Gonadotropin-releasing hormone and TGF-β activate MAP kinase and differentially regulate fibronectin expression in endometrial epithelial and stromal cells

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Luo, Xiaoping, Li Ding, and Nasser Chegini. Gonadotropin-releasing hormone and TGF-β activate MAP kinase and differentially regulate fibronectin expression in endometrial epithelial and stromal cells. Am J Physiol Endocrinol Metab 287: E991–E1001, 2004. First published July 20, 2004; doi:10.1152/ajpendo.00200.2004.—Gonadotropin-releasing hormone analog (GnRHa) is used for medical management of endometriosis and premature luteinizing hormone surge during controlled ovarian stimulation. Human endometrium expresses GnRH receptors, and GnRHa alters the expression of transforming growth factor-β (TGF-β) and receptors in endometrial cells. Because the diverse biological actions of GnRHa and TGF-β are mediated in part through the MAPK pathway, we determined whether utilization of MAPK/ERK and transcriptional activation of immediate early genes c-fos and c-jun result in differential regulation of fibronectin, known as a key regulator of embryo implantation and endometriosis progression. Using endometrial stromal cells (ESC) and the endometrial epithelial cell line HES, we demonstrated that GnRHa and TGF-β, in a dose-, time-, and cell-dependent manner, increased the level of phosphorylated ERK1/2 (pERK1/2). GnRH antagonist Antide also increased pERK1/2 induction in ESC and HES, whereas pretreatment reduced GnRHa-induced pERK2 in ESC but not in HES. Cotreatments with GnRHa plus TGF-β1 did not have an additive or an inhibitory effect on pERK1/2 induction compared with GnRHa or TGF-β1 action alone. TGF-β1 and GnRHa increased ERK1/2 nuclear accumulation and inversely regulated the expression of c-fos and c-jun and that of fibronectin in a cell-specific manner. Pretreatment with U-0126, a MEK1/2 inhibitor, blocked basal, as well as GnRHa- and TGF-β1-induced pERK1/2; however, it differentially affected c-fos, c-jun, and fibronectin expression. In conclusion, the results indicate that GnRHa and TGF-β signaling through MAPK/ERK results in differential regulation of fibronectin expression in endometrial cells, a molecular mechanism where short- and long-term GnRHa therapy and locally expressed TGF-β could influence embryo implantation and endometriosis implants, respectively.

endometrial cells; endometriosis; extracellular signal-regulated kinase; fibronectin; gonadotropin-releasing hormone analog; transforming growth factor-β; regulation

GONADOTROPIN-RELEASING HORMONE ANALOG (GnRHa) therapy is often used for medical management of several uterine abnormalities, including endometriosis (47, 49). Short-term administration of GnRHa is used to prevent premature luteinizing hormone (LH) surge in women undergoing controlled ovarian stimulation (2, 3, 25, 29, 40). GnRHa therapy is traditionally believed to act at the level of the pituitary-gonadal axis, regulating the ovarian steroid production and steroid-dependent activities of the target tissues such as endometrium and endometriosis implants. Clinical observations indicate that GnRHa administration to prevent premature LH surge is associated with a reduced rate of embryo implantation (2, 3, 25, 29, 40). Although changes in hormonal milieu affecting endometrial preparation could account for lower implantation rate, accumulating evidence suggests that GnRHa acts directly on endometrium and other peripheral tissues expressing GnRHa receptors (12, 21, 28). GnRHa administration is reported to alter the endometrial expression of ovarian steroid receptors and to induce antimotopic effects compared with endometrium of the natural cycle (3). Other in vivo and in vitro studies also reported GnRHa-induced alteration in cell growth and expression of cell cycle proteins, growth factors, cytokines, proteases, and protease inhibitors in several peripheral tissues/cells, including endometrial carcinoma cell lines, stromal cells, ectopic endometrial cells, and leiomyoma and myometrial cells (5–11, 14, 15, 18, 19, 21–24, 26, 28, 33, 36, 43, 52, 54). These observations and identification of GnRHa and GnRHa receptor expression in these tissues and cells have led to the proposal of an autocrine/paracrine role for GnRHa and a direct action for GnRHa in peripheral tissues (11–13, 17, 22, 28, 33, 42).

Ovarian steroids are key regulators of many uterine growth factor, cytokine, and chemokine expressions, including transforming growth factor-β (TGF-β), a multifunctional cytokine expressed in human endometrium throughout the menstrual cycle. TGF-β is known to regulate various cellular activities, including cell growth and differentiation, apoptosis, inflammatory and immune responses, and extracellular matrix (ECM) turnover (1, 38, 48, 56). Altered expression of TGF-β has been associated with several abnormalities, including endometriosis and endometrial cancer (1, 11, 34, 38, 44, 45, 48, 56). In the endometrium, TGF-β regulates its own expression and that of ECM, adhesion molecules, proteases, and protease inhibitors, thus regulating trophoblast invasion, angiogenesis, and tumor metastasis, which occur during embryo implantation, endometriosis, and endometrial cancer, respectively (4, 9, 33, 34, 44, 45). GnRHa suppresses the expression of TGF-β and TGF-β receptors (11), ovarian steroid-induced TGF-β expression in leiomyoma and myometrial smooth muscle cells, and matrix metalloproteinases and their inhibitors in endometrial stromal and decidual cells (10, 14–17, 43).

Signaling pathways activated by GnRHa receptors include protein kinase A (PKA), PKC, G protein-coupled receptor kinases, calcium-calmodulin (Ca2+-CaM), and the mitogen-activated protein kinase (MAPK) cascade and may also involve...
epidermal growth factor receptor tyrosine and c-Src kinases (12, 28, 30). In contrast, TGF-β receptor signaling is mediated mainly through the Smad pathway, although activation of other pathways, including MAPKs, and their functional interactions with Smads have been documented in several cell types (38, 48, 56). Functional interactions between GnRH and TGF-β receptors involving MAPK and Smad pathways have been reported to result in differential regulation of LHI, GnRH, GnRH receptor, and Smad expression in pituitary gonadotropes, leiomyoma and myometrial smooth muscle cells, and endometrial stromal cells, respectively (20, 33, 39, 54, 55).

Fibronectin is a major component of ECM whose expression is regulated by ovarian steroids and TGF-β (4, 41, 50, 53). Fibronectin is a key regulator of various cellular activities including cell-cell and cell-ECM communications that are central to endometrial preparation for embryo implantation, trophoblast invasion, angiogenesis, and tissue turnover (4, 50, 53). Attachment of endometriosis implants to the peritoneal surface also involves cellular adhesion, invasion, angiogenesis, and ECM turnover (45). Because of the diverse actions of GnRHa and TGF-β in the endometrium, we hypothesized that their receptors signaling through MAPK/ERK and transcriptional activation of immediate early response genes c-fos and c-jun result in differential regulation of fibronectin expression. To test our hypothesis, we used primary culture of endometrial stromal cells and an endometrial epithelial cell line (HES).

MATERIALS AND METHODS

Materials. All the materials used for isolation and culturing of endometrial stromal cells, GnRH analogs, leuprolide acetate and Antide, α-smooth muscle actin, and vimetine antibodies were purchased from Sigma Chemical (St. Louis, MO). The materials for immunoblotting, real-time PCR, and immunocytochemistry were purchased from Bio-Rad (Hercules, CA), Applied Biosystems (Foster City, CA), and Vector Laboratories (Burlingame, CA), respectively. Recombinant human TGF-β1 was purchased from R&D Systems (Minneapolis, MN), and affinity-purified monoclonal anti- phosphospecific ERK1/2 and rabbit anti-ERK1 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). MEK1/2 synthetic inhibitors U-0126 and PD-98059, and the p38 MAPK inhibitor SB-203580 were purchased from Calbiochem (San Diego, CA).

Tissue collection and cell culture. Portions of endometrial tissues were collected from premenopausal women (n = 3) who were undergoing hysterectomy for medically indicated reasons, excluding endometrial cancer. The patients had not received any medications (including hormonal therapy) during the previous 3 mo before surgery. The tissues were collected at the University of Florida-affiliated Shands Hospital with the approval of the Institutional Review Board. Immediately after collection, small portions of endometrial tissues were prepared for isolation of endometrial stromal cells (ESC) and cultured in DMEM-F-12 until reaching visual confluence, as previously described (33). Before use in these experiments, the isolated cells were seeded in eight-well culture slides (Nalge Nunc, Naperville, IL) and after 24 h of culturing were characterized by immunofluorescence microscopy using antibodies to vimentin and α-smooth muscle actin, as previously described (34). In addition, a human endometrial epithelial cell line (HES), derived from spontaneous transformation of isolated endometrial surface epithelial cells from benign proliferative endometrium [16] kindly provided by Dr. D. Kniss at Ohio State University, Columbus, Ohio] was used in parallel with ESC. HES cells were cultured in DMEM containing 10% FBS.

GnRH receptor I and II mRNA expression. Total cellular RNA isolated from ESC and HES was subjected to RT-PCR to determine GnRH I and II receptor mRNA by use of the following primers, respectively: sense 5'-GATCAACAGACATCCCCC-3’ and antisense 5'-CTCGTGGGAGAGGATCTG-3’. product size 247 bp, and sense 5'-GCAAGAGCACCCTATAACCT-3’ and antisense 5'-GGTCCCAAGAGGATGAAGCTAG-3’, product size 660 bp (37, 51).

MAPK activity. HES and ESC were seeded in six-well plates at an approximate density of 106 cells/well in supplemented medium containing 10% FBS. After reaching visual confluence, the cells were washed with serum-free medium and incubated under serum-free condition for 24 h. pERK1 activation was determined following treatment with TGF-β1 (2.5 ng/ml) and/or GnRHa (0.1 μM) for 5, 15, and 30 min (time dependence) or with TGF-β1 (1, 2.5, 5, and 10 ng/ml) and/or GnRHa (0.01, 0.1, 1, and 10 μM) for 15 min (dose dependence). The specificity of GnRHa on ERK1/2 activation was determined after pretreatment of HES and ESC with 10 μM of the GnRH antagonist Antide for 2 h followed by GnRHa (0.1 μM) for 15 min. To determine possible autocrine/paracrine action of TGF-β1 on ERK activation, HES and ESC were treated with TGF-β type II receptor antisense or sense oligonucleotides (1 μM) for 24 h, and then the cells were washed and treated with TGF-β1 (2.5 ng/ml) for 15 min. The specificity of TGF-β1- and GnRHa-induced pERK1/2 was assessed using MEK1- and MEK1/2-specific inhibitors PD-98059 and U-0126. A parallel experiment was also performed using the p38 MAPK inhibitor SB-203580. HES and ESC were treated with 20 μM PD-98059, U-0126, or SB-203580 for 2 h before treatment with TGF-β1 (2.5 ng/ml) or GnRHa (0.1 μM) for 15 min. The cell lysate was prepared and subjected to immunoblotting.

Western blot analysis. Cell lysates were centrifuged, the supernatants were collected, and their total protein content was determined using a conventional method (Pierce, Rockford, IL), as previously described (33, 54). Equal amounts of sample proteins were subjected to SDS-PAGE and transferred to polyvinylidene difluoride membrane, as previously described (33, 54). Immunostained proteins were visualized using enhanced chemiluminescence reagents (Amersham Pharmacia Biotech, Piscataway, NJ), and the band intensity corresponding to pERK1/2 (44/42 kDa) and ERK1/2 was determined as previously described (33, 54).

Immunostaining. TGF-β1- and GnRHa-induced pERK1/2 translocation into the nucleus was assessed in HES and ESC cultured in eight-well culture slides for 24 h. The cells were washed and further incubated under serum-free, phenol red-free condition for 24 h and then treated with TGF-β1 (2.5 ng/ml) or GnRHa (0.1 μM) for 5, 15, and 30 min. The cells were immunostained with anti-ERK antibodies by FITC-labeled indirect method and Vectashield with 4,6-diamidino-2-phenylindole (DAPI, Vector Laboratories) as the mounting medium (33).

c-fos, c-jun, and fibronectin expression. HES and ESC were cultured as above and treated with TGF-β1 (2.5 ng/ml) and GnRHa (0.1 μM) for 0.5, 1, 2, 4, 6, and 12 h. Additional experiments were performed by pretreating the cells with PD-98059, U-0126, and SB-203580 at the aforementioned doses for 2 h followed by treatment with TGF-β1 (2.5 ng/ml) and GnRHa (0.1 μM) for 1 or 6 h selected from the time course study. Total RNA was isolated from treated and untreated control and subjected to real-time PCR.

Real-time PCR. cDNA was generated from 2 μg of total RNA by use of Taqman reverse transcription (RT) reagent. The RNA was incubated in 100 μl of RT reaction mixture (1× RT buffer, 5.5 mM MgCl2, 2 mM DNTP, 2.5 μM random hexamers, 0.4 U RNasin, and 1.25 U MultiScribe reverse transcriptase) for 10 min at 25°C and for 30 min at 48°C. The reverse transcriptase was inactivated by heating at 95°C for 5 min. PCR was performed in 96-well optical reaction plates on cDNA equivalent to 100 ng of RNA in a volume of 50 μl, containing 25 μl of TaqMan Universal Master Mix and optimized concentrations of FAM-labeled probe, forward and reverse primers selected from Assay on Demand (Applied Biosystems). Real-time PCR was performed for c-fos, c-jun, fibronectin, and 18S ribosomal RNA gene by means of ABI-Prism 7700 Sequence System at the
following conditions: 2 min at 50°C and 10 min at 95°C for 1 cycle, and 15 s at 95°C and 1 min at 60°C for 40 cycles. The cycle number at which fluorescence emission crossed the automatically determined threshold level (C_T) was determined using Applied Biosystems software. The results were analyzed using the comparative method, and the values were normalized to the 18S rRNA expression by subtracting mean C_T of 18S rRNA from mean target C_T for each sample to obtain the mean ΔC_T. The mean ΔC_T values were then converted into fold change based on a doubling of PCR product in each PCR cycle, according to the manufacturer’s guidelines.

Statistical analysis. All the experiments were performed at least three times in duplicate, using independent cell cultures. Where appropriate, the results are expressed as means ± SE and were statistically analyzed using unpaired Student’s t-test and Tukey’s test (ANOVA). A probability level of P < 0.05 was considered significant.

RESULTS

GnRHa- and TGF-β-induced MAPK/ERK activation in ESC and HES. Human endometrial tissues and their isolated epithelial and stromal cells, as well as HES, an endometrial surface epithelial cell line, express GnRH I and II as well as GnRH I and II receptor mRNA (Refs. 17, 22; Fig. 1). Because these cells also express TGF-β isoforms and TGF-β receptors, and MAPK/ERK pathway is activated by both GnRH and TGF-β receptors as part of their intracellular signaling in other cell types, we determined the involvement of this pathway in mediating GnRHa and TGF-β actions in HES and ESC. The

Fig. 1. RT-PCR of total RNA isolated from endometrial epithelial cell line HES and endometrial stromal cells (ESC) cultured for 48 h in supplemented medium containing 10% FBS. The predicted 247- and 660-bp fragments of gonadotropin-releasing hormone (GnRH) I and GnRH II receptors (arrows) are shown for both cell types. M, DNA marker.

Fig. 2. Dose-dependent action of transforming growth factor (TGF)-β1 (1, 2.5, 5, and 10 ng/ml) and GnRHa analog (GnRHa; 0.01 to 10 μM) on the rate of phosphorylated extracellular signal-regulated kinase 1/2 (pERK1/2) and total ERK (ERK) induction in HES and ESC. Top: serum-starved cells were treated with TGF-β1 (A) and GnRHa (B) for 15 min, and cell lysates were prepared from treated and untreated control (Ctrl) cells and subjected to immunoblotting using pERK and ERK antibodies. Bottom: bar graphs show means ± SE of fold change in pERK1/2 induction in HES and ESC from 3 different experiments. * and **Significantly different from untreated controls; * differs from ** and ** from *** (P < 0.05).
Fig. 3. Time-dependent action of TGF-β1 (2.5 ng/ml) and GnRHa (0.1 μM) on the rate of pERK1/2 and total ERK1/2 induction in HES and ESC. Top: serum-starved cells were treated with TGF-β1 (A) and GnRHa (B) for 5, 15, and 30 min, and cell lysates from treated and untreated control cells (time 0) were prepared and subjected to immunoblotting using pERK1/2 and ERK antibodies. Bottom: bar graphs show means ± SE of fold change in pERK1/2 induction in HES and ESC from 3 different experiments. *Significantly different from controls (P < 0.05).

Fig. 4. Effect of GnRH antagonist (Antide, An), GnRHa, and GnRHa + Antide (An+GnRH) on the rate of pERK1/2 induction in HES (left) and ESC (right). Top: serum-starved cells were pretreated with 10 μM Antide for 2 h and then treated with 0.1 μM GnRHa for an additional 15 min. Cell lysates from treated and untreated control cells were prepared and subjected to immunoblot analysis using pERK1/2 and ERK antibodies. Bottom: bar graphs show means ± SE of fold change in pERK1/2 induction from 3 different experiments. *Significantly different from controls (P < 0.05).
Fig. 5. Effect of TGF-β1 on the rate of pERK1/2 induction in HES and ESC following pretreatment with TGF-β type II receptor antisense (1 μM) and sense (1 μM) oligonucleotides. Top: cells were treated with antisense (A) or sense (S) oligomers for 24 h (medium contained 2% FBS), washed, and then treated with TGF-β1 (2.5 ng/ml) for 15 min. Cell lysates were prepared from TGF-β (TGF), TGF-β1 + TGF-β type II receptor antisense (T+A) and TGF-β1 + TGF-β type II receptor sense (T+S) treated and untreated (Ctrl) cells and subjected to immunoblotting using pERK1/2 and ERK antibodies. Bottom: bar graphs show means ± SE of fold change in pERK1/2 induction in HES and ESC from 3 different experiments. *Significantly different from untreated controls; **P < 0.05.

Fig. 6. Effect of TGF-β1 and GnRHa on pERK1/2 induction following pretreatment of HES and ESC with MEK1/2 synthetic inhibitors PD-98059 (PD) and U-0126 (U) and p38 MAPK inhibitor SB-203580 (SB). Top: cells were treated with PD, U, or SB at 20 μM for 2 h, washed, and then treated without (-) or with TGF-β1 (T, 2.5 ng/ml) or GnRHa (G, 0.1 μM) for 15 min. Cell lysates were subjected to immunoblotting using pERK1/2 and ERK antibodies. Bottom: bar graphs show means ± SE of fold change in pERK1/2 induction in HES and ESC from 3 different experiments. *Significantly different from untreated controls; **P < 0.05.
results show that HES and ESC contain varying levels of constitutively activated ERK1/2, and treatments of serum-starved cells with GnRHa and TGF-β1 in a dose- (Fig. 2, A and B) and time- (Fig. 3, A and B) dependent manner increased the level of pERK1/2 in both cell types compared with untreated controls (P < 0.05). Cotreatment of HES and ESC with GnRHa plus TGF-β1 did not have an additive and/or inhibitory effect on pERK1/2 induction compared with levels induced by TGF-β1 or GnRHa (not shown). Treatment of HES and ESC with Antide also increased pERK1/2 induction (P < 0.05); however, pretreatment with Antide had a minimal effect on GnRHa-induced pERK1/2 in ESC as opposed to HES (Fig. 4). Pretreatment of HES and ESC with TGF-β receptor type II antisense oligonucleotide also reduced TGF-β1-induced pERK1/2 (P < 0.05), although a partial inhibition also occurred with TGF-β receptor type II sense oligomer, particularly in ESC (Fig. 5).

Pretreatment with U-0126 (MEK1/2 inhibitor) inhibited basal as well as TGF-β1- and GnRHa-induced pERK1/2 in HES and ESC (P < 0.05; Fig. 6). However, pretreatment with PD-98059 (MEK1 inhibitor) was less effective in inhibiting basal and did not affect TGF-β1-induced pERK1/2 in HES but prevented GnRHa-induced pERK1/2 in both cells and TGF-β1 action in ESC (P < 0.05; Fig. 6). Pretreatment of HES with SB-203580, a p38 MAPK inhibitor, had no significant effect on TGF-β1 but increased GnRHa-induced pERK1/2 (P < 0.05; Fig. 6). In contrast, pretreatment of ESC with SB-203580 increased TGF-β1-induced pERK1/2, while reducing that in GnRHa-treated cells (P < 0.05, Fig. 6).

GnRHa and TGF-β differently regulate transcriptional activation of c-fos and c-jun genes and fibronectin expression. By use of indirect fluorescent immunocytochemistry, ERK1/2 was localized in the cytoplasmic and nuclear regions of HES and ESC. Treatments with TGF-β1 (2.5 ng/ml) or GnRHa (0.1 μM) for 5, 15, and 30 min resulted in an increase in ERK1/2 nuclear labeling (Fig. 7, shown for 15 min). Nuclear translocation of activated ERK1/2 is accompanied by transcriptional activation of immediate early response genes, including c-fos and c-jun. GnRHa and TGF-β1 induced a rapid and time-dependent expression of c-fos mRNA in ESC, which was inhibited in HES after prolonged exposure (P < 0.05; Fig. 8). GnRHa did not significantly alter c-fos expression during the early time points; however, prolonged exposure resulted in gradual inhibition in both ESC and HES (P < 0.05; Fig. 8).

Unlike c-fos, TGF-β1 significantly increased c-jun expression in HES, whereas in ESC TGF-β1 action was limited, with only a slight, but significant, increase in c-jun expression after 2–6 h (P < 0.05; Fig. 8). GnRHa treatment resulted in a rapid induction of c-jun expression in HES while inhibiting that in ESC (P < 0.05; Fig. 8).

Transcriptional activation of c-fos and c-jun leads to regulation of various genes possessing activating protein (AP)-1 binding sites on their promoters such as fibronectin. As expected, TGF-β increased the expression of fibronectin mRNA in HES and ESC. With a progressive inhibition by GnRHa compared with untreated controls (P < 0.05; Fig. 8). Pretreatment with U-0126 and PD-98059 resulted in differential expression of basal and TGF-β1 and GnRHa actions on c-fos, c-jun, and fibronectin mRNA expression (P < 0.05; Fig. 8). PD-98059 had only a slight, but significant, effect on TGF-β1-induced c-fos, c-jun, and fibronectin expression in HES and ESC but reversed the inhibitory action of GnRHa on fibronectin expression (P < 0.05; Fig. 9). Furthermore, pretreatment with SB-203580 was equally effective in altering TGF-β1 and GnRHa actions on c-fos, c-jun and fibronectin expression, suggesting a potential cross talk between ERK1/2 and p38 MAPK in mediating their actions (Fig. 9). Overall the influence of TGF-β1 on c-fos, c-jun, and fibronectin expression in HES

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![Fig. 7. Immuno-fluorescence localization of ERK in HES and ESC.](image-url)

HES

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ESC

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Note: The figure shows immunofluorescence localization of ERK in HES and ESC. Cells were incubated under serum-free condition for 24 h and then treated with TGF-β1 (2.5 ng/ml) or GnRHa (0.1 μM) for 5, 15, and 30 min. Note cytoplasmic/nuclear localization of ERK1/2 in untreated control (A and G) and increased nuclear localization in TGF-β1- (B and H) and GnRHa- (C and I) treated cells shown after 15 min of treatments. FITC staining was used to localize ERK and 4,6-diamidino-2-phenylindole (DAPI) staining for the nuclei (D, E, F, J, K, and L).
and ESC differed by 2- to 20-fold compared with GnRHa action (Figs. 8 and 9).

DISCUSSION

In the present study, we demonstrated that human endometrial epithelial and stromal cells, as well as HES, an endometrial surface epithelial cell line, express GnRH I and II receptors, and we showed that GnRHa and TGF-β1 activates the MAPK/ERK1/2 pathway, which translocated into the nucleus and differentially regulated the expression of c-fos, c-jun, and fibronectin in these cells. Because of the sensitivity of GnRHa- and TGF-β1-induced pERK1/2 as well as c-fos, c-jun, and fibronectin expression to U-0126, the results suggest the involvement of MAPK/ERK signaling downstream from MEK1/2 in mediating their actions in endometrial cells. In support of our observations with ESC and HES are previous reports demonstrating the expression of GnRH and GnRH receptors in endometrium and endometrial cell lines (17, 22, 42) and direct action of GnRHa in several cell types, including endometrial cell lines, endometrial stromal cells, ectopic endometrial cells, and leiomyoma and myometrial smooth muscle cells. Treatment of these cells with GnRHa resulted in alteration of their rate of cell growth and apoptosis and the expression of cell cycle proteins, growth factors, cytokines, proteases, and protease inhibitors (5–11, 14, 15, 18, 19, 21–24, 26, 33, 36, 52, 54). Furthermore, human endometrium expresses all the components of the TGF-β family and TGF-β receptors throughout the menstrual cycle, and TGF-β1, through an autocrine/paracrine action, influences various endometrial biological activities, including cell growth and differentiation, apoptosis, inflammatory and immune responses, and ECM turnover (9, 34, 44, 45).

Several specific signaling pathways, including MAPK/ERK, are utilized by GnRH receptors to regulate these processes in several cell types, including endometrial cancer cell lines; however, the signaling pathway activated by GnRH receptor in the endometrium has not been investigated (12, 23, 24, 28, 33, 35, 39, 46, 54). In contrast, TGF-β receptor signaling occurs mainly through the activation of the Smad pathway; it may also involve MAPKs in certain cell types (38, 48, 56). We (33) have recently reported the endometrial expression of Smads and their regulation by GnRHa and demonstrated the activation of Smad3 by TGF-β1 in ESC and HES. To our knowledge, our observation is the first to demonstrate the activation of MAPK/ERK by GnRHa and TGF-β1 in ESC and HES, suggesting that both Smad and MAPK pathways are involved in mediating
GnRH and TGF-β receptor signaling in the endometrium (Fig. 10). We found that, under the culture condition of our study, ESC and HES contain constitutively active ERK, possibly induced by many autocrine/paracrine growth factors and cytokines expressed by these cells, thus contributing toward moderate activation of ERK1/2 after GnRH and TGF-β1 treatments. Because GnRHa therapy results in inhibition of TGF-β isoform, TGF-β receptor, and Smad expression (19, 33), as such we expected cotreatment with GnRHa to inhibit/reduce TGF-β-induced pERK in HES and ESC. Lack of inhibitory and/or additive effect of GnRHa on TGF-β-induced pERK1/2 suggests that GnRHa inhibitory action on TGF-β and TGF-β receptor expression may occur through pathways independent of MAPK/ERK. Because GnRH and TGF-β receptors utilize signaling pathways including PKC, Ca²⁺-CaM, and other members of the MAPK pathway, such as p38 MAPK and c-Jun NH₂-terminal protein kinase (JNK), their activation and/or cross talk could mediate GnRH inhibitory action on TGF-β and TGF-β receptor expression. Additionally, we have recently reported that, in HES and ESC, GnRHa alters the expression of Smads, specifically antagonist Smad, Smad7, which interacts with TGF-β type I receptor kinase and receptor-activated Smad3 and, through a feedback regulatory mechanism, controls TGF-β receptor signaling (Refs. 38, 48, and 56 and Fig. 10). GnRHa-induced Smad7 expression could alternatively influence TGF-β self-regulatory action, including ERK activation. GnRHa is also reported to regulate Smad expression and activation in leiomyoma and myometrial smooth muscle cells (54) and by interacting with TGF-β and activin, through a mechanism involving Smad and MAPK pathways, regulate the expression of LH, GnRH, and GnRH receptor in pituitary gonadotropes (20, 39, 55). Using U-0126, PD-98059, and SB-203580 to identify the specificity of MAPK signaling in mediating GnRHa and TGF-β actions implicated MEK1/2 with possible cross talk with p38 MAPK in HES and ESC.

Despite the similarity between GnRHa- and TGF-β1-induced pERK1/2 in ESC and HES, their effects on transcriptional regulation of c-fos, c-jun, and fibronectin differed significantly and occurred in cell-specific manners. Although TGF-β resulted in a rapid induction of c-fos expression in ESC, GnRHa action was a delayed response. This contrasted with c-jun expression, as GnRHa, and specifically TGF-β, caused a rapid induction of c-jun in HES, whereas TGF-β action in ESC was a delayed response, with GnRHa inhibiting c-jun expression. MAPK signaling involving MEK1/2 with possible interaction with p38 MAPK appears to mediate GnRHa and TGF-β actions on c-fos and c-jun transcriptional activation in HES and ESC. We have recently reported a similar interaction between GnRHa and TGF-β actions in leiomyoma and myometrial smooth muscle cells (16a). In pituitary-derived αT3-1 cells,
cells is needed before any conclusion is reached, the results provide support for the presence of GnRH receptor subtypes with different ligand selectivity in HES and ESC cells. Interestingly, Antide acting through the well-characterized GnRH I receptors is reported to convert an antagonistic action into an agonist (12, 35). Because GnRH I and II receptors are expressed in the endometrium with surface and glandular epithelial cells as the major sites of GnRH I receptor (Refs. 8, 13, 17, 22 and 23 unpublished data), both receptors may participate in mediating GnRHa actions in ESC and HES. With respect to TGF-β action, blocking/reducing TGF-β type II receptor expression in part reduced TGF-β-induced pERK1/2 in HES, reaching near control levels in ESC. The results support that TGF-β-induced pERK1/2 is receptor mediated; however, inadequate inhibition, or overexpression, of TGF-β receptor type II in HES may have been a limiting factor in preventing the action of exogenously added TGF-β1 and endogenously expressed TGF-β isoforms. TGF-β receptor content, MAPK activation by other autocrine/paracrine factors, and the extent of ERK1/2 activation in response to TGF-β could also account for the differences between HES and ESC.

TGF-β is known to regulate the expression of a wide range of genes in a variety of cell types (1, 38, 48, 56). However, only a limited number of genes are known to be the target of GnRHa action in gonadotropes and tumor cells (31, 32, 35, 46, 57, 58). Recent reports indicate that GnRH can also target the expression of a large number of genes, including several components of ECM and proteases (10, 14, 15, 18, 43). Fibronectin is a major component of ECM whose expression is regulated by ovarian steroids and TGF-β (41, 50, 53). Fibronectin is involved in various cellular activities, including cell-cell and cell-ECM communications that are central to endometrial preparation for embryo implantation, trophoblast invasion, angiogenesis, and tissue turnover (33, 41, 48, 50, 53). Endometriosis implant adherence to the peritoneal surface also involves cellular invasion, angiogenesis, and ECM turnover (26, 45). Thus the interaction between GnRH and TGF-β receptor signaling may serve to direct their diverse actions in their target tissues including endometrium, reflected in their gene expression profile. As expected, we found that TGF-β increases the expression of fibronectin and discovered an inhibitory action for GnRHa both occurring through a signaling pathway downstream from MEK1/2 and potential cross talk with p38 MAPK. Fibronectin and its receptor components, integrins, are important elements in endometrial preparation for embryo implantation and endometriosis implant attachment (50, 53), and its inhibition may represent a molecular mechanism by which short- and long-term GnRH therapies influence the outcome of these events. GnRHa is reported to induce an imbalance in endometrial ovarian steroid receptor expression with an antimotic effect, altering the endometrial preparation for embryo implantation in patients who received GnRHa therapy to prevent premature LH surge (2, 3, 25, 29, 40).

In conclusion, we demonstrated that GnRHa and TGF-β signaling through MAPK/ERK downstream from MEK1/2 with potential interaction with p38 MAPK results in differential regulation of c-fos and c-jun transcriptional activation, altering the endometrial expression of fibronectin in a cell-specific manner. Because fibronectin plays a key role in endometrial preparation for embryo implantation and endometriosis implant attachment into the peritoneal cavity, alteration of
fibronectin expression may represent a molecular mechanism whereby short- and long-term GnRHa therapies and locally expressed TGF-β could influence embryo implantation and endometriosis implants, respectively.

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REFERENCES


