estrone serum levels range between 15 and 20 pg/ml (around $10^{-11}$ M) and can peak up to $10^{-10}$ M in a minority of EC patients (3). Therefore, types 5 and 12 17β-HSD could generate 17β-estradiol in some patients with extremely high (serum/tissue) estrone levels.

### Other steroidogenic enzymes

From the present study, it can be concluded that local aromatase plays little role in estrogen generation in EC, which is in line with other studies in endometrial disorders (11, 17), EC (6, 13, 28, 32), and endometrial cell lines (30). The sulfatase pathway controls estrogen availability in EC (13, 28, 33), and its inhibition has shown promising results (34, 35). Here the sulfatase pathway was not addressed.

## Conclusions

The key enzyme determining high exposure to 17β-estradiol among, at least, low-grade EC is type 1 17β-HSD. Due to the low number of high-grade ER-α-positive lesions analyzed, the present study does not exclude that unbalanced estrogen metabolism can play a role among estrogen-sensitive, high-grade EC. The major role of 17β-HSD type 1 in controlling estrogen metabolism has been shown in distinct tissues and disorders (9, 11, 24, 36). Inhibitors of 17β-HSD type 1 (37–39) have been used in mouse models of breast cancer (40) and endometrial hyperplasia (36) and represent future therapeutic options.

Despite endocrine treatments have high tolerability/few side effects, a number of trials have indicated the need to tailor them to preselected patients (18). The wide distribution of enzyme activity and expression levels of the distinct enzymes observed among patients in the present study underscores this need. Therefore, clarifying the contribution of the local and the systemic estrogen metabolism is important to both indicate novel possible therapeutic targets and improve our selection of patients that can benefit from hormonal therapies.

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