Gynecologic Oncology **78,** 194–202 (2000)
doi:10.1006/gyno.2000.5863, available online at http://www.idealibrary.com on IDEAL®

Luteinizing Hormone–Releasing Hormone Agonist Triptorelin and Antagonist Cetrorelix Inhibit EGF-Induced c-*fos* Expression in Human Gynecological Cancers

Carsten Gründker, Ph.D.,* Peter Völker, Ph.D.,* Klaus-Dieter Schulz, M.D.,† and Günter Emons, M.D., Ph.D.*

**Department of Gynecology and Obstetrics, Georg-August University, Göttingen, D-37075 Germany; and*
†Department of Gynecology and Obstetrics, Philipps University, Marburg, Germany

Received January 4, 2000

Objectives. **Spontaneous and epidermal growth-factor-induced proliferation of human gynecological cancer cell lines is dose- and time-dependently reduced by treatment with the luteinizing hormone–releasing hormone (LHRH) agonist triptorelin and antagonist Cetrorelix. This antiproliferative activity is probably directly mediated through the LHRH receptors expressed by the tumor cells interacting with growth-factor-dependent mitogenic signal transduction. We have examined whether epidermal growth-factor (EGF)-induced expression of the early response gene c-*fos* is reduced by LHRH analogs.**

Methods. **Human endometrial (Ishikawa, Hec-1A), ovarian (EFO-21, EFO-27, SK-OV-3), and breast cancer cell lines (MCF-7) were rendered quiescent by incubation (72 h) in the absence of fetal calf serum and phenol red. This was followed by a 15-min incubation in the absence or presence of the LHRH agonist triptorelin (100 nM) or the antagonist Cetrorelix (100 nM) before the cells were stimulated for 10 min with EGF (100 nM). C-*fos* mRNA expression was determined by semi-quantitative RT-PCR using a synthetic DNA fragment as internal standard. C-Fos protein synthesis was determined by SDS–PAGE and semi-quantitative Western blotting.**

Results. **In cells derived from endometrial and ovarian cancer, maximal c-*fos* mRNA expression (seven- to ninefold over basal level) was obtained 30 min after EGF stimulation. In the breast cancer cell line MCF-7 this effect was obtained 60 min after EGF treatment. In all of the lines expressing LHRH receptor, EGF-induced c-*fos* mRNA expression as well as c-Fos protein synthesis was dose-dependently reduced by treatment with LHRH agonists and antagonists. At 100 nM concentrations of the LHRH analogs, c-*fos* expression was reduced to baseline levels. No effect of LHRH analogs on EGF-induced c-*fos* expression was observed in the ovarian cancer cell line SK-OV-3, which does not express the LHRH receptor.**

Conclusions. **These results suggest that the binding of LHRH agonists and antagonists to their receptors inhibits the mitogenic signal transduction pathway of the EGF receptor in endometrial, ovarian, and breast cancer cell lines. The coupling of both signal transduction systems mediates the antiproliferative effect of LHRH analogs.** **© 2000 Academic Press**

INTRODUCTION

The hypothalamic decapeptide luteinizing hormone–releasing hormone (LHRH) plays a key role in the control of mammalian reproduction. It is released from the hypothalamus in a pulsatile manner and stimulates the synthesis and release of luteinizing hormone and follicle stimulating hormone (FSH). In addition to these well-documented classic hypophysiotropic actions, LHRH might play a role in the brain and a variety of peripheral organs. An autocrine/paracrine function of LHRH has been suggested to exist, for instance, in the placenta, granulosa cells, myometrium, and lymphoid cells. It is probable that such LHRH-based autocrine systems are present in a number of human malignant tumors including cancers of the breast, ovary, endometrium, and prostate (for review see [1–3]).

In a series of recent studies, it could be demonstrated that endometrial, ovarian, and breast cancer cell lines and primary tumors of these organs express LHRH immuno- and bioactivity as well as the mRNA for LHRH [4–7]. In addition, specific high-affinity binding sites for LHRH and the expression of the mRNA for the pituitary LHRH receptor have been detected in endometrial, ovarian, and breast cancer cell lines and in over 80 (endometrial and ovarian cancer) or 50% (breast cancer) of biopsy specimens of these cancers, respectively [7–12]. The function of the expression of LHRH and its receptor is still unclear.

The proliferation of human endometrial, ovarian, and breast cancer cell lines which express LHRH receptors was inhibited by both agonistic and antagonistic analogs of LHRH. These antiproliferative effects were evident at nanomolar concentrations of the LHRH analogs, suggesting that they are mediated through the LHRH receptors in the tumor cells [13–15]. The exact mechanism of action of this antiproliferative effect is still obscure.

In view of the apparent similarity of LHRH receptors in endometrial, ovarian, and breast cancers to those in the pituitary [16], it seemed reasonable to speculate also that LHRH

0090-8258/00 $35.00

signal transduction pathways in the tumors might be the same as those operating in pituitary gonadotrophs, including phospholipase C or protein kinase C [17]. Previous findings from our laboratory, however, suggested that these classical LHRH receptor signal transduction mechanisms are not involved in the mediation of antiproliferative effects of LHRH analogs in endometrial and ovarian cancer cells [18]. Reports from different laboratories, including ours, rather suggest that antiproliferative effects of LHRH analogs are mediated through interaction with growth-factor-induced mitogenic pathways [e.g., epidermal growth factor (EGF) and insulin-like growth factor (IGF)], as LHRH analogs antagonized growth-factor-induced proliferation, tyrosine phosphorylation, and activity of mitogen-activated protein kinase (MAP-kinase) [1]. To further corroborate this hypothesis, the effects of LHRH agonist and antagonist treatment on EGF-induced c-*fos* expression were assessed in this study. Induction of the immediate early gene c-*fos* is one of the most downstream events in mitogenic signal transduction and should be reduced if LHRH analogs interacted with this pathway.

MATERIAL AND METHODS

Cell lines and culture conditions. The human endometrial cancer cell lines used were derived from an endometrial adenocarcinoma (Ishikawa) [19] or a moderately differentiated papillary adenocarcinoma (Hec-1A) [20]. The human ovarian cancer cell lines used were derived from a poorly differentiated serous adenocarcinoma (EFO-21) [21], a mucinous papillary adenocarcinoma of intermediate differentiation (EFO-27) [21], or an adenocarcinoma derived from ascites (SK-OV-3) [22]. The human breast cancer cell line used was derived from a mammary gland adenocarcinoma (MCF-7) [23]. The cells were cultured as described in detail previously [13]. For determination of EGF-induced c-*fos* mRNA expression, the cells were cultured for 72 h in the absence of fetal calf serum (FCS) and phenol red. The quiescent cells were incubated with 100 nM bovine EGF (Sigma, Deisenhofen, Germany) for 10 min with or without a previous incubation (15 min) with 10 μM, 100 nM, 1 nM, and 10 pM of the LHRH agonist triptorelin or the LHRH antagonist Cetrorelix. C-*fos* mRNA expression was determined after 5, 15, 30, 60, and 120 min by semi-quantitative RT-PCR (see below). For determination of EGF-induced c-Fos protein synthesis, the cells were cultured as described below. To analyze interactions between LHRH agonist triptorelin and LHRH antagonist Cetrorelix, quiescent cells were incubated with 100 nM EGF for 10 min with a previous incubation (15 min) with 100 nM of LHRH agonist triptorelin. In addition the cells were simultaneously exposed to an increasing concentration of LHRH antagonist Cetrorelix (10 μM, 100 nM, and 1 nM) and c-*fos* mRNA expression was determined as described above.

LHRH analogs. The LHRH agonist [D-Trp^{6}]–LHRH (triptorelin) was kindly provided by Ferring Arzneimittel (Kiel, Germany). The LHRH antagonist Cetrorelix ([Ac-D-Nal(2)^{1}, D-Phe(4Cl)^{2}, D-Pal^{3}, D-Cit^{6}, D-Ala^{10}]LHRH) was kindly provided by Asta Medica (Frankfurt, Germany).

Isolation of RNA and cDNA synthesis. Total RNA was prepared from cells grown in a monolayer using the RNeasy protocol (Qiagen, Hilden, Germany). The concentration of RNA in each sample was determined by photospectroscopy. First-strand cDNA was generated by reverse transcription of 4 μg of total RNA using p(dT)$_{15}$ primers (Boehringer Mannheim, Mannheim, Germany) with MMLV reverse transcriptase according to the instructions of the suppliers (Gibco BRL, Karlsruhe, Germany). After determining the concentration of the cDNAs, the samples were used for semi-quantitative PCR analysis. The integrity of the samples was tested by RT-PCR of the housekeeping gene GAPDH (forward primer: 5′ CAT CAC CAT CTT CCA GGA GCG AGA 3′, backward primer: 5′ GTC TTC TGG GTG GCA GTG ATG G 3′).

Internal standard synthesis. For semi-quantitative RT-PCR of c-*fos*, a 161-bp internal standard was generated by PCR in three steps. In the first PCR step we used primers specific for a synthetic DNA template (Institute for Molecular Biology and Tumor Research, Marburg, Germany), amplifying a 141-bp DNA fragment of the synthetic DNA template. In the second PCR step, hybrid primers were used. The first hybrid primer (hybrid forward primer: 5′ GAG ATT GCC AAC CTG CTG AAC GCA AGT GAA ATC TCC TCC G 3′) consisted of nucleotides 1–20 of the c-*fos* cDNA sequence (positions 2730–2749) and a primer sequence specific for the synthetic DNA in positions 21–40. The second hybrid primer (hybrid backward primer: 5′ AGA CGA AGG AAG ACG TGT AAT CTG TCA ATG CAG TTT GTA G 3′) consisted of the second primer sequence specific for the synthetic DNA in positions 21–40. Nucleotides 1–20 are identical to the c-*fos* cDNA sequence from positions 3194 to 3213 in reverse orientation. Using these hybrid primers, together with the 141-bp product from the first PCR step, the second PCR product has a total length of 161 bp, containing synthetic DNA and c-*fos*-specific primer sites. This secondary PCR product was then amplified in a third PCR step with the c-*fos* primers only (forward primer: 5′ GAG ATT GCC AAC CTG CTG AA 3′, backward primer: 5′ AGA CGA AGG AAG ACG TGT AA 3′), resulting in an internal standard for c-*fos* quantification of 161 bp.

Semi-quantitative PCR amplification. The cDNAs (2 ng) were amplified in a 50-μl reaction volume containing 10 mM Tris–HCl (pH 8.3), 50 mM potassium chloride, 1.5 mM magnesium chloride, 200 μM of each of the dNTPs including [^{32}P]ATP (3000 Ci/mmol; Amersham-Buchler, Braunschweig, Germany), 1 μM of the appropriate primers (see above), and 1.25 U Taq polymerase (Boehringer Mannheim) in a Perkin–Elmer DNA thermal cycler 2400 (Weiterstadt, Germany). Twenty-five cycles of amplification were carried out: denaturation at 94°C for 30 s, annealing at 54°C for 30 s, followed by extension at 72°C for 60 s. The PCR product amplified with the

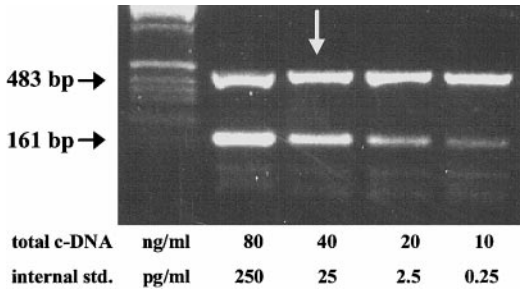

FIG. 1. Optimization experiment for internal standard DNA. Oligonucleotide primers for human c-*fos* were used. The gel was stained with ethidium bromide, and bands were visualized with UV light. A serial dilution of cDNA obtained from total RNA of the serum-induced endometrial cell line Hec-1A (upper bands) and an inverse dilution of c-*fos* internal standard DNA (lower bands) were coamplified in a single tube by PCR. Using 25 pg/ml of c-*fos* internal standard DNA and 40 ng/ml of total cDNA, signals of equal intensity were obtained (arrow).

c-*fos* primers has a total length of 483 bp. For testing of the optimal concentration of the internal standard used in semiquantitative PCR, the internal standard and target cDNA were added to the PCR tubes in inverse serial dilutions. PCR products were separated by gel electrophoresis in 1.5% agarose. PCR reactions yielding standard and target signals of identical intensity were used for PCR analysis for determination of c-*fos* expression levels. The respective DNA products were run on 1.5% agarose gels and bands were visualized by ethidium bromide staining on an UV transilluminator. For quantification the bands were cut out of the gel and radioactivity was determined as counts per minute using a β-liquid scintillation counter (Beckman, Munich, Germany). Expression levels of c-*fos* were calculated in comparison with the basal c-*fos* expression levels.

Restriction enzyme analysis. PCR products were digested with restriction endonucleases *Hin*fI (cut in position 2886) and *Fok*I (cut in positions 2771, 3016, and 3121) under the conditions recommended by the supplier (Boehringer Mannheim). The digested products along with untreated aliquots of each PCR sample were then fractionated on 1.5% agarose gels and stained using ethidium bromide.

Western blotting. The cells were plated at a density of 10^6 cells in 100-mm dishes and grown under standard conditions. After 2 days, culture media were changed to FCS-free and phenol-red-free medium for 72 h. The quiescent cells were incubated with 100 nM bovine EGF (Sigma) for 10 min with or without previous incubation (15 min) with 100 nM of the LHRH agonist triptorelin or the LHRH antagonist Cetrorelix. After 4 h the cells were detached with 1 ml of a solution containing 0.5 g trypsin (Biochrom) and 5 mmol EDTA in 1 L PBS/BSA and then lysed using a lysis buffer containing 9.5 M urea, 2.0% NP-40, and 5.0% β-mercaptoethanol. The cell lysates were electrophoresed on SDS–PAGE (7.5%) under reducing conditions and transferred to nitrocellulose. The nitrocellulose membranes were blocked in 3% BSA (Sigma) in TBST (10 mM Tris, pH 8, 500 mM NaCl, 0.1% Tween 20) for 2 h; incubated with polyclonal rabbit anti-human c-Fos (Calbiochem, Bad Soden, Germany) in a 1:1500 dilution in 1% BSA in TBST for 1 h; and then, following washings, incubated with horseradish peroxidase-conjugated anti-rabbit IgG in an 1:1000 dilution in 1% BSA in TBST (Amersham, Buckinghamshire, UK) for 1 h. After the washings, specifically bound antibody was detected using the enhanced chemiluminescence kit (Amersham). The bands were analyzed using the Kodak 1D image system (Kodak, New Haven, CT).

Statistical analysis. All experiments were reproduced three times in different passages of the respective cell lines. Data were tested for significant differences using the Mann–Whitney *U* test.

RESULTS

The PCR amplification of c-*fos* encoding DNA used here was sensitive enough to detect a minimal concentration of 0.25 pg/ml of c-*fos* cDNA (data not shown).

To obtain the correct dilution of the internal standard, c-*fos* mRNA was quantified by RT-PCR using total RNA from the serum-induced endometrial cell line Hec-1A in the presence of c-*fos* internal standard DNA. Serial dilution of cDNA and an inverse dilution of c-*fos* internal standard were coamplified in a single tube by PCR. Using 25 pg/ml of internal standard and 40 ng/ml of total cDNA, signals of equal intensity were obtained (Fig. 1). This concentration of internal standard was used in the following experiments.

The expression levels of c-*fos* in LHRH agonist- and LHRH antagonist-treated tumor cells were quantified by this semiquantitative RT-PCR system.

By cultivation of the cells under serum- and phenol-red-free conditions for 72 h, c-*fos* expression of all analyzed cell types could be downregulated to basal expression levels (Fig. 2).

Treatment of the quiescent cells with 100 nM EGF (10 min) resulted in a marked increase of the c-*fos* expression levels in all cell lines analyzed. In the endometrial (Ishikawa, Hec-1A) and ovarian cancer cell lines (EFO-21, EFO-27, SK-OV-3), a

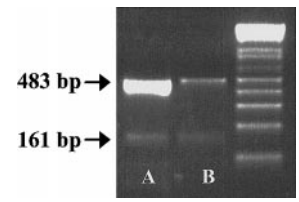

FIG. 2. PCR amplification of first-strand cDNA from ovarian cancer cell line EFO-21. Oligonucleotide primers for human c-*fos* were used. The gel was stained with ethidium bromide, and bands were visualized with UV light. Upper bands represent c-*fos* amplification and lower bands represent internal standard amplification. Cells grown under normal conditions (10% FCS) showed a high c-fos expression (A). In the serum-starved cells the c-*fos* expression was decreased to low levels (B).

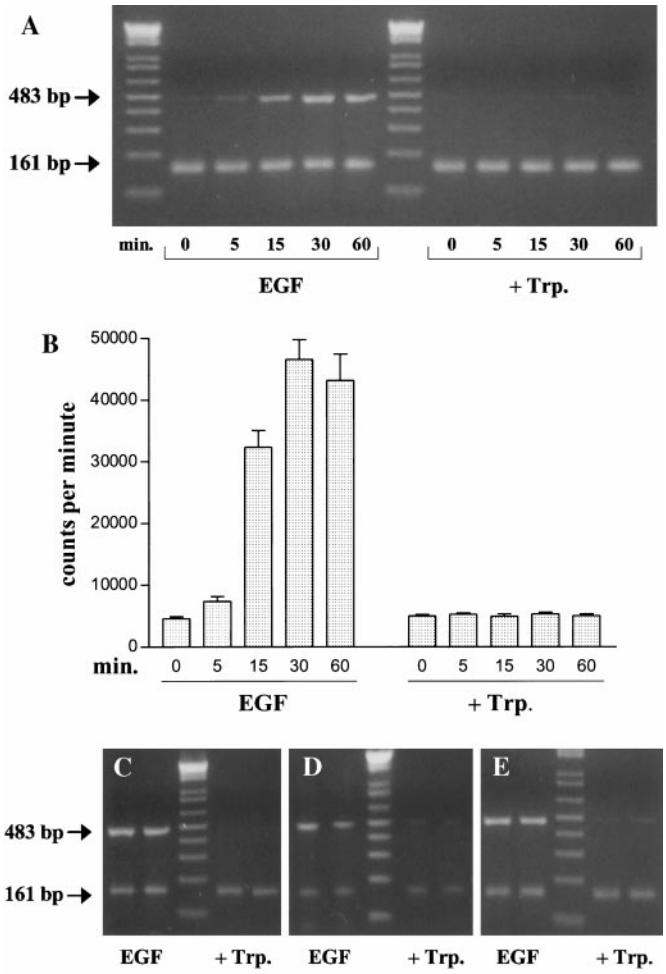

FIG. 3. PCR amplification of first-strand cDNA from ovarian cancer cell line EFO-21 (A, B), endometrial cancer cell lines Hec1A (C) and Ishikawa (D), and breast cancer cell line MCF-7 (E). Oligonucleotide primers for human c-*fos* were used. The gel was stained with ethidium bromide, and bands were visualized with UV light. Upper bands represent c-*fos* amplification and lower bands represent internal standard amplification. Quiescent cells were incubated in the absence or presence (+Trp) of the LHRH agonist triptorelin (100 nM) for 15 min followed by treatment with 100 nM EGF for 10 min. Quantitative data obtained from three independent experiments of ovarian cancer cell line EFO-21 are shown (B).

maximal c-*fos* expression level (seven- to ninefold increase) was reached 30 min after EGF treatment. In the MCF-7 cell line, maximal c-*fos* expression (eightfold increase) was reached 60 min after EGF treatment.

To analyze whether LHRH analogs are able to affect EGF-induced c-*fos* expression, quiescent cells were kept for 15 min in the absence or presence of either the LHRH agonist triptorelin (100 nM) or the LHRH antagonist Cetrorelix (100 nM) before they were incubated for 10 min with 100 nM EGF. In the ovarian cancer cell line EFO-21, the endometrial cancer cell lines Hec1A and Ishikawa, and the breast cancer cell line MCF-7, EGF-induced c-*fos* expression was completely inhibited by the LHRH agonist triptorelin (Figs. 3A–3E) as well as by the LHRH antagonist Cetrorelix (data not shown).

In the ovarian cancer cell line EFO-21 (Figs. 3A and 3B), c-*fos* expression was increased ninefold over the basal level by EGF treatment. In cells treated with the LHRH agonist triptorelin (Figs. 3A and 3B) or the LHRH antagonist Cetrorelix (data not shown) this EGF-induced c-*fos* expression was completely inhibited and remained on basal levels.

In the endometrial cancer cell lines Hec-1A (Fig. 3C) and Ishikawa (Fig. 3D) as well as in the breast cancer cell line MCF-7 (Fig. 3E), the EGF-induced c-*fos* expression was seven- or eightfold higher, respectively, than the basal c-*fos* expression levels. After treatment with the LHRH agonist triptorelin as well as with the LHRH antagonist Cetrorelix, the EGF-induced c-*fos* expression remained on basal expression levels.

In the ovarian cancer cell line SK-OV-3, which does not express LHRH receptors, LHRH agonists or antagonists had no effects on the EGF-induced c-*fos* expression. In the absence or presence of either the LHRH agonist triptorelin or the LHRH antagonist Cetrorelix, EGF-induced c-*fos* expression was increased up to eightfold of the basal c-*fos* expression (Fig. 4).

The ovarian cancer cell line EFO-27 showed a different behavior than other cancer cell lines expressing LHRH receptors. EGF-induced c-*fos* expression was inhibited by the LHRH agonist triptorelin but not by the LHRH antagonist Cetrorelix (Fig. 5A). Instead, LHRH agonist triptorelin-induced (100 nM) inhibition of EGF-induced c-*fos* expression was partly antagonized by the LHRH antagonist Cetrorelix (10 μM, 100 nM, and 1 nM). This antagonistic activity was clearly dose-dependent but did not result in a complete inhibition of the triptorelin-induced inhibition of EGF-induced c-*fos* expression (Fig. 5B).

To analyze whether the inhibitory effects of the LHRH agonist triptorelin and the LHRH antagonist Cetrorelix on EGF-induced c-*fos* expression are dose-dependent, the cells were treated with a decreasing concentration of the LHRH

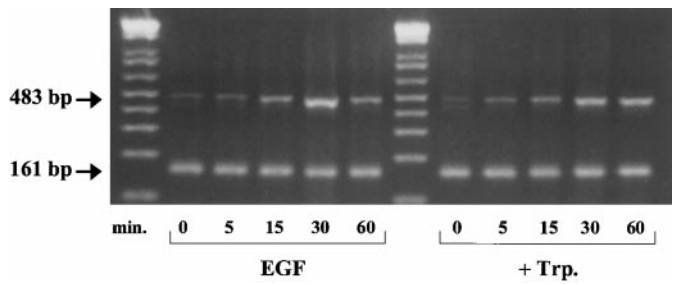

FIG. 4. PCR amplification of first-strand cDNA from the ovarian cancer cell line SK-OV-3. Oligonucleotide primers for human c-*fos* were used. The gel was stained with ethidium bromide, and bands were visualized with UV light. Upper bands represent c-*fos* amplification and lower bands represent internal standard amplification. Quiescent cells were incubated in the absence or presence (+Trp) of the LHRH agonist triptorelin (100 nM) for 15 min followed by treatment with 100 nM EGF for 10 min.

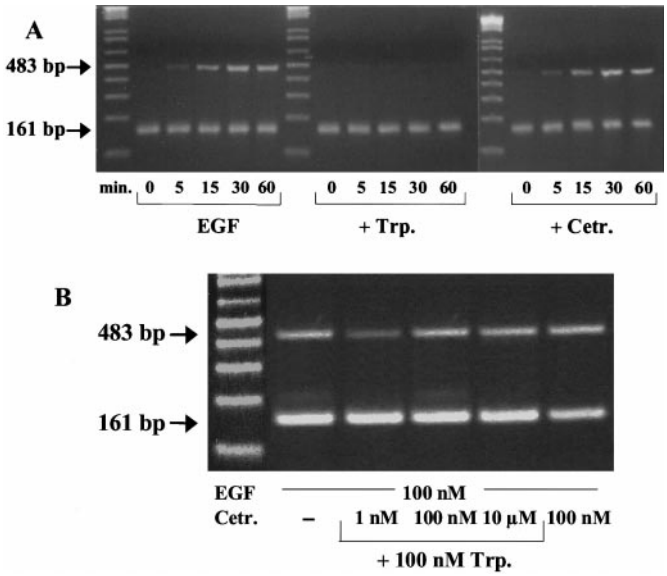

FIG. 5. PCR amplification of first-strand cDNA from the ovarian cancer cell line EFO-27. Oligonucleotide primers for human c-*fos* were used. The gel was stained with ethidium bromide, and bands were visualized with UV light. Upper bands represent c-*fos* amplification and lower bands represent internal standard amplification. Quiescent cells were incubated in the absence or presence of the LHRH agonist triptorelin (100 nM, + Trp) or of the LHRH antagonist Cetrorelix (100 nM, + Cetr) for 15 min followed by treatment with 100 nM EGF for 10 min (A). To analyze interactions between the LHRH agonist triptorelin and the LHRH antagonist Cetrorelix, quiescent cells were incubated with 100 nM EGF for 10 min with previous incubation (15 min) with 100 nM of the LHRH agonist triptorelin. In addition the cells were simultaneously exposed to an increasing concentration of the LHRH antagonist Cetrorelix (1 nM, 100 nM, and 10 μM) (B).

agonist triptorelin or the LHRH antagonist Cetrorelix. As shown in Fig. 6 for the ovarian cancer cell line EFO-21 as an example, EGF-induced c-*fos* expression was decreased to basal levels after treatment with 10 μM and 100 nM of the LHRH agonist triptorelin. Using a concentration of 1 nM triptorelin EGF-induced c-*fos* expression was decreased, but not to basal levels. The c-*fos* expression reached fourfold of the basal expression level. Using a concentration of 10 pM triptorelin the EGF-induced c-*fos* expression was reduced to half of the maximum expression levels or fivefold of basal expression levels, respectively. Identical results were obtained using the LHRH antagonist Cetrorelix (data not shown). Comparable dose–response relations of LHRH-induced suppression were found in all analyzed cell lines that expressed LHRH receptors (data not shown).

To analyze whether LHRH analog treatment affects c-Fos protein synthesis, quiescent cells were kept for 15 min in the absence or presence of either the LHRH agonist triptorelin (100 nM) or the LHRH antagonist Cetrorelix (100 nM) before they were incubated for 10 min with 100 nM EGF.

The amount of c-Fos protein was evaluated by SDS–PAGE and Western blotting of the cell lysates. Figure 7 shows that a protein band of 62 kDa, corresponding to c-Fos protein, is present in the cell lysates. In the serum-starved cells the amount of c-Fos protein is very small and is increased strongly after EGF treatment (Figs. 7A and 7B, lanes 1 and 2). In the ovarian cancer cell line EFO-21, EGF-induced c-Fos protein synthesis was inhibited by the LHRH agonist triptorelin (Fig. 7A, lanes 3 and 4) as well as by the LHRH antagonist Cetrorelix (data not shown). Identical results were obtained using the endometrial cancer cell lines Hec-1A and Ishikawa and the breast cancer cell line MCF-7 (data not shown). In the ovarian cancer cell line SK-OV-3, LHRH agonists or antagonists had no effects on the EGF-induced c-Fos protein synthesis (Fig. 7B, lanes 3 and 4).

DISCUSSION

In pituitary gonadotrophs, LHRH receptor signaling, inducing exocytosis of gonadotrophins, is essentially mediated through the activation of phospholipase C (PLC), leading to the rapid hydrolysis of membrane phospholipids, the liberation of inositol phosphates, subsequent mobilization of intracellular Ca^{2+}, and activation of protein kinase C (PKC) [17].

In view of the apparent similarity of LHRH receptors in peripheral cancers to those in the pituitary, it seemed reason-

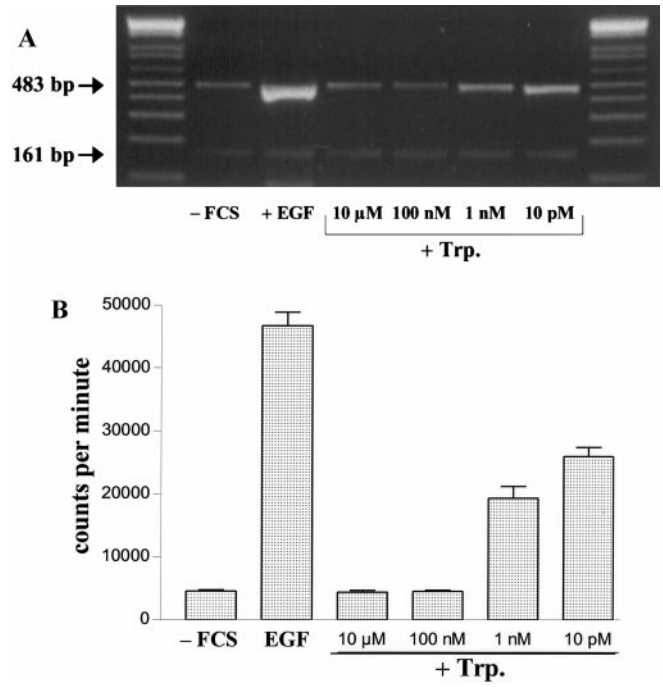

FIG. 6. PCR amplification of first-strand cDNA from the ovarian cancer cell line EFO-21. Oligonucleotide primers for human c-*fos* were used. The gel was stained with ethidium bromide, and bands were visualized with UV light. Upper bands represent c-*fos* amplification and lower bands represent internal standard amplification. (A) Quiescent cells were incubated in the absence or presence of decreasing concentrations (10 μM, 100 nM, 1 nM, and 10 pM) of the LHRH agonist triptorelin for 15 min followed by treatment with 100 nM EGF for 10 min (+Trp). Quantitative data obtained from three independent experiments are shown (B).

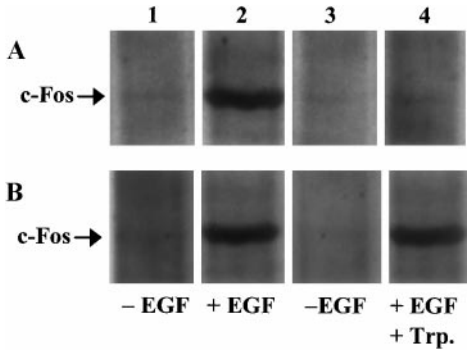

FIG. 7. Immunoblotting of c-Fos protein in ovarian cancer cell lines EFO-21 (A) and SK-OV-3 (B) using a polyclonal rabbit anti human c-Fos antibody. Quiescent cells were kept for 15 min in the absence or presence of the LHRH agonist triptorelin (100 nM) before they were incubated for 10 min with 100 nM EGF. In the serum-starved cells the amount of c-Fos protein is very small (A and B, lane 1) and is increased strongly after EGF treatment (A and B, lane 2). In the ovarian cancer cell line EFO-21, EGF-induced c-Fos protein synthesis was inhibited by the LHRH agonist triptorelin (A, lanes 3 and 4). In the ovarian cancer cell line SK-OV-3, LHRH agonists or antagonists had no effects on the EGF-induced c-Fos protein synthesis (B, lanes 3 and 4).

able to speculate also that LHRH signal transduction pathways in tumors might be comparable to those operating in pituitary gonadotrophs, such as PLC and PKC. Early reports on LHRH signal transduction in rat mammary tumors, human breast cancer cell lines, and membranes from ovarian cancer biopsies supported this concept [24–26]. Our group performed extensive studies in human ovarian (EFO-21, EFO-27) and endometrial (HEC-1A, Ishikawa) cancer cell lines. These cell lines express LHRH receptors, and their proliferation is inhibited by LHRH analogs [7, 13, 14]. Although we could clearly demonstrate the activation of phospholipase C, protein kinase C, and adenylyl cyclase in the tumor cells by pharmacological stimuli, the LHRH agonist triptorelin, at concentrations that were clearly inhibitory on proliferation, had no effects on activity of these signaling systems [18]. We found, however, that the mitogenic effect of epidermal growth factor in these cell lines could be counteracted by triptorelin, indicating an interaction with the mitogenic signal transduction pathway [18]. Similar results were obtained when the proliferation of EFO-21, EFO-27, Hec-1A, and Ishikawa cells was stimulated with IGF-1 [18]. Direct measurement of EGF-induced net tyrosine phosphorylation by membrane preparations from EFO-21, EFO-27, and Hec-1A cells revealed that it was markedly reduced in the presence of the LHRH agonist triptorelin. A similar reduction of EGF-induced net tyrosine phosphorylation was observed when the tumor cells were pretreated for 48 h with 100 nM triptorelin. In the presence of 100 μM sodium vanadate, an inhibitor of phosphotyrosine phosphatase, the reduction of EGF-induced net tyrosine phosphorylation by triptorelin treatment was much less marked than in the absence of vanadate. This suggests that the reduction of EGF-induced net tyrosine phosphorylation by triptorelin could be mediated through the activation of a phosphotyrosine phosphatase [18]. Comparable data were obtained by Moretti *et al.* [27] in human prostatic cancer cell lines LNCaP and DU 145. These findings are in accord with reports that LHRH analogs reduce growth-factor-induced tyrosine kinase activity [1, 18, 25, 27, 28]. Growth-factor-induced tyrosine phosphorylation is supposed to be counteracted by LHRH analogs through activation of a phosphotyrosine phosphatase [1, 18, 27], which is probably coupled to the LHRH receptor through a Gi protein in human reproductive tract tumors [29]. Imai *et al.* [29] speculated that the Gi protein that couples the LHRH receptor to the effector may be responsible for the difference in the response in peripheral tumors and those of the anterior pituitary. The concept of an inhibition of mitogenic signal transduction by LHRH analogs in human cancer cells was further corroborated by the demonstration that EGF-induced activation of mitogen-activated protein kinase, an enzyme further downstream in the growth factor signaling cascade [30], was virtually blocked in ovarian and endometrial cancer cells treated with the LHRH agonist triptorelin [18]. In the ovarian and endometrial cancer cell lines tested, stimulation with 100 nM EGF for 5 min produced an approximately fivefold increase in MAP-kinase acticity. This dramatic increase of MAP-kinase activity was almost completely nullified when the cells were exposed for 15 min to 10 μM triptorelin [18]. By quantitative RT-PCR we now show that the EGF-induced expression of the immediate early gene c-*fos*, a mechanism still further downstream in mitogenic signaling, is completely abrogated in breast, ovarian, and endometrial cancer cells that express LHRH receptors by treatment with the LHRH agonist triptorelin as well as with the LHRH antagonist Cetrorelix. These results could be confirmed by Western blotting analysis of c-Fos protein synthesis.

Neither the LHRH agonist triptorelin nor the LHRH antagonist Cetrorelix had an impact on EGF-induced c-*fos* mRNA expression as well as c-Fos protein synthesis in the ovarian cancer cell line SK-OV-3. We had expected this result since this cell line does not express LHRH receptors and its proliferation is not reduced by LHRH analogs (unpublished results). These findings support the hypothesis that the effect of the LHRH analogs on the EGF-induced c-*fos* expression is mediated through the LHRH receptor.

In most cancer cell lines tested by us, the LHRH antagonist Cetrorelix acts as an LHRH agonist, indicating that the dichotomy of LHRH agonists and antagonists does not exist in tumor cells. The reason for this discrepancy to findings in the pituitary is still obscure and is the subject of ongoing investigations in our laboratory.

In the ovarian cancer cell line EFO-27 the LHRH agonist triptorelin clearly suppressed EGF-induced c-*fos* mRNA expression and c-Fos protein synthesis, while the LHRH antagonist Cetrorelix was inactive. These findings are well in line with earlier observations that in this cell line triptorelin dose- and time-dependently reduced proliferation, while Cetrorelix had no antiproliferative activity although the antagonist bound to the LHRH receptor in this cell line with identical affinity as

the LHRH agonist [13]. Cetrorelix, however, was shown to dose-dependently antagonize triptorelin-induced reduction of proliferation as well as triptorelin-induced inhibition of EGF-induced c-*fos* mRNA expression in EFO-27 cells, indicating that it acts as a classical LHRH antagonist in this cell line [13]. The reason for this particular behavior of EFO-27 ovarian cancer cells remains to be elucidated.

Our results that endometrial cancer cell lines including Ishikawa and Hec-1A showed both LHRH and LHRH receptor gene transcription [7] were recently corroborated by Chatzaki *et al.* [31]. But, in contrast to our results and those of other laboratories [1, 17, 32], they could detect neither LHRH immunoreactivity nor high-affinity binding sites for LHRH [7]. Maybe the use of different analogs and variations in the methodology of the binding assays account for these discrepancies. Our results that LHRH analogs have no effects on the activity of LHRH signaling mechanisms as found in the pituitary [18] were also corroborated by Chatzaki *et al.* [31]. In contrast to us and other groups they could not detect any antiproliferative effects of LHRH analogs [1–3]. Therefore they concluded that LHRH has no biological effects on endometrial cancer cells [31]. However, our results on LHRH signal transduction and interaction with EGF-induced mitogenic signal transduction [1–3, 16], including the data we present here, clearly demonstrate receptor-mediated biological effects of LHRH in endometrial cancer cells.

In prostatic cancer cells LHRH agonists inhibit proliferation by interfering with some of the cellular mechanisms mediating the stimulatory action of the EGF and the IGF system [27, 33]. Dondi *et al.* [34] found that LHRH agonists exert significant and dose-dependent antiproliferative action on DU-145 prostate cancer cells. Both LHRH and its receptor are expressed in this cell line indicating that an autocrine/paracrine LHRH loop is present in androgen-independent prostate cancer cells and may participate in the regulation of tumor growth. As in gynecological tumors [18], LHRH agonists inhibited the proliferation of human prostatic cancer cells by interfering with the stimulatory actions of EGF [27]. Both LHRH receptors and EGF receptors are present in a high percentage of human ovarian, endometrial, breast, and prostatic tumors indicating that these cancer cells might have local regulatory systems for their proliferation based on LHRH and EGF [35]. Lamharzi *et al.* [36] already showed that prolonged administration of the LHRH antagonist Cetrorelix is accompanied by a marked decrease in LHRH and EGF receptors in DU-145 prostate tumor xenografts. Szepeshazi *et al.* [37] demonstrated the anti-tumorigenic effect of Cetrorelix in hamsters afflicted with experimental pancreatic cancer, concomitant with a fall in EGF receptors. Other possible molecular mechanisms that might be involved in the mediation of anti-tumor effects of LHRH analogs such as apoptosis or interaction with the mitogenic effects of steroids have been also suggested [38]. Kim *et al.* showed that the antiproliferative effect of LHRH agonists in ovarian cancer is mainly attributed to cytostatic activities resulting in blocking cell cycle activities rather than to the induction of apoptosis [39]. The reasons for the differences in LHRH signal transduction in the pituitary and peripheral cancers are still unclear. Experimentally induced mutations of the LHRH receptor have altered LHRH binding, G-protein–receptor interaction, or proper membrane incorporation [40, 41]. Mutations of the coding region of the LHRH receptor gene, however, were not found in the EFO-21 and EFO-27 ovarian cancer cell lines or the Ishikawa and Hec-1A endometrial cancer cell lines or the MCF-7 breast cancer cell line (unpublished results). Therefore the LHRH receptor itself cannot be responsible for the variant LHRH signal transduction pathway in cancer cells. On the other hand, some normal and neoplastic human tissues were found to express differential splice variants of the LHRH receptor gene in a tissue-dependent manner [42]. It is not yet clear whether these splice variants can be translated into active membrane receptors. In the tumor cell lines analyzed by us, however, no signs for alternative LHRH receptor splice variants were seen (unpublished results). Active mutations of G-proteins have been implicated in the pathogenesis of some human neoplasms including ovarian tumors [29, 43]. It is possible that G-protein mutations or unknown subtypes of G-proteins are responsible for the specific LHRH signaling in tumors and therefore for its antiproliferative actions.

Our present results on c-*fos* expression show that the mitogenic signaling of the EGF receptor is completely inhibited downstream in the mitogenic pathway by binding of LHRH agonists and antagonists to their receptors in endometrial, ovarian, and breast cancer cell lines. This clearly demonstrates that the coupling of both signal transduction systems mediates the antiproliferative LHRH effects of LHRH analogs.

ACKNOWLEDGMENTS

We thank Dr. M. Krause, Institute for Molecular Biology and Tumor Research, Marburg, Germany, for the preparation of the oligonucleotide primers. We are grateful to Ferring Arzneimittel, Kiel, Germany, for supplying the LHRH agonist triptorelin and to Asta Medica AG, Frankfurt, Germany, for the gift of the LHRH antagonist Cetrorelix. This study was supported by the Bundesministerium für Bildung und Forschung and Asta Medica AG, Frankfurt, Germany.

REFERENCES

1. Gründker C, Emons G: GnRH—Mechanisms of Action, in Lunenfeld B (ed): GnRH Analogues, The State of the Art at the Millennium. Parthenon, Carnforth, 1999, pp 7–29
2. Emons G, Schally AV: The use of luteinizing hormone–releasing hormone agonists and antagonists in gynecological cancers. Hum Reprod 9:1364–1379, 1994
3. Emons G, Ortmann O, Schulz KD, Schally AV: Growth-inhibitory actions of analogues of luteinizing hormone releasing hormone on tumor cells. Trends Endocrinol Metab 8:155–362, 1997
4. Ohno T, Imai A, Furui T, Takahashi K, Tamaya T: Presence of gonadotropin-releasing hormone and its messenger ribunucleic acid in human ovarian epithelial carcinoma. Am J Obstet Gynecol 169:605–610, 1993
5. Eidne KA, Flanagan CA, Harris NS, Millar RP: Gonadotropin-releasing

hormone (GnRH)-binding sites in human breast cancer cell lines and inhibitory effects of GnRH antagonists. J Clin Endocrinol Metab 64:425–432, 1987

6. Irmer G, Bürger C, Ortmann O, Schulz KD, Emons G: Expression of luteinizing hormone–releasing hormone and its mRNA in human endometrial cancer cell lines. J Clin Endocrinol Metab 79:916–919, 1994
7. Irmer G, Bürger C, Müller R, Ortmann O, Peter U, Kakar S, Neill J, Schulz KD, Emons G: Expression of the messenger RNAs for luteinizing hormone–releasing hormone (LHRH) and its receptor in human ovarian epithelial carcinoma. Cancer Res 55:817–822, 1995
8. Emons G, Pahwa GS, Brack C, Sturm R, Oberheuser F, Knuppen R: Gonadotropin releasing hormone binding sites in human epithelial ovarian carcinomata. Eur J Cancer Clin Oncol 25:215–221, 1989
9. Imai A, Ohno T, Iida K, Fuseya T, Furui T, Tamaya T: Presence of gonadotropin-releasing hormone receptor and its messenger ribonucleic acid in endometrial carcinoma and endometrium. Gynecol Oncol 55:114–118, 1994
10. Baumann K, Kiesel L, Kaufmann M, Bastert G, Runnebaum B: Characterization of binding sites for GnRH-agonist (buserelin) in human breast cancer biopsies and their distribution in relation to tumor parameters. Breast Cancer Res Treat 25:37–46, 1993
11. Srkalovic G, Schally AV, Wittliff JL, Day TG Jr, Jenison EL: Presence and characteristics of receptors for [D-Trp6]luteinizing hormone releasing hormone and epidermal growth factor in human ovarian cancer. Int J Oncol12:489–498, 1998
12. Fekete M, Wittliff JL, Schally AV: Characteristics and distribution of receptors for [D-Trp^6]-luteinizing-hormone–releasing hormone, somatostatin, epidermal growth factor and sex steroids in 500 biopsy samples of human breast cancer. J Clin Lab Anal 3:137–147, 1989
13. Emons G, Ortmann O, Becker M, Irmer G, Springer B, Laun R, Hölzel F, Schulz KD, Schally AV: High affinity binding and direct antiproliferative effects of LHRH analogues in human ovarian cancer cell lines. Cancer Res 54:5439–5446, 1993
14. Emons G, Schröder, B, Ortmann O, Westphalen S, Schulz KD, Schally AV: High affinity binding and direct antiproliferative effects of luteinizing hormone–releasing hormone analogs in human endometrial cancer cell lines. J Clin Endocrinol Metab 77:1458–1464, 1993
15. Yano T, Pinski J, Radulovic S, Schally AV: Inhibition of human epithelial ovarian cancer cell growth in vitro by agonistic and antagonistic analogues of luteinizing hormone-releasing hormone. Proc Natl Acad Sci USA 91:1701–1705, 1994
16. Kakar SS, Grizzle WE, Neill JD: The nucleotide sequences of human GnRH receptors in breast and ovarian tumors are identical with that found in pituitary. Mol Cell Endocrinol 106:145–149, 1994
17. Stojilkovic SS, Catt KJ: Expression and signal transduction pathways of gonadotropin-releasing hormone receptors. Rec Prog Horm Res 30:161–205, 1995
18. Emons G, Müller V, Ortmann O, Grossmann G, Trautner U, v Stuckrad B, Schulz KD, Schally AV: Luteinizing hormone–releasing hormone agonist triptorelin antagonizes signal transduction and mitogenic activity of epidermal growth factor in human ovarian and endometrial cancer cell lines. Int J Oncol 9:1129–1137, 1996
19. Nishida M, Kasahara K, Kaneko M, Iwasaki H: Establishment of a new endometrial adenocarcinoma cell line, Ishikawa cells, containing estrogen and progesterone receptors. Acta Obstet Gynaecol Jpn 37:1103–1111, 1985
20. Kuramoto H, Tamura S, Notake Y: Establishment of a cell line of human endometrial adenocarcinoma in vitro. Am J Obstet Gynecol 114:1012–1019, 1972
21. Simon WE, Albrecht M, Hänsel M, Dietel M, Hölzel F: Cell lines derived from human ovarian carcinomas: growth stimulation by gonadotropic and steroid hormones. J Natl Cancer Inst 70:839–845, 1983
22. Fogh J, Wright WC, Loveless JD: Absence of HeLa cell contamination in 169 cell lines derived from human tumors. J Natl Cancer Inst 58:209–214, 1977
23. Soule HD, Vasques J, Long A, Albert S, Brennan M: A human cell line from a pleural effusion derived from breast carcinoma. J Natl Cancer Inst 51:1409–1416, 1973
24. Segal-Abramson T, Giot J, Levy J, Sharoni Y: Guanine nucleotide modulations of high affinity gonadotropin-releasing hormone receptors in rat mammary tumors. Mol Cell Endocrinol 85:105–116, 1992
25. Kéri G, Balogh A, Szöke B, Téplan J, Csika O: Gonadotropin-releasing hormone analogues inhibit cell proliferation and activate signal transduction pathways in MDA-MB-231 human breast cancer cell lines. Tumor Biol 12:61–67, 1991
26. Imai A, Ohno T, Furui T, Takahashi K, Matsuda T, Tamaya T: Gonadotropin-releasing hormone stimulates phospholipase C but not protein phosphorylation/dephosphorylation in plasma membrane from human epithelial ovarian cancer. Int J Gynecol Cancer 3:311–317, 1993
27. Moretti RM, Montagnani-Marelli M, Dondi D, Poletti A, Martini L, Motta M, Limonta P: Luteinizing hormone–releasing hormone agonists interfere with the stimulatory actions of epidermal growth factor in human prostatic cancer cell lines, LNCaP and DU 145. J Clin Endocrinol Metab 81:3930–3937, 1996
28. Hershkovitz E, Marbach M, Bosin M, Levy J, Roberts C Jr, Le Roith D, Schally AV, Sharoni Y: Luteinizing hormone-releasing hormone antagonists interfere with autocrine and paracrine growth stimulation of MCF-7 mammary cancer cells by insulin like growth factors. J Clin Endocrinol Metab 77:963–968, 1993
29. Imai A, Takagi H, Horibe S, Fuseya T, Tamaya T: Coupling of gonadotropin releasing hormone receptor to Gi protein in human reproductive tract tumors. J Clin Endocrinol Metab 81:3249–3253, 1996
30. Hunter T: Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signaling. Cell 80:243–236, 1995
31. Chatzaki E, Bax CMR, Eidne KA, Anderson L, Grudzinskas JG, Gallagher CJ: The expression of gonadotropin-releasing hormone and its receptor in endometrial cancer, and its relevance as an autocrine growth factor. Cancer Res 56:2059–2065, 1996
32. Srkalovic G, Wittlif J, Schally AV: Detection and partial characterization of receptors for [D-Trp^6]-luteinizing hormone–releasing hormone and epidermal growth factor in human endometrial carcinoma. Cancer Res 50: 1841–1846, 1990
33. Montagnani-Marelli M, Moretti RM, Dondi D, Motta M, Limonta P: Luteinizing hormone–releasing hormone agonists interfere with mitogenic activity of the insulin-like growth factor system in androgen-independent prostate cancer cells. Endocrinology 140:329–334, 1999
34. Dondi D, Limonta P, Moretti RM, Marelli MM, Garattini E, Motta M: Antiproliferative effects of luteinizing hormone–releasing hormone (LHRH) agonists on human androgen-independent prostate cancer cell line DU 145: evidence for an autocrine-inhibitory LHRH loop. Cancer Res 54:4091–4095, 1994
35. Lamharzi N, Halmos G, Armatis P, Schally AV: Expression of mRNA for luteinizing hormone–releasing hormone receptors and epidermal growth factor recptors in human cancer cell lines. Int J Oncol 12:671–675, 1998
36. Lamharzi N, Halmos G, Jungwirth A, Schally AV: Decrease in the level and mRNA expression of LHRH and EGF receptors after treatment with LHRH antagonist cetrorelix in DU-145 prostate tumor xenografts in nude mice. Int J Oncol 13:429–435, 1998

37. Szepeshazi K, Halmos G, Schally AV, Arencibia JM, Groot K, Vadillo-Buenfil M, Rodriguez-Martin E: Growth inhibition of experimental pancreatic cancers and sustained reduction in epidermal growth factor receptors during therapy with hormonal peptide analogs. J Cancer Res Clin Oncol 125:444–452, 1999

38. Emons G, Ortmann O, Schulz KD: GnRH Analogues in Ovarian, Breast and Endometrial Cancers, in Lunenfeld B, Insler V (eds): GnRH Analogues, The State of the Art. Parthenon, Carnforth, 1996, pp 95–120

39. Kim JH, Park DC, Kim JW, Choi YK, Lew YO, Kim DH, Jung JK, Lim YA, Namkoong SE: Antitumor effect of GnRH agonist in epithelial ovarian cancer. Gynecol Oncol 74:170–180, 1999

40. Arora KK, Cheng Z, Catt KJ: Mutations of the conserved DRS motif in the second intracellular loop of the gonadotropin-releasing hormone receptor affect expression, activation, and internalization. Mol Endocrinol 11: 1203–1212, 1997

41. Flanagan CA, Zhou W, Chi l, Yuen T, Rodic V, Robertson D, Johnson M, Holland P, Millar RP, Weinstein H, Mitchell R, Sealfon SC: The functional microdomain in transmembrane helices 2 and 7 regulates expression, activation, and coupling pathways of the gonadotropin-releasing hormone receptor. J Biol Chem 274:28880–28886, 1999

42. Kottler ML, Bergametti F, Carré MC, Morice S, Decoret E, Lagarde JP, Starzec A, Counis R: Tissue-specific pattern of variant transcripts of the human gonadotropin-releasing hormone receptor gene. Eur J Endocrinol 140:561–569, 1999

43. Raymond JR: Multiple mechanisms of receptor-G protein signaling specificity. Am J Physiol 269:F141–F158, 1995