PERGAMON

Journal of Steroid Biochemistry & Molecular Biology 84 (2003) 513–526

The Journal of Steroid Biochemistry & Molecular Biology

www.elsevier.com/locate/jsbmb

Endometrial effects of selective estrogen receptor modulators (SERMs) on estradiol-responsive gene expression are gene and cell-specific☆

Yuhua Z. Farnell, Nancy H. Ing*

Departments of Animal Science, and Veterinary Anatomy and Public Health, Faculties of Genetics and Reproductive Biology, Texas A&M University, College Station, TX 77843-2471, USA

Received 10 June 2002; accepted 23 January 2003

Abstract

Three selective estrogen receptor modulator (SERM) drugs which included 4-OH-tamoxifen (Tam), EM-800 (EM) and GW 5638 (GW) were investigated to determine their ability to inhibit estradiol-responsive gene expression in sheep endometrium. The uteri of ovariectomized ewes (10 ewes per SERM group) were infused with 10^{-7} M SERMs for 24 h prior to hysterectomy. Five ewes from each group received 50 μg 17β-estradiol (E2) and the remaining five ewes received vehicle 18 h prior to hysterectomy. Northern blot analyses and in situ hybridization demonstrated that E2 treatment increased estrogen receptor (ER), progesterone receptor (PR), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and cyclophilin (CYC) mRNA levels in most endometrial cells examined. Tam and GW exhibited characteristics similar to E2 by increasing ER gene expression, but they antagonized the E2-induced increases in PR and CYC mRNA levels. EM acted as an E2-agonist of GAPDH gene expression, but antagonized the E2 up-regulation of ER, PR and CYC gene expression in most endometrial cells. Immunohistochemistry determined that EM decreased ER protein levels in the glandular epithelium, and the SERMs investigated antagonized increases in PR protein levels in endometrium. In conclusion, GW and EM exhibit fewer agonist effects than Tam on endometrial gene expression. EM demonstrated the greatest antagonism of E2-enhanced levels of ER, PR and CYC, likely due to the inhibition of ER gene expression at both mRNA and protein levels.

Keywords: Selective estrogen receptor modulator; Estradiol; Endometrium; Gene expression

1. Introduction

Estrogens play important roles in the growth regulation, development, and differentiation of the reproductive tract, mammary glands, and central nervous system (reviewed in [1]). Estrogens bind to estrogen receptor (ER) proteins, which in association with cofactors, and activate transcription of estrogen-responsive genes [2,3]. Selective estrogen receptor modulators (SERMs) are synthetic compounds developed to bind ER proteins and act as estrogen (E2)-antagonists in mammary gland (such as tamoxifen; Tam) or E2-agonists in bone (such as raloxifene) [3,4]. Tam, one of SERMs currently used in the treatment and prevention of breast cancer [5], has been shown to express mixed estrogenic/anti-estrogenic activity in uteri of women and different rodent species [6,7]. In women, its use to prevent breast cancer growth is correlated to an increased risk of endometrial cancer [8,9]. In sheep endometrium, Tam acts as an E2-agonist in the up-regulation of ER and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA concentrations [10].

Two new SERMs, GW 5638 (GW) and EM-800 (EM) are currently being tested in clinical trials as “pure anti-estrogens”. As shown in Fig. 1, GW is a triphenylethylene derivative, structurally related to tamoxifen, while EM is a derivative of benzopyran structurally related to raloxifene [11,12]. In the rat, GW antagonized E2-increased uterine weight, while displaying minimal uterotropic activity when used alone [13]. Also, GW acted as an E2-agonist in both bone and cardiovascular system, thus demonstrating a high degree of tissue specificity [11]. EM is an orally active anti-tumor agent for mammary cancer in the rat [14]. EM displayed pure anti-estrogenic effects in mouse uterus, vagina, mammary gland, and hypothalamic–pituitary axis as well as in Ishikawa cells, a human endometrial carcinoma cell line [15,16]. EM also prevented bone loss and lowered serum cholesterol levels without estrogenic effects on the endometrium in ovariectomized rats [17–19].

☆ This is the first paper in a set, with the second concerning myometrial effects in the same ewes.

* Corresponding author. Tel.: +1-979-862-2790; fax: +1-979-862-3399.
E-mail address: ning@cvm.tamu.edu (N.H. Ing).

0960-0760/03/$ – see front matter © 2003 Elsevier Science Ltd. All rights reserved.
doi:10.1016/S0960-0760(03)00076-1

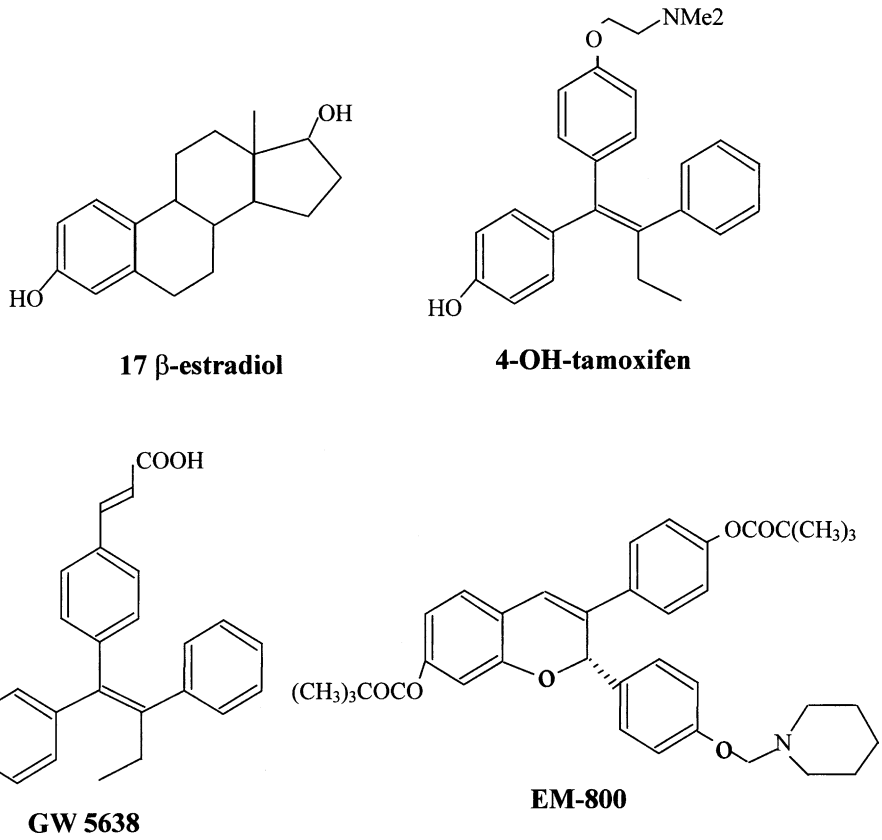

Fig. 1. Chemical structures of 17β-estradiol and SERMs used in this study. 4-OH tamoxifen (Tam), a triphenylethylene SERM, is the active metabolite of tamoxifen. The compound GW 5638 (GW) is a new SERM that is structurally related to tamoxifen. EM-800 (EM) is the derivative of benzopyran.

During estrous and menstral cycles, the pre-ovulatory surge of estrogen alters gene expression in endometrium to facilitate the preparatory development of pre-implantation. Our previous work has demonstrated that a single physiological dose of E2 mimics the pre-ovulatory surge of estrogen and up-regulates ER, progesterone receptor (PR), GAPDH and cyclophilin (CYC) mRNA levels at 18 h post-injection in sheep endometrium [20,21]. In this paper, the E2-responsive genes [20,21] found in sheep endometrium are evaluated in vivo based on the agonist and antagonist effects of the three SERMs: Tam, GW and EM.

2. Materials and methods

2.1. Chemicals

All chemicals (including Tam) were purchased from Sigma (St. Louis, MO), unless otherwise indicated. EM and GW were obtained from Dr. Fernand Labrie (Laval University; Qué., Canada) and Dr. David C. Morris (Glaxo Wellcome Research and Development; Durham, NC), respectively. Tam, GW and EM were dissolved in ethanol to make 10^{-3} M stock solutions. They were diluted to 10^{-7} M in 0.1% ovine serum albumin (OSA) in phosphate-buffered saline (PBS) for infusion. E2 was dissolved in a minimal amount of ethanol prior to dilution to 100 μg/ml in charcoal-stripped corn oil (Kodak; Rochester, NY).

2.2. Animals, treatments and sample collection

After confirmation of estrous cycles of normal duration (16–18 days), cross-bred Rambouillet ewes were ovariectomized and their uterine horns were fitted with the catheters [22]. Fifteen days after ovariectomy, four groups of ewes (10 ewes per group) were infused with 10^{-7} M SERMs or drug vehicle (0.1% ovine serum albumin in phosphate-buffered saline, OSA in PBS) at rate of 3 ml/h for 24 h in each uterine horn via indwelling catheters. Eighteen hours prior to hysterectomy, five ewes in each group were injected intramuscularly with E2 (50 μg) and remaining five ewes received vehicle (0.5 ml charcoal-stripped corn oil). The time for E2 treatment was chosen for its rapid ER mRNA accumulation [20]. For subsequent histochemistry, a 1 cm cross-section was removed from each uterine horn distal to the external bifurcation and fixed in 4% paraformadehyde [23]. The endometrium was dissected from the remaining uterus, minced, snap-frozen in liquid nitrogen, and stored at −80 °C. The Texas A&M University Laboratory Animal Care and Use Committee approved all animal procedures.

2.3. Total RNA preparation and Northern blot analysis

Total cellular RNA was extracted from 0.5 g of endometrium using Tripure Reagent according to the manufacturer's instructions (Boehringer Mannheim; Indianapolis, IN). Northern blot analysis of total cellular RNA (8 μg per lane) was performed as previously described [23]. Antisense cRNA probes were generated for probing estrogen-responsive (ER, PR, GAPDH and CYC) mRNAs, as previously described [20,21]. Blot hybridization and washing were performed according to Ing et al. [20]. Hybridization signals were captured by directly scanning blots on InstantImager (Packard, Meriden, CT).

2.4. Identifying the endometrial cells that change the expression of E2-responsive genes by in situ hybridization

Adjacent uterine cross-sections (7 mm) from each ewe were placed on Superfrost Plus slides (Curtin Matheson Scientific, Houston, TX) less than 1 week before histochemical development. In situ hybridization was performed to identify the response of specific uterine cells to SERMs and E2 as described previously [21]. Antisense and sense cRNA probes were generated with (^{35}S)-UTP in place of (^{32}P)-UTP for in vitro transcription reactions. Slides were dipped in NTB-2 autoradiography emulsion (Eastman, Kodak) and exposed for 5 weeks for ER and PR mRNA data or 8 weeks for GAPDH and CYC mRNA data. Sections were counterstained with hematoxylin. Quantitative analyses of silver grains (relating to amount of mRNAs) were assessed using the Reichert MicroStar IV Microscope (Diagnostic Instrument; MI) and NIH image 1.61 software [21]. For each mRNA examined, pixel densities were assessed on sections probed with antisense and sense cRNAs. Pixel densities of silver grain were analyzed for five regions containing 15–25 cells of a cell compartment in three view fields for each uterine section. The in situ hybridization signals for an mRNA were analyzed alongside the sense signals (non-specific) on the same area of an adjacent section, used as a covariate in the analyses.

2.5. Immunohistochemistry to characterize regulation of ER and PR protein levels by SERMs

Immunohistochemical procedures were performed as described previously [23]. Briefly, uterine cross-sections were treated with 0.3% H_2O_2 for 30 min at room temperature, 0.5 mg/ml of Pronase for 8 min at 37 °C and then subjected to protein blocker (Biostain Super ABC kit; Biomeda, Foster City, CA). Sections were incubated with rat anti-ER antibody H222 (4 μg/ml), which is a monoclonal antibody raised against human ERα (a gift from Dr. G. Greene, University of Chicago, Chicago, IL), biotinylated goat anti-rat IgG and peroxidase-labeled avidin (Biostain Super ABC kit). The chromagen used for peroxidase localization was 3,3′-diaminobenzidine tetrahydrochloride. Another monoclonal antibody, mouse anti-human ER 1D5 (DAKO A/S, Glostrup, Denmark), which, like H222, does not cross-react with ERβ [24], was also used in immunohistochemistry. For detection of PR protein, we used the MA1-411 antibody (Affinity BioReagents, Golden, CO) and microwave antigen retrieval [23]. Non-immune rat and mouse IgG replaced the antibodies, respectively, on adjacent sections for negative controls.

2.6. Statistical analysis

All quantitative data were analyzed by least-squares ANOVA using the general linear model procedure of SAS. Data analysis of gene expression used 18S rRNA hybridization signals to correct for unequal RNA loading. Data are presented as least-squares means and standard errors of the mean for each treatment group. The E2 treatment group data was compared to control (Con) group data to identify E2 effects. Data from SERM treatment alone groups were compared to those from the Con group to identify E2-agonist effects of SERMs. Data from SERM + E2 groups were compared to E2 group to identify E2-antagonistic effects. Level of statistical significance was considered to be comparisons with *P*-values less than or equal to 0.05, unless otherwise indicated.

3. Results

3.1. Northern blot analysis identified gene-specific effects of SERMs

We quantitated the effects of E2 with and without SERMs on the regulation of E2-responsive genes in the endometrium of ovariectomized ewes with Northern blot analyses. Hybridization signals for ER, PR, GAPDH, and CYC mRNAs and 18S rRNA are shown in Fig. 2A for all of the ewes. Quantitation of these results demonstrated that ER gene expression was up-regulated by Tam and GW alone treatments by more than 100% compared to those in the Con group ewes, similar to the up-regulation by E2 (indicated by asterisks in Fig. 2B). Only EM antagonized E2's up-regulation of ER gene expression (indicated by double asterisks in Fig. 2B) and it did so completely. Results from the PR gene indicated that SERMs tested alone showed no agonist action or replication of E2's 23% up-regulation of PR gene expression. However, both Tam and GW antagonized this E2 response. For the expression of GAPDH and CYC genes, Tam and EM acted as E2-agonists, inducing an average 45% increase in GAPDH and CYC mRNA levels, similar to the increase in response to E2. Northern blot analysis detected no antagonistic effect towards the E2 up-regulation of either GAPDH or CYC mRNA levels in endometrium by any SERM. Thus, all SERMs exhibited partial agonist/partial antagonist effects in endometrium that were gene-specific.

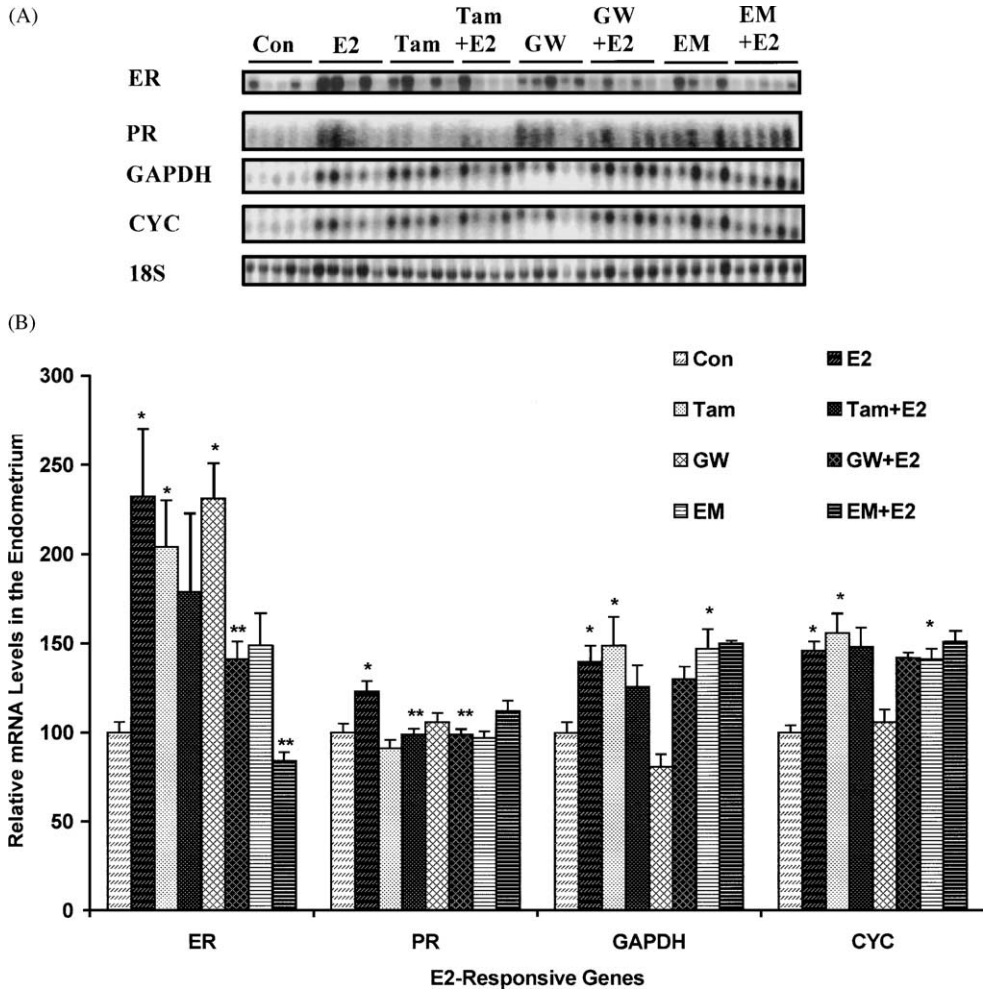

Fig. 2. Effects of SERMs with and without E2 on estrogen receptor (ER), progesterone receptor (PR), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and cyclophilin (CYC) mRNA concentrations in endometrium. Total cellular RNA was prepared from endometrium from each ewe in the eight treatment groups: control (Con), E2, Tam, Tam + E2, GW, GW + E2, EM, EM + E2 ($n = 5$ ewes/group). Replicate Northern blots were hybridized with ^{32}P-labeled antisense cRNA probes for ER, PR, GAPDH, and cyclophilin mRNAs and 18S rRNA. Panel A shows the raw data while panel B presents the quantitative analysis of their hybridization signals as least-squares means ± standard error of the means. For treatment groups: Con (open, hatched), E2 (filled, hatched), Tam (open, stippled), Tam + E2 (filled, stippled), GW (open, cross-hatched), GW + E2 (filled, cross-hatched), EM (open, horizontal strips), and EM + E2 (filled, horizontal stripes). Values are normalized to those of Con group, set at 100. Significant E2-agonist effects (differences compared to the Con group) are indicated by asterisks (*) while antagonist effects (compared to E2 group) are indicated by asterisks (**) over the bars.

3.2. Cell-specific effects of SERMs on endometrial gene expression

In situ hybridization identified the regulation of gene expression in the specific endometrial cells that responded to SERMs ± E2 treatments. Representative images of signals for ER, PR, GAPDH and cyclophilin mRNAs on sheep endometrial cross-sections are shown in Figs. 3–6, respectively, for ewes from each treatment group. Brightfield images show the pinpoint black silver grains from in situ hybridization over the cell nuclei stained lightly with hematoxylin. Each panel spans the endometrium from luminal epithelium (LE) at the upper part of the panel to deep glandular epithelium (DGE) and surrounding stroma (SS) at lower part of the picture. These cell compartments, along with stratum compactum (SC) and superficial glandular epithelium (SGE), are labeled in the upper left panel from a Con ewe in Fig. 3. This panel shows very little hybridization signal for ER mRNA over the gray cell nuclei. E2 treatment increases the ER mRNA signals strongly in all but the LE cells. The effect is not antagonized by Tam or GW, but it is antagonized by EM. In Fig. 4, E2 enhancement of PR mRNA levels is obvious when comparing the top two panels. The primary response was in the epithelial and superficial stromal cells. All SERMS tested antagonized the effect of E2 but EM did this most completely across the different endometrial cell types. GAPDH mRNA (Fig. 5) was up-regulated by E2, Tam and EM treatments. Although it was strongly expressed in the epithelial cells, the stromal cells also responded. SERM treatment did

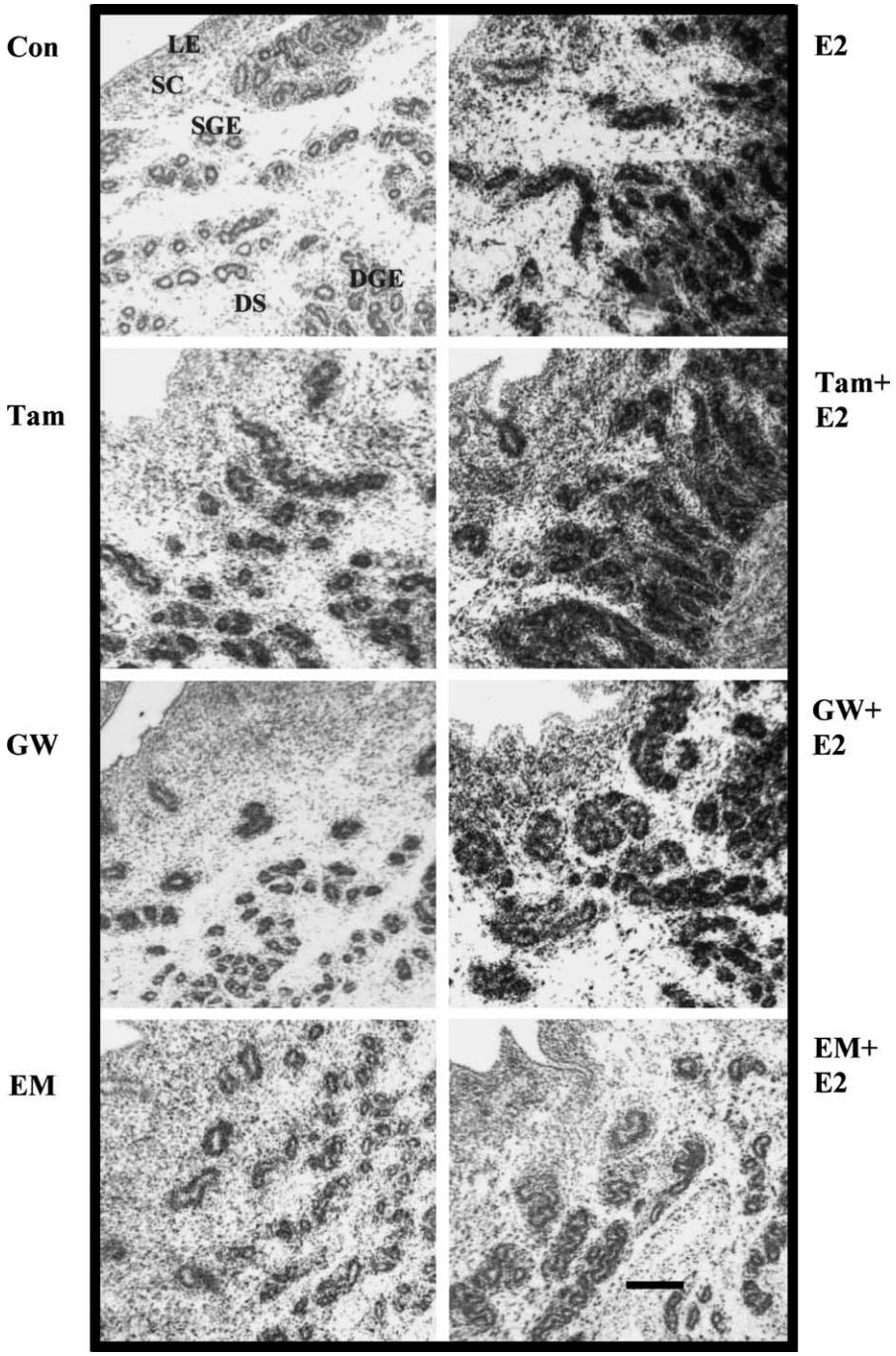

Fig. 3. In situ hybridization of ER mRNA demonstrates EM antagonism of its up-regulation by E2. Paraformaldehyde-fixed cross-sections of sheep uteri were mounted onto glass slides, pre-hybridized, and hybridized with antisense CYC cRNA probe. The ^{35}S labeling of the cRNA probe exposed the autoradiographic emulsion so that tiny black silver grains represent hybridization signals of CYC mRNA on brightfield images. Nuclei lightly counterstained with hematoxylin appear as gray enlarged areas. Representative views are shown for ewes from Con, E2, Tam, Tam + E2, GW, GW + E2, EM and EM + E2 treatment groups. Panels span the endometrium from luminal epithelium (LE) at the top to deep glandular epithelium (DGE) and deep stroma (DS) at the bottom. Cell compartments, including stratum compactum (SC) and superficial glandular epithelium (SGE) are labeled in the "Con" ewe panel (upper left). ER mRNA increased in most endometrial cells with E2 treatment, but EM antagonized the effect. The bar in the "EM + E2" panel represents 100 μm.

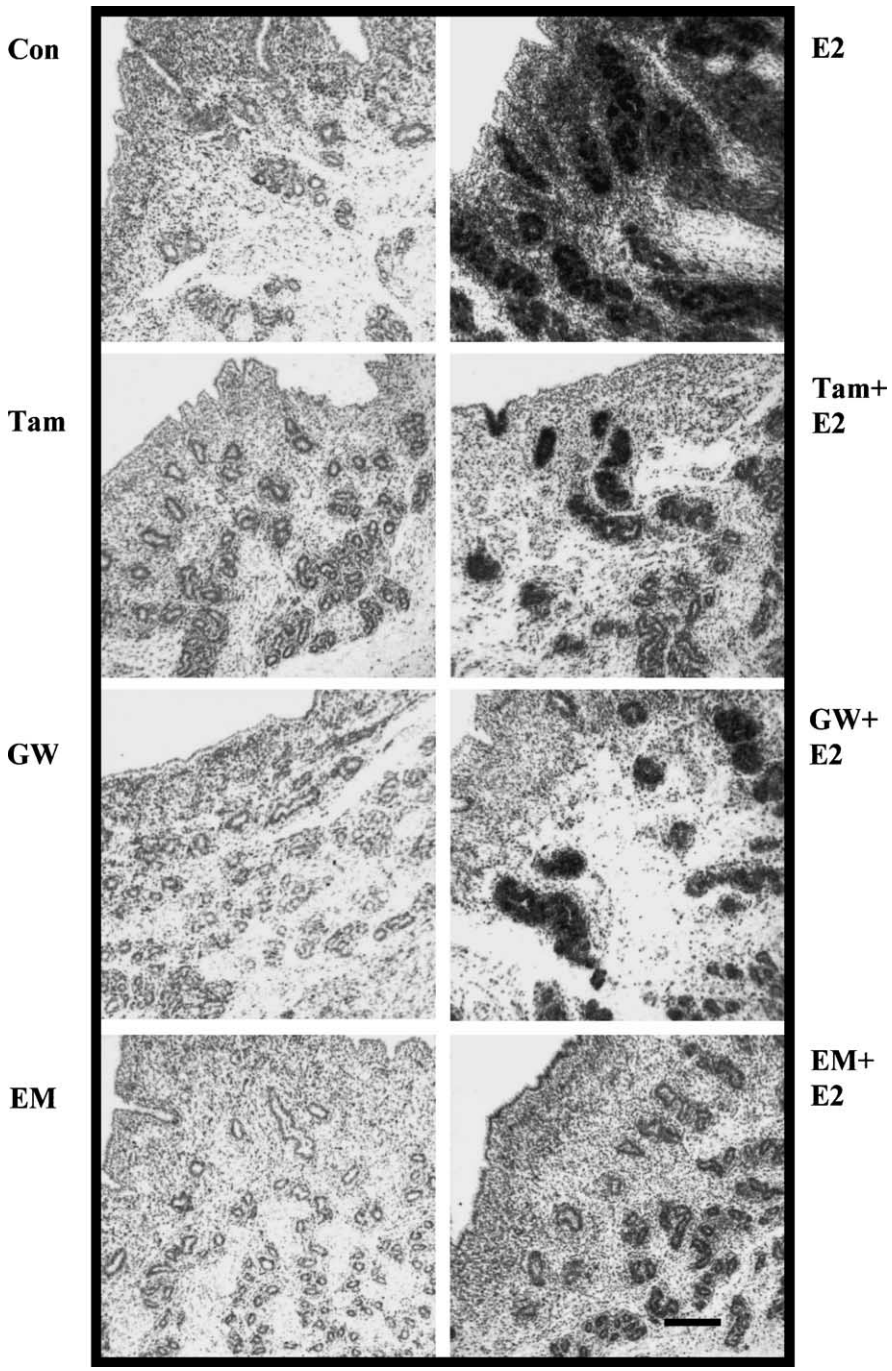

Fig. 4. In situ hybridization demonstrates that all three SERMs antagonize E2 up-regulation of PR mRNA levels. In situ hybridization was performed and results are presented as described in Fig. 3. The bar in the "EM + E2" panel represents 100 μm. E2 increased PR mRNA levels in most endometrial cells, but SERMs antagonized the effect especially in the deep endometrium.

not antagonize or add to the E2 effect on GAPDH gene expression.

While similar data for ER, PR, and GAPDH gene expression have been shown for Con and E2 ewes in previous works [21,23], this is the first description of CYC gene expression in uterine cross-sections. Cyclophilin mRNA appeared most prevalent in superficial epithelium, LE and SGE in Con, GW and EM ewes (Fig. 6). Consistent with Northern blot results, E2 treatment resulted in strong, uniform CYC gene expression in all endometrial epithelium as well as stromal cells ("E2" panel in Fig. 6). Tam acted as a partial E2-agonist by enhancing CYC mRNA levels in deep endometrium when used alone ("Tam" panel), but prevented some up-regulation by E2 in deep endometrium ("Tam+E2"

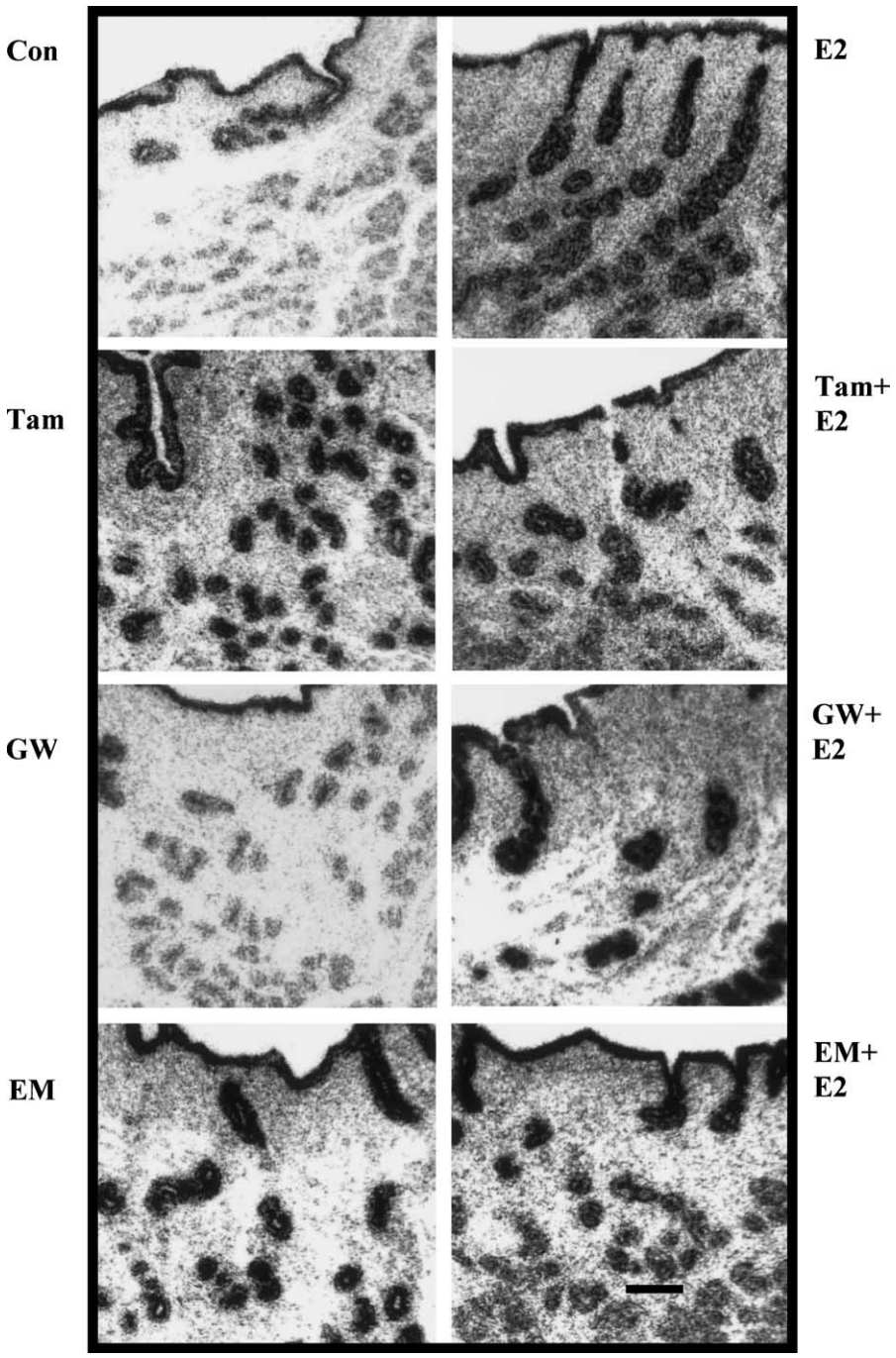

Fig. 5. In situ hybridization demonstrates that Tam and EM act as E2-agonists by up-regulating GAPDH mRNA levels in endometrium. In situ hybridization was performed and results are presented as described in Fig. 3. The bar in the "EM + E2" panel represents 100 μm. E2, Tam and EM increased GAPDH mRNA levels in most endometrial cells, and no SERM antagonized the effect of E2.

panel). Both GW and EM showed no agonist activity ("GW" and "EM" panels), but they antagonized E2's increase in CYC gene expression in deep endometrium ("GW + E2" and "EM + E2" panels). In addition, EM also antagonized the E2-induced increases in CYC mRNA levels in LE and SGE ("EM + E2" panel). Semi-quantitative analyses of the in situ hybridization results are presented in the following section.

3.3. Semi-quantitative analyses of in situ hybridization identified both gene- and cell-specific effects of SERMs

In situ hybridization signals for ER, PR, GAPDH and CYC mRNA were quantitated in the five endometrial cell compartments labeled in the "Con" panel of Fig. 3 as well as in epithelium (CE) and stroma (CS) of caruncles, which are endometrial evaginations unique to ruminant species. As

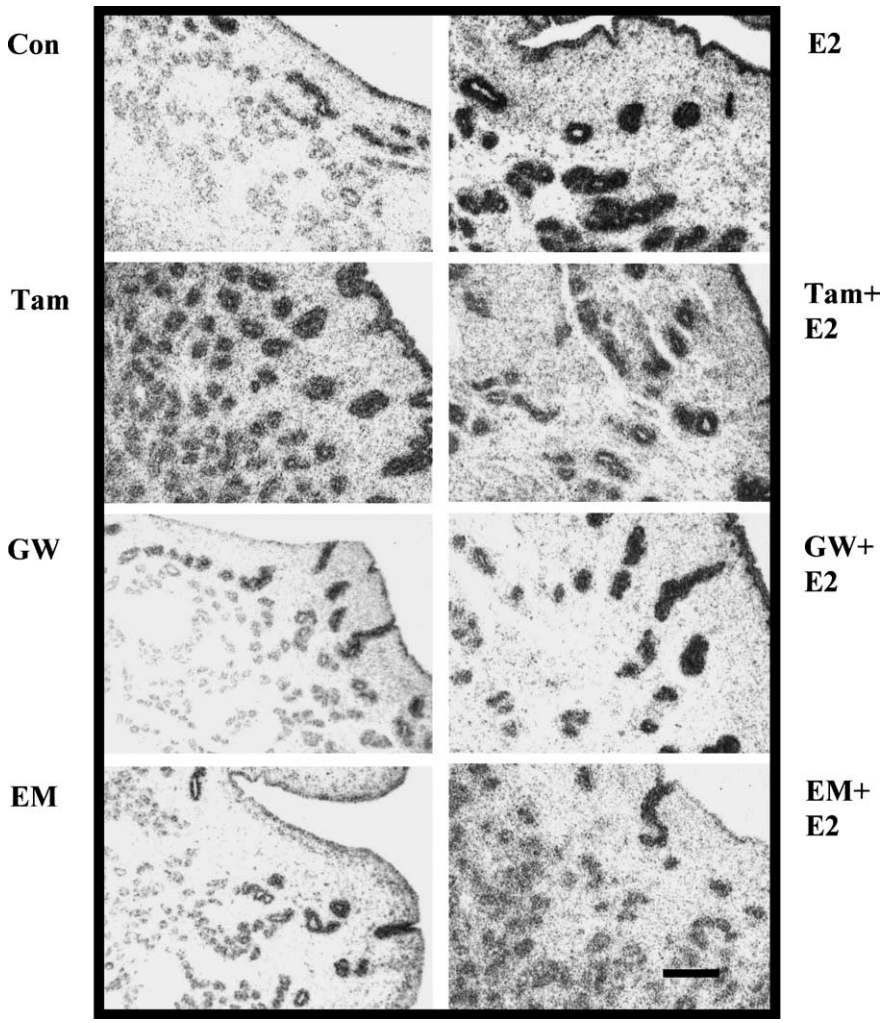

Fig. 6. In situ hybridization demonstrates that all three SERMs antagonize E2 up-regulation of CYC mRNA levels in the deep endometrium. In situ hybridization was performed and results are presented as described in Fig. 3. The bar in the "EM + E2" panel represents 100 μm. E2 increased CYC mRNA levels in most endometrial cells, but SERMs antagonized the effect especially in the deep endometrium.

shown in the top section of Table 1, E2 treatment increased ER mRNA levels compared to Con values in all endometrial cell compartments except CE and LE. Agonist actions of the SERMs were more limited. In ewes treated with Tam, GW or EM alone, ER mRNA levels increased in only one cell compartment: DGE, SGE or CS, respectively. Only EM antagonized E2-upregulation of ER gene expression and was most effective in CS, SGE and DGE cell compartments.

E2 enhanced PR gene expression in CS, SC, SGE, DGE and probably DS ($P = 0.07$) cell compartments (Table 1). As demonstrated in Northern analyses, no SERM up-regulated PR gene expression when it was used alone. On the contrary, GW and EM tested alone decreased PR mRNA levels in CE cells compared to those in Con ewes. Also, EM examined alone decreased PR gene expression in CS and SC. Tam and GW markedly antagonized up-regulation of PR gene expression by E2 in DS and DGE cells. EM completely ablated E2's up-regulation of PR mRNA levels uniformly across all endometrial cells.

Consistent with Northern blot results, GAPDH mRNA hybridization signals on uterine cross-sections were enhanced by E2, Tam and EM treatments (Table 1). E2 treatment enhanced GAPDH gene expression in most endometrial cells, which was consistent with our previous study [21]. Tam used alone increased GAPDH mRNA concentrations primarily in SC, SGE, DGE and DS cells. EM also acted as an E2-agonist by enhancing GAPDH gene expression in all cell compartments of the endometrium. GW did not display any effect on GAPDH gene expression. No SERMs tested antagonized E2's up-regulation of GAPDH mRNA levels in any cell compartment.

E2 increased CYC gene expression in all cell compartments, although LE and SC analyses had *P*-values of 0.1 and 0.08, respectively (Table 1). Tam enhanced CYC mRNA levels strongly in DS cells and significantly inhibited E2's up-regulation of CYC mRNA in DGE cells. GW and EM used alone had no effect on CYC gene expression. However, GW inhibited E2's up-regulation of CYC gene expression

Table 1
Quantitative results of in situ hybridization for ER, PR, GAPDH and CYC mRNA in specific endometrial cell compartments: caruncular epithelium (CE), caruncular stroma (CS), luminal epithelium (LE), stratum compactum (SC, dense stroma right beneath the LE), superficial glandular epithelium (SGE), deep glandular epithelium (DGL) and deep stroma (DS)

	Con	E2	Tam	TE	GW	GE	EM	EE
ER								
CE	12.3 ± 1.2	14.0 ± 1.2	11.8 ± 2.0	15.3 ± 0.9	10.7 ± 2.9	12.3 ± 3.7	12.7 ± 2.2	14.3 ± 2.46
CS	49.3 ± 12.3	98.3 ± 2.0[a]	50.0 ± 4.6	83.0 ± 6.7	40.0 ± 6.3	102 ± 3.9	84.7 ± 0.2[a]	66.71 ± 6.3[b]
LE	13.0 ± 2.4	14.41 ± 0.8	13.6 ± 4.5	13.5 ± 3.2	13.0 ± 3.5	13.8 ± 4.1	12.5 ± 2.6	13.4 ± 1.1
SC	31.2 ± 5.3	53.2 ± 5.6[a]	31.6 ± 5.7	37.8 ± 4.3	20.0 ± 7.1	53.2 ± 4.7	43.3 ± 6.0	46.0 ± 5.3
SGE	39.6 ± 5.7	68.6 ± 9.0[a]	49.4 ± 4.1	58.5 ± 6.9	58.4 ± 9.1[a]	55.2 ± 6.6	49.0 ± 7.2	46.2 ± 4.1[b]
DGE	26.0 ± 6.8	77.0 ± 8.2[a]	45.0 ± 7.6[a]	62.2 ± 8.5	33.0 ± 8.1	67.2 ± 6.7	38.8 ± 8.2	34.1 ± 5.7[b]
DS	10.0 ± 2.2	25.1 ± 3.2[a]	13.6 ± 2.1	26.5 ± 3.0	15.2 ± 3.9	31.6 ± 6.0	20.7 ± 7.7	21.4 ± 20
PR								
CE	19.2 ± 2.4	22.4 ± 2.1	17.3 ± 4.4	19.0 ± 1.0	9.0 ± 1.2[a]	16.5 ± 4.5	7.7 ± 3.8[a]	8.7 ± 2.3[b]
CS	41.0 ± 1.4	57.0 ± 4.3[a]	34.0 ± 8.7	45.2 ± 9.5	39.1 ± 4.4	48.0 ± 2.9	18.0 ± 8.0[a]	15.0 ± 3.8[b]
LE	15.0 ± 2.6	15.0 ± 3.4	14.0 ± 2.4	11.0 ± 2.9	8.2 ± 1.4	14.0 ± 1.2	12.1 ± 2.5	7.8 ± 2.1[b]
SC	33.2 ± 2.4	43.8 ± 2.7[a]	26.8 ± 2.4	27.0 ± 5.7[b]	31.0 ± 34	37.0 ± 2.0	18.0 ± 4.1[a]	14.0 ± 2.5[b]
SGE	25.0 ± 1.8	61.0 ± 5.6[a]	38.0 ± 5.4	60.0 ± 5.6	23.0 ± 3.2	49.0 ± 1.2	33.2 ± 5.3	18.0 ± 2.1[b]
DGE	16.4 ± 4.1	37.4 ± 6.6[a]	20.6 ± 3.8	12.8 ± 1.1[b]	10.8 ± 4.0	12.5 ± 3.6[b]	10.5 ± 6.1	5.2 ± 1.3[b]
DS	13.0 ± 2.1	20.0 ± 3.1	12.0 ± 2.6	11.0 ± 2.3[b]	8.0 ± 2.6	11.0 ± 1.8	12.0 ± 3.9	4.8 ± 2.2[b]
GAPDH								
CE	95.6 ± 11.8	123.8 ± 2.3	89.9 ± 28.8	123.2 ± 8.0	92.9 ± 19.9	141.0 ± 2.6	144.6 ± 9.3[a]	142.7 ± 6.2
CS	45.5 ± 7.4	91.6 ± 0.4[a]	52.7 ± 20.3	93.0 ± 9.8	62.5 ± 4.6	93.3 ± 7.9	89.6 ± 12.8[a]	105.4 ± 5.2
LE	94.7 ± 8.4	129.4 ± 7.8[a]	104.8 ± 18.6	135.0 ± 4.0	97.8 ± 19.4	130.8 ± 9.8	133.3 ± 4.6[a]	134.7 ± 1.5
SC	32.6 ± 1.3	65.2 ± 6.2[a]	55.5 ± 8.7[a]	65.2 ± 4.6	37.3 ± 4.5	77.4 ± 9.2	63.6 ± 15[a]	67.4 ± 8.4
SGE	103.5 ± 4.6	142.3 ± 2.4[a]	129.4 ± 8.2[a]	140.5 ± 5.7	113.2 ± 2.3	149.7 ± 3.6	139.7 ± 4.8[a]	144.7 ± 5.9
DGE	60.0 ± 5.1	105.7 ± 8.3[a]	83.6 ± 12.9[a]	102.1 ± 7.9	66.0 ± 4.0	104.4 ± 0.9	109.1 ± 0.8[a]	106.0 ± 5.7
DS	17.3 ± 3.4	38.1 ± 4.9[a]	37.2 ± 9.3[a]	39.2 ± 5.8	24.4 ± 2.6	42.8 ± 4.2	48.2 ± 5.5[a]	44.8 ± 5.4
CYC								
CE	36.2 ± 2.1	60.6 ± 7.5[a]	33.2 ± 11.1	56.3 ± 6.4	17.6 ± 9.3	42.8 ± 4.6	36.3 ± 9.9	50.1 ± 5.8
CS	17.9 ± 13.4	41.4 ± 9.2[a]	23.5 ± 8.0	39.2 ± 4.0	10.5 ± 5.6	26.6 ± 2.7	27.7 ± 5.1	35.6 ± 10.3
LE	44.9 ± 2.2	61.0 ± 6.1	44.3 ± 11.0	66.5 ± 5.9	27.9 ± 8.0	46.3 ± 3.5	40.0 ± 5.2	41.1 ± 8.4[b]
SC	16.0 ± 4.3	27.5 ± 4.0	21.4 ± 6.1	24.7 ± 2.9	20.5 ± 4.6	19.4 ± 5.3	5.3 ± 2.6	15.2 ± 5.4
SGE	62.0 ± 6.8	98.9 ± 5.3[a]	69.7 ± 9.9	86.0 ± 4.5	54.4 ± 6.5	88.1 ± 5.7	58.5 ± 3.4	73.9 ± 6.1[b]
DGE	34.9 ± 6.1	75.1 ± 6.4[a]	46.9 ± 5.7	56.5 ± 0.8[b]	28.5 ± 6.4	54.3 ± 5.5[b]	43.5 ± 4.1	45.2 ± 1.9[b]
DS	3.8 ± 1.3	25.2 ± 5.3[a]	15.0 ± 5.4[a]	17.1 ± 5.8	2.6 ± 3.2	9.0 ± 2.8[b]	4.1 ± 2.3	3.6 ± 0.6[b]

Treatment groups are Con, E2, TE ("Tam + E2"), GW, GE ("GW + E2"), EM and EE ("EM + E2"). Data are expressed as the mean ± standard error of the mean (S.E.M.).
[a] Indicates a difference of treatment group compared to Con group.
[b] Indicates a difference of treatment group compared to E2 group.

in both DS and DGE cell compartments. EM also acted as E2-antagonist and inhibited the E2-induced increase in CYC gene expression in LE, SC ($P = 0.07$), SGE, DS and DGE cells.

Our overall conclusions from the in situ hybridization data are that GW had fewer actions as an E2-agonist than did Tam or EM in sheep endometrium. Of the SERMs tested, EM showed the strongest antagonism of E2 effects and did so in all endometrial cell types. The greater effectiveness of EM was likely due to its antagonism of ER gene expression in many endometrial cell compartments.

3.4. EM decreased ER protein levels in epithelial and stromal cells of endometrium

To examine how ER protein levels changed in response to SERM treatment, immunohistochemistry was performed using two ER-alpha-specific antibodies. Both resulted in similar immunostaining for ER protein that was predominantly in the nuclei of epithelial and stromal cells of the endometrium. Representative results of ER immunostaining with the H222 antibody are shown in Fig. 7. These ER protein levels appeared to be down-regulated by E2 in the epithelial cells and not changed in the stromal compartments (compare "Con" and "E2" panels). Tam and GW used with or without E2 did appear to change ER protein levels (middle panels of Fig. 7). However, EM treatment down-regulated ER protein levels in both epithelial and stromal cells of the endometrium (Fig. 7, lowest panels). Negative control sections developed with non-immune IgG in place of H222 antibody lacked staining, similar to the EM panels shown. Interestingly, of the three SERMs evaluated, only EM effectively down-regulates ER protein levels throughout the endometrium.

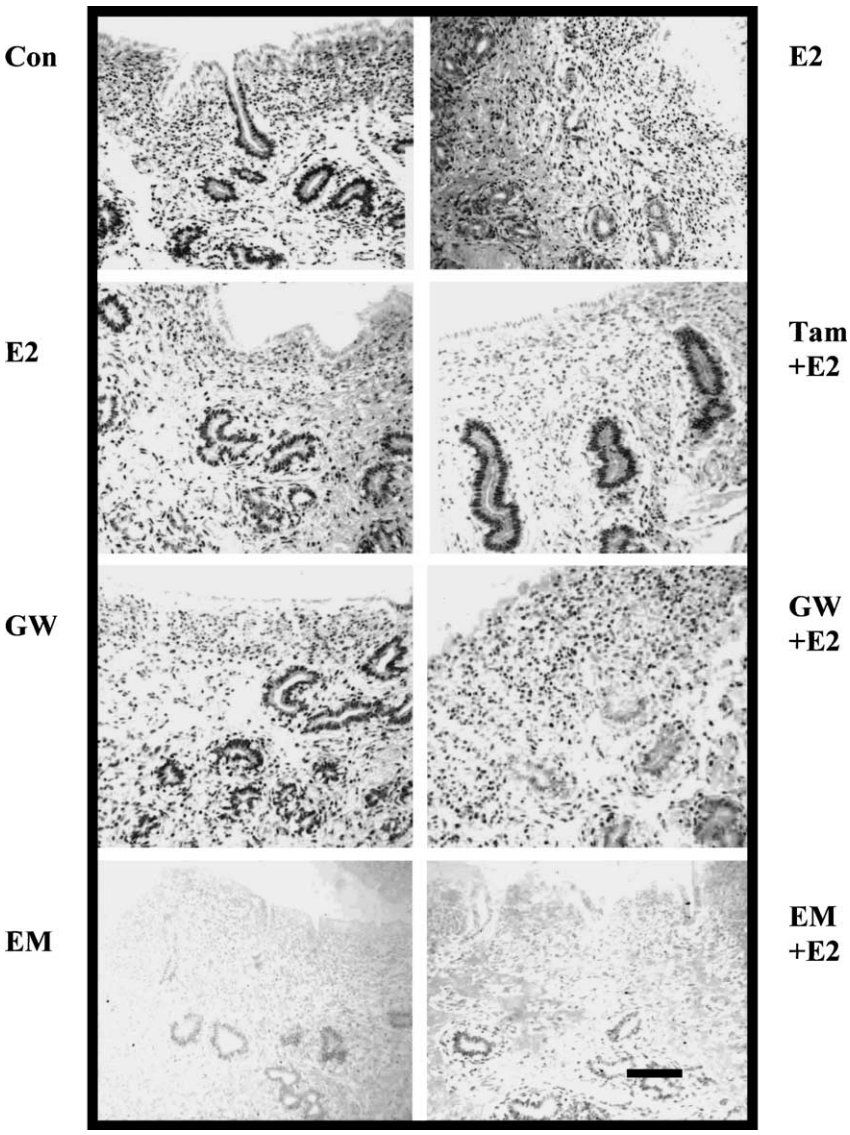

Fig. 7. SERM effects on ER protein levels in endometrium. Representative data from endometrial cross-sections are shown for ewes from all eight treatment groups. Immunohistochemistry with H222 antibody demonstrated nuclear staining of ER protein in endometrial cells (see "Con" panel). LE is located at the top of each panel and the deep endometrium is at the bottom. E2 decreased ER protein in glandular epithelial cells, while EM decreased ER protein in both epithelial and stromal cells. The bar in the "EM + E2" panel represents 100 μm.

3.5. All of the SERMs antagonized the E2-induced increases in PR protein levels

PR immunostaining was predominantly nuclear in endometrial cells of Con ewes (Fig. 8, "Con" panel). Intensity of immunostaining ranged from moderate in the luminal epithelium (at the upper left of each panel) and stroma to faint in glandular epithelium. Nuclear PR staining was strongly enhanced by E2 in the glandular epithelial cells and stromal cells ("E2" panel). Cytoplasmic PR staining was also increased by E2 treatment. Used alone, none of the SERMs tested altered PR immunostaining in endometrial cells. However, all three SERMs tested antagonized the E2-induced increases in PR protein levels in the nuclei but not in the cytoplasm of the epithelial cells of the middle and deep glands. Tam + E2 and EM + E2 treatments also appeared to reduce PR protein levels in the stromal cells relative to PR protein levels in E2-treated ewes. Therefore, SERMs decrease PR protein levels, as they do the PR mRNA levels, and these effects are primarily in the deep glandular epithelium.

4. Discussion

Previously, we reported that E2 acutely increased ER, PR, GAPDH and CYC gene expression in the endometrium of ovariectomized sheep [21,23]. Consistent across all of our studies, ER mRNA levels had the greatest magnitude of up-regulation in response to E2. Tam acted as E2-agonist, up-regulating ER and GAPDH gene expression when

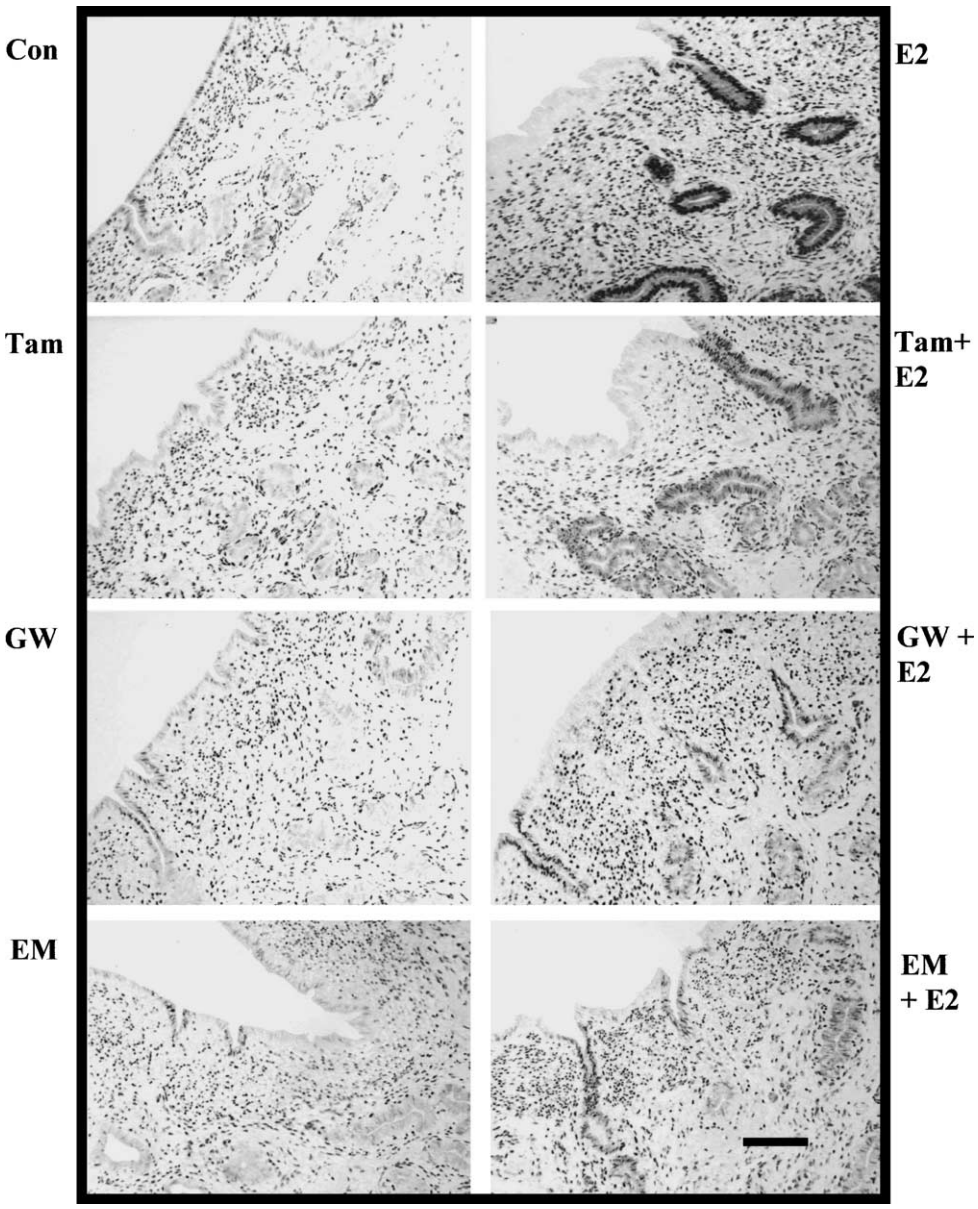

Fig. 8. All three SERMs antagonized E2 up-regulation of PR protein levels in DGE. PR immunohistochemistry demonstrated mainly nuclear staining in endometrial cells. Representative endometrial cross-sections are shown for Con, E2, Tam, Tam + E2, GW, GW + E2, EM and EM + E2 ewes. The uterine lumen and LE are located at the upper left corner of each panel. E2 intensified PR immunostaining in glandular epithelial cells and all three SERMs inhibited this effect. The bar in the "EM + E2" panel represents 100 μm.

delivered systemically by injection [10]. Here, we infused 10^{-7} M of Tam directly into the uterine lumen for 24 h and found it had similar E2-agonist actions that up-regulated expression of ER and GAPDH genes. Along with the many SERM effects identified in the deep endometrial compartments (this report), as well as in myometrium (accompanying paper), this confirms the efficacy of intrauterine delivery of SERMs to the entire endometrium. This report of Tam antagonism of E2 induction of PR gene expression is in agreement with Tam antagonism of E2-induced PR gene expression in the ovariectomized rat uterus and other tissues, as well as in breast cancer cell lines [25–29,41]. It is noteworthy that our E2 and SERM treatments were acute, so as to minimize changes in the endometrial cell populations. Most other SERM trials in animals employ chronic administration that simulates women taking the drugs chronically.

In our sheep, GW treatment alone had E2-agonist effects on expression of the ER gene at mRNA levels in very few endometrial cell compartments. Others have shown no effect of GW on ER mRNA or protein levels in human breast and endometrial cancer cell lines MCF-7 and ECC-1 cells, respectively [28]. Ours is the first report of GW's antagonism of the E2 up-regulation of PR and CYC gene expression. However, EM also showed some E2-agonist effects,

e.g. increases in ER gene expression in CS cells and enhanced GAPDH gene expression in most of the endometrial cells. In this study, EM appears to exert superior antagonistic effects of E2-induced expression of ER, PR, and CYC genes among the SERMs examined. It is important to note that E2 up-regulation of ER gene expression occurs by a post-transcriptional mechanism, by stabilizing ER mRNA [30,31], and this is the first report of a SERM blocking it. The superior antagonism is likely to be due to EM antagonizing the up-regulation of ER mRNA and decreasing ER protein levels in most endometrial cells. Our data agrees with a report that EM decreased rat uterine ER and PR levels, measured with a ligand binding assay [14,32].

GAPDH and CYC have been thought of as "housekeeping genes", expressed constitutively in tissues and cells. However, that is not the case in the sheep and rat uterus and breast cancer cell lines after an E2 challenge, where expression of GAPDH and CYC genes increases [33–35]. Since GAPDH and CYC have important roles in metabolism and protein folding, perhaps it should not be surprising to see them up-regulated by E2 [36,37]. Tam acts as an E2-agonist on GAPDH gene expression, which agrees with a previous study in sheep endometrium [10]. All three SERMs tested showed antagonism of E2 up-regulation of CYC mRNA levels, primarily in deep cell compartments of the endometrium. These SERM effects confirm that GAPDH and CYC genes are regulated, not constitutive, in sheep endometrium.

In this study, E2 increased ER mRNA but decreased ER protein levels in glandular epithelial cells (Figs. 3 and 7). Several reports have stated changes in ER protein levels were not directly correlated to those of ER mRNA levels in rat, ewe and lamb uterus, which were treated with E2 for 8 h, or 12 h [12,23,38,39]. Acute ER protein degradation in response to E2 treatment has been reported in rat, mouse and sheep uterus, as well as MCF-7 cells [12,23,40,41]. We have found that time of E2 treatment greatly affects ER protein in cells: immunoreactive ER was absent at 12 h post-injection, but increased to levels greater than those in untreated control ewes at 24 and 48 h post-injection [23]. Estradiol binding triggers rapid ER degradation via ubiquitin-proteasome pathway [42–44], with the later rise in ER protein levels due to increased translation from increased levels of ER mRNA. EM may similarly affect ER protein levels to result in the profound down-regulation demonstrated here. Sampling time in this study (18 h post-estradiol and immediately after the 24 h infusion of EM) is during the up-regulation/recovery phase of ER mRNA and protein levels. Several SERMs have been tested for regulation of ER mRNA or protein levels in other tissues or cell lines. Alteration of ER gene expression is dependent on the specific SERM, tissue or cell, and length of treatment. Another SERM, ICI 182,780, also causes loss of ER protein levels, while ER mRNA levels are maintained in MCF-7 cells [12]. The fact that the combined treatment of E2 and EM has the lowest level of ER gene expression may relate to both potent ligands down-regulating the ER protein [43].

Endometrium is composed of heterogeneous cell populations including luminal and glandular epithelial cells, stromal cells, immune cells, and cells that form blood vessels [45]. The majority of epithelial and stromal endometrial cells respond to estrogens (endogenous and exogenously applied) by altering gene expression. We and others have seen the greatest up-regulation of ER and PR genes by estrogen in the deep endometrial cells and inner layer of myometrium ([23], accompanying paper). The SERMs that were most potent as E2-agonists and antagonists (Tam and EM, respectively) acted in the majority of endometrial cell compartments. In other cases, however, compartment-specific effects were observed. For example, the deep endometrial region and the outer myometrium appeared more sensitive to E2-antagonism by Tam and GW than the other regions. These data imply paracrine regulation of gene expression between the deep endometrium and the myometrial layers which have yet to be elucidated.

In conclusion, of the two new SERMs, GW has fewer E2-agonist effects in endometrium than the older Tam drug does. Although EM mimicked E2 in up-regulating GAPDH mRNA levels, it showed the most effective and widespread antagonism of E2-enhanced levels of ER, PR and CYC gene expression. This is probably due to EM down-regulating ER mRNA and protein levels in the majority of endometrial cells.

Acknowledgements

We gratefully acknowledge the generous gifts of EM from Dr. Fernand Labrie of Laval University, GW from Dr. David C. Morris of Glaxo Wellcome Research and Development, and the H222 anti-ER antibody from Dr. Geoffery Greene of University of Chicago, IL. The authors also thank Ms. Cindy Balog, Dr. Lora Lindahl as well as members of Dr. Fuller Bazer's laboratory for assistance with animal work. We also acknowledge the assistance of the image analysis core facility of Dr. Robert C. Burghardt. Funding for this research was received from the USDA NRI-CGP grant # 98-35203-6272.

References

[1] C.M. Klinge, Estrogen receptor interaction with co-activators and co-repressors, Steroids 65 (2000) 227–251.
[2] J.F. Couse, K.S. Korach, Estrogen receptor null mice: what have we learned and where will they lead us, Endocrinol. Rev. 20 (1999) 358–417.
[3] V.C. Jordan, M. Morrow, Tamoxifen, raloxifene, and the prevention of breast cancer, Endocrinol. Rev. 20 (1999) 253–278.
[4] C. Ribot, F. Tremollieres, Anti-estrogens and bone tissue, Ann. Endocrinol. 56 (1995) 603–608.
[5] G.H. Eltabbakh, S.L. Mount, Tamoxifen and the female reproductive tract, Expert Opin. Pharmacother. 2 (2001) 1399–1413.
[6] J.I. MacGregor, V.C. Jordan, Basic guide to the mechanism of anti-estrogen action, Pharmacol. Rev. 50 (1998) 151–196.

[7] B.S. Katzenellenbogen, I. Choi, R. Delage-Mourroux, T.R. Ediger, P.G. Martini, M. Montano, J. Sun, K. Weis, J.A. Katzenellenbogen, Molecular mechanisms of estrogen action: selective ligands and receptor pharmacology, J. Steroid Biochem. Mol. Biol. 74 (2000) 279–285.
[8] D.C. Robinson, J.D. Bloss, M.A. Schiano, A retrospective study of tamoxifen and endometrial cancer in breast cancer patients, Gynecol. Oncol. 59 (1995) 186–190.
[9] Y. Daniel, M. Inbar, A. Bar-Am, M.R. Peyser, J.B. Lessing, The effects of tamoxifen treatment on the endometrium, Fertil. Steril. 65 (1996) 1083–1089.
[10] J.A. Robertson, S. Bhattacharyya, N.H. Ing, Tamoxifen up-regulates oestrogen receptor-alpha, c-fos and glyceraldehyde 3-phosphate dehydrogenase mRNA in ovine endometrium, J. Steroid Biochem. Mol. Biol. 67 (1998) 285–292.
[11] T.M. Willson, B.R. Henke, T.M. Momtahen, P.S. Charifson, K.W. Batchelor, D.B. Lubahn, L.B. Moore, B.B. Oliver, H.R. Sauls, J.A. Triantafillou, 3-[4-(1,2-Diphenylbut-1-enyl)phenyl]acrylic acid: a non-steroidal estrogen with functional selectivity for bone over uterus in rats, J. Med. Chem. 37 (1994) 1550–1552.
[12] J.I. Schafer, H. Liu, D.A. Tonetti, V.C. Jordan, The interaction of raloxifene and the active metabolite of the anti-estrogen EM-800 (SC 5707) with the human estrogen receptor, Cancer Res. 59 (1999) 4308–4313.
[13] T.M. Willson, J.D. Norris, B.L. Wagner, I. Asplin, P. Baer, H.R. Brown, S.A. Jones, B. Henke, H. Sauls, S. Wolfe, D.C. Morris, D.P. McDonnell, Dissection of the molecular mechanism of action of GW5638, a novel estrogen receptor ligand, provides insights into the role of estrogen receptor in bone, Endocrinology 138 (1997) 3901–4101.
[14] S. Luo, M. Stojanovic, C. Labrie, F. Labrie, Inhibitory effect of the novel anti-estrogen EM-800 and medroxyprogesterone acetate on estrone-stimulated growth of dimethylabenz[α]anthracene-induced mammary carcinoma in rats, Int. J. Cancer 73 (1997) 580–586.
[15] S. Luo, A. Sourla, C. Labrie, S. Gauthier, Y. Merand, A. Belanger, F. Labrie, Effect of twenty-four-week treatment with the anti-estrogen EM-800 on estrogen-sensitive parameters in intact and ovariectomized mice, Endocrinology 139 (1998) 2645–2656.
[16] J. Simard, R. Sanchez, D. Poirier, S. Gauthier, S.M. Singh, Y. Merand, A. Belanger, C. Labrie, F. Labrie, Blockage of the stimulatory effect of estrogens, OH-tamoxifen, OH-toremifene, droloxifen, and raloxifene on alkaline phosphatase activity by the anti-estrogen EM-800 in human endometrium adenocarcinoma Ishikawa cells, Cancer Res. 57 (1997) 3494–3497.
[17] S. Gauthier, B. Caron, J. Cloutier, Y.L. Dory, A. Favre, D. Larouche, J. Mailhot, C. Ouellet, A. Schwerdtfeger, G. Leblanc, C. Martel, J. Simard, Y. Merand, A. Belanger, C. Labrie, F. Labrie, (S)-(+)-4-[7-(2,2-dimethyl-1-oxopropoxy)-4-methyl-2-[4-[2-(1-piperidinyl)-ethoxy]phenyl]-2H-1-benzopyran-3-yl]-phenyl 2,2-dimethylpropanoate (EM-800): a highly potent, specific, and orally active nonsteroidal anti-estrogen, J. Med. Chem. 40 (1997) 2117–2122.
[18] F. Labrie, C. Labrie, A. Belanger, J. Simard, S. Gauthier, V. Luu-The, Y. Merand, V. Giguere, B. Candas, S. Luo, C. Martel, S.M. Singh, M. Fournier, A. Coquet, V. Richard, R. Charbonneau, G. Charpenet, A. Tremblay, G. Tremblay, L. Cusan, R. Veilleux, EM-652 (SCH 57068), a third generation SERM acting as pure anti-estrogen in the mammary gland and endometrium, J. Steroid Biochem. Mol. Biol. 69 (1999) 51–84.
[19] C. Martel, S. Picard, V. Richard, A. Belanger, C. Labrie, F. Labrie, Prevention of bone loss by EM-800 and raloxifene in the ovariectomized rat, J. Steroid Biochem. Mol. Biol. 74 (2000) 45–56.
[20] N.H. Ing, T.E. Spencer, F.W. Bazer, Estrogen enhances endometrial estrogen receptor gene expression by a post-transcriptional mechanism in the ovariectomized ewe, Biol. Reprod. 54 (1996) 591–599.
[21] J.A. Robertson, Y. Zhang, N.H. Ing, ICI 182,780 acts as partial agonist and antagonist of estradiol effects in specific cells of the sheep uterus, J. Steroid Biochem. Mol. Biol. l77 (2001) 281–287.
[22] J.L. Vallet, F.W. Bazer, Effect of ovine trophoblast protein-1, oestrogen and progesterone on oxytocin-induced phosphatidylinositol turnover in endometrium of sheep, J. Reprod. Fertil. 87 (1989) 755–761.
[23] N.H. Ing, M.B. Tornesi, Estradiol up-regulates estrogen receptor and progesterone receptor gene expression in specific ovine uterine cells, Biol. Reprod. 56 (1997) 1205–1215.
[24] M. Tomanek, C. Pisselet, P. Monget, T. Madigou, M.L. Thieulant, D. Monniaux, Estrogen receptor protein and mRNA expression in the ovary of sheep, Mol. Reprod. Dev. 48 (1997) 53–62.
[25] P.J. Shughrue, M.V. Lane, I. Merchenthaler, Regulation of progesterone receptor messenger ribonucleic acid in the rat medial preoptic nucleus by estrogenic and anti-estrogenic compounds: an in situ hybridization study, Endocrinology 138 (1997) 5476–5484.
[26] S.L. Fitzpatrick, T.J. Berrodin, S.F. Jenkins, D.M. Sindoni, D.C. Deecher, D.E. Frail, Effect of estrogen agonists and antagonists on induction of progesterone receptor in a rat hypothalamic cell line, Endocrinology 140 (1999) 3928–3937.
[27] E. Castellano-Diaz, M.I. Gonzalez-Quijano, J.M. Liminana, B.N. Diaz-Chico, Tamoxifen decreases the estradiol-induced progesterone receptors by interfering with nuclear estrogen receptor accumulation, J. Steroid Biochem. 33 (1989) 133–139.
[28] D. Bentrem, R. Dardes, H. Liu, J. MacGregor-Schafer, J. Zapf, V. Jordan, Molecular mechanism of action at estrogen receptor alpha of a new clinically relevant anti-estrogen (GW 7604) related to tamoxifen, Endocrinology 142 (2001) 838–846.
[29] U. Karck, F. Kommoss, Does tamoxifen change oestrogen and progesterone receptor expression in the endometrium and breast, Eur. J. Cancer 36 (2000) S45–46.
[30] N.H. Ing, T.L. Ott, Estradiol up-regulates estrogen receptor-α messenger ribonucleic acid in sheep endometrium by increasing its stability, Biol. Reprod. 60 (1999) 134–139.
[31] J. A Robertson, Y. Farnell, L.S. Lindahl, N.H. Ing, Estradiol up-regulates estrogen receptor messenger ribonucleic acid in endometrial carcinoma (Ishikawa) cells by stabilizing the message 29 (2002) 125–135.
[32] C. Martel, C. Labrie, A. Belanger, S. Gauthier, Y. Merand, X. Li, L. Provencher, B. Candas, F. Labrie, Comparison of the effects of the new orally active anti-estrogen EM-800 with ICI 182780 and toremifene on estrogen-sensitive parameters in the ovariectomized mouse, Endocrinology 139 (1997) 2486–2492.
[33] K. Zou, N.H. Ing, Oestradiol up-regulates oestrogen receptor, cyclophilin, and glyceraldehyde phosphate dehydrogenase mRNA concentrations in endometrium, but down-regulates them in liver, J. Steroid Biochem. Mol. Biol. 64 (1998) 231–237.
[34] P. Diel, T. Schulz, K. Smolnikar, E. Strunck, G. Vollmer, H. Michna, Ability of xeno- and phytoestrogens to modulate expression of estrogen-sensitive genes in rat uterus: estrogenicity profiles and uterotropic activity, J. Steroid Biochem. Mol. Biol. 73 (2000) 1–10.
[35] P. Kumar, P.J. Mark, B.K. Ward, R.F. Minchin, T. Ratajczak, Estradiol-regulated expression of the immunophilin cyclophilin 40 and FKBP52 in MCF-7 breast cancer cells, Biochem. Biophys. Res. Commun. 284 (2001) 219–225.
[36] E. Nagy, W.F. Rigby, Glyceraldehyde-3-phosphate dehydrogenase selectively binds AU-rich RNA in the NAD^+-binding region, J. Biol. Chem. 270 (1995) 2755–2763.
[37] L. Andreeva, R. Heads, C.J. Green, Cyclophilins and their possible role in the stress response, Int. J. Pathol. 80 (1999) 305–315.
[38] Y. Zhou, L.P. Chorich, V.B. Mahesh, T.F. Ogle, Regulation of estrogen receptor protein and messenger ribonucleic acid by estradiol and progesterone in rat uterus, J. Steroid Biochem. Mol. Biol. 46 (1993) 687–698.
[39] A. Meikle, M. Forsberg, L. Sahlin, B. Masironi, C. Tasende, M. Rodriguez-Pinon, E.G. Garofalo, A biphasic action of estradiol on estrogen and progesterone receptor expression in the lamb uterus, Reprod. Nutr. Dev. 40 (2000) 283–293.

[40] K.L. Medlock, C.R. Lyttle, N. Kelepouris, E.D. Newman, D.M. Sheehan, Estradiol down-regulation of the rat uterine estrogen receptor, Proc. Soc. Exp. Biol. Med. 196 (1991) 293–300.
[41] T.A. Tibbetts, M. Mendoza-Meneses, B.W. O'Malley, O.M. Conneely, Mutual and intercompartmental regulation of estrogen receptor and progesterone receptor expression in the mouse uterus, Biol. Reprod. 59 (1998) 1143–1152.
[42] P.B. Nirmala, R.V. Thampan, Ubiquitination of the rat uterine estrogen receptor: dependence on estradiol, Biochem. Biophys. Res. Commun. 213 (1995) 24–31.
[43] Z. Nawaz, D.M. Lonard, A.P. Dennis, C.L. Smith, B.W. O'Malley, Proteasome-dependent degradation of human estrogen receptor, Proc. Natl. Acad. Sci. U.S.A. 96 (1999) 1858–1862.
[44] A.L. Wijayaratne, D.P. McDonnell, The human estrogen receptor-alpha is a ubiquitinated protein whose stability is affected differentially by agonists, antagonists, and selective estrogen receptor modulators, J. Biol. Chem. 276 (2001) 35684–35692.
[45] C.A. Gray, F.F. Bartol, B.J. Tarleton, A.A. Wiley, G.A. Johnson, F.W. Bazer, T.E. Spencer, Developmental biology of uterine glands, Biol. Reprod. 65 (2001) 1123–1311.