Gonadotropin-Releasing Hormone Promotes Ovarian Cancer Cell Invasiveness through c-Jun NH2-Terminal Kinase–Mediated Activation of Matrix Metalloproteinase (MMP)-2 and MMP-9

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Abstract
Gonadotropin-releasing hormone (GnRH) receptor is present in 80% of ovarian cancer, and numerous studies have provided evidence for a role of GnRH in cell proliferation. In this study, the effect of GnRH on the invasion potential of ovarian cancer cells was investigated. In vitro migration and cell invasion assays with the ovarian cancer cell lines Caov-3 and OVCAR-3 revealed the biphasic nature of GnRH; low concentrations of GnRH agonist (GnRHa) increased the cell motility and invasiveness of these cells, but at increased concentrations, the stimulatory effect was insignificant. Reverse transcription-PCR, Western blot, and gelatin zymography showed that the expression of metastasis-related proteases, matrix metalloproteinase (MMP)-2 and MMP-9, was up-regulated and activated by GnRHa. Moreover, we observed that GnRHa was able to transactivate the MMP-2 and MMP-9 promoters. The invasive/migratory phenotype activated by GnRHa can be blocked by specific inhibitors or neutralizing antibodies to MMP-2 and MMP-9. Knockdown of the GnRH receptor using small interfering RNA significantly inhibited the GnRH-mediated up-regulation of MMP-2 and MMP-9, as well as the stimulatory effect of GnRH on the invasion of ovarian cancer cells. We also present evidence suggesting that the activation of MMP-2 and MMP-9 is required for GnRH-mediated invasion and migration. In addition, we showed that the c-Jun NH2-terminal kinase, but not extracellular signal-regulated kinase 1/2 or p38 mitogen-activated protein kinase, signaling pathway was critical for GnRH-mediated up-regulation of MMP, cell invasion, and motility. These results indicate for the first time an expanded role for GnRH in other aspects of ovarian tumor progression, such as metastasis, via activation of MMP and the subsequent increase in cell migration and invasion. (Cancer Res 2006; 66(22): 10902–10)

Introduction
The hypothalamic decapeptide gonadotropin-releasing hormone (GnRH) plays a key role in the ovary and ovarian cancer (reviewed in ref. 1). GnRH receptor expression has been shown in 80% of human ovarian tumors and numerous cancer cell lines, and the analogues inhibit proliferation of the GnRH receptor-bearing tumor cells both in vivo and in vitro, supporting evidence for a direct antiproliferative effect (1–3). Of particular interest, levels of GnRH receptor seem to be associated with cancer grading and have been reported to be elevated in advanced-stage (stages III and IV) ovarian carcinomas (4). This opens the possibility that GnRH could directly regulate tumor progression of ovarian cancer cells.

However, a role of GnRH in ovarian epithelial cell migration/invasion has not been established and the mechanism by which GnRH contributes to ovarian cancer invasiveness is also unknown.

Ovarian cancer is the most lethal gynecologic malignant tumor in the Western world. This is in part due to the fact that most (60%) of the patients are diagnosed with already widespread intraabdominal metastasis and ascites and the lack of effective therapies for advanced-stage disease (5). Thus, there is a need for new therapeutic targets and a better understanding of the mechanisms involved in the spread of ovarian carcinoma. The factors that regulate the metastatic process of ovarian cancer, however, are poorly understood.

Matrix metalloproteinases (MMP), a family of secreted or transmembrane enzymes, can collectively digest almost all extracellular matrix (ECM) and basement membrane components (6, 7). Thus, MMPs are largely implicated in promoting angiogenesis and tumor metastasis. Above all, MMP-2 and MMP-9, which can degrade collagen IV, the major ECM component of the basement membranes, have been suggested to be critical for the invasive and metastatic potential in ovarian carcinoma. Elevated expression of MMP-2 and MMP-9 has been detected in ovarian cancer ascites, tissues, and cancer cells in culture (8–11), and experimental metastasis is suppressed by a synthetic MMP inhibitor (12). MMP activity is often controlled by a specific inhibitor known as the tissue inhibitor of metalloproteinases (TIMP). TIMP-1 selectively binds pro-MMP-9, whereas TIMP-2 associates with pro-MMP-2 in a crucial step in the cell-mediated activation of MMPs (13). By inhibiting active MMPs, TIMPs inhibit cell invasion in vitro and tumorigenesis and metastasis in vivo (14–16).

In the present study, we show for the first time a role for GnRH in the invasive phenotype and motility of human ovarian cancer cells. We also present evidence suggesting that the activation of metastasis-related proteases, especially MMP-2 and MMP-9, through the c-Jun NH2-terminal kinase (JNK) mediated signaling pathway in this process.

Materials and Methods
Cell culture. The human ovarian epithelial carcinoma cell lines Caov-3 and OVCAR-3 (kindly provided by Dr. N. Auersepsg, University of British Columbia, Vancouver, British Columbia, Canada) were grown in medium 199 (Sigma, St. Louis, MO) supplemented with 5% fetal bovine serum and 100 units/mL penicillin-streptomycin (Invitrogen, San Diego, CA) in a humidified atmosphere of 5% CO2, at 37°C.
Antibodies, reagents, and plasmids. GnRH agonist (GnRHa; D-Ala6) was purchased from Sigma, P998059, SB203580, SP600125, MMP-2 inhibitor (OA-Hy), MMP-9 inhibitor (SB-3CT), and monoclonal antibodies to MMP-2 and MMP-9 were purchased from Calbiochem (San Diego, CA). Anti-TIMP-1 and anti-TIMP-2 were purchased from Chemicon International, Inc. (Temecula, CA). Anti-extracellular signal-regulated kinase 1/2 (ERK1/2),
anti-p38 mitogen-activated protein kinase (MAPK), anti-JNK, anti-phosphorylated ERK1/2, anti-phosphorylated p38 MAPK, and anti-phosphorylated JNK were obtained from Cell Signaling Technology, Inc. (Beverly, MA). The dominant-negative mutant of human JNK was kindly provided by Dr. R. Davis (University of Massachusetts Medical School, Worcester, MA). The MMP-2 promoter region between positions –1,659 and –57 was cloned into promoterless luciferase reporter vector pGL3-Basic as described previously (17). The MMP-9 promoter plasmid was a generous gift of Dr. D. Boyd (M. D. Anderson Cancer Center, Houston, TX).

Western blot analysis. Equal amounts of protein (50 μg) were subjected to SDS-PAGE (7.5%) and then transferred to nitrocellulose membrane. Membranes were blocked using 5% nonfat dry milk in PBS containing 0.05% Tween 20 and incubated overnight at 4°C with the appropriate primary antibody. The immunocomplex was detected with horseradish peroxidase–conjugated IgG as secondary antibody (Bio-Rad, Hercules, CA), and blots were developed with an enhanced chemiluminescence kit (Amersham Biosciences, Little Chalfont, United Kingdom). The experiment was repeated at least twice with independent protein samples.

Small interfering RNA transfection. The pSUPER vector encoding 19-mer hairpin small interfering RNA (siRNA) duplex specific to GnRH receptor sequence 5'-AGATCGCGAGCTCGGTTAC-3' (nucleotides 107-125 downstream of the start codon) has been described elsewhere (18). As a non-specific siRNA control, scrambled siRNA duplex was used. Transfection was done using LipofectAMINE 2000 reagent (Invitrogen) following the manufacturer’s instruction.

Gelatin zymography. The activities of MMP-2 and MMP-9 in the conditioned medium were determined by gelatin zymography. Briefly, the media were collected and clarified by centrifugation to remove cells and debris. Samples (80 μg) were loaded under nonreducing conditions onto SDS-polyacrylamide gel polymerized with 1 mg/mL gelatin. Following electrophoresis, the gels were washed with 2.5% Triton X-100 to remove SDS and then incubated in a developing buffer [50 mmol/L Tris-HCl buffer (pH 7.4), 10 mmol/L CaCl2 overnight at 37°C. Gels were stained with 0.25% Coomassie Brilliant Blue R-250 and destained in the same solution without dye. Gelatinase activity was visualized as clear bands against the blue-stained gelatin background. Molecular sizes were determined from mobility of gelatinase standards (Chemicon International). Three individual experiments were conducted with independent protein samples.

Semiquantitative reverse transcription-PCR. Total RNA was isolated from cultured cells using the Trizol reagent (Invitrogen) according to the manufacturer’s procedure. First-strand cDNA synthesis was done from 2.5 μg of total RNA using SuperScript Reverse Transcriptase (Life Technologies). The cDNA was used for subsequent PCR using primers specific for human GnRH receptor, MMP-2, MMP-9, TIMP-1, and TIMP-2 as

Figure 1. GnRH promotes ovarian cancer cell migration and invasion. A, ovarian cancer cells (1.5 × 10^5/mL) were seeded on a Matrigel-precoated filter (8-μm pore) of Transwell chambers in the presence or absence of increasing concentrations (0.1 nmol/L to 1 μmol/L as indicated) of GnRHa. B, cell motility under same treatment was measured through uncoated filters, except that conditioned medium from NIH-3T3 fibroblast culture was used in the lower chamber as chemoattractant. After 24 hours of incubation, cells in the upper side of the filter were removed and the invaded or migrated cells were fixed, stained, and counted. Left, representative pictures. Columns, mean number of invaded or migrated cells of five fields of triplicate wells from three independent experiments; bars, SD. C, expression of GnRH receptor (GnRHR) in ovarian cancer cell lines was detected by semiquantitative PCR using primers specific for GnRH receptor and β-actin. D, cells were transiently transfected with vector expressing short hairpin RNA directed against GnRH receptor (2 μg) or nonspecific siRNA (NS siRNA) as control. Transfected cells were collected for invasion assay through Matrigel (left) and migration assay (right) through uncoated filter in the presence or absence of 0.1 nmol/L GnRHa. *, P < 0.05; **, P < 0.005, compared with untreated controls.
reported previously (19, 20). β-Actin was used as internal control to normalize the relative amount of cDNA in each reaction. The logarithmic phase of PCR amplification for each target gene had been determined in initial experiments. The reproducibility of the quantitative measurements was evaluated by three independent cDNA syntheses and PCR ran from each preparation of RNA.

**Reporter gene assay.** Cells were transiently transfected with 1 µg of the construct using LipofectAMINE (Invitrogen) as directed by the manufacturer. After 6 hours, the cells were incubated with or without GnRHa (0.1 nmol/L) for an additional 24 hours. The pSV-β-galactosidase plasmid was cotransfected as internal control. Luciferase units were calculated as luciferase activity/β-galactosidase activity and are presented as the mean ± SD of three independent experiments with triplication. The fold change was calculated by comparison with the promoterless luciferase vector (pGL3-Basic).

**Invasion and migration assays.** Twenty-four-well Transwell inserts with 8-µm pores coated with Matrigel (50 µL/well; BD Biosciences, Palo Alto, CA) were used to assess cell invasion. Cells (1 × 10⁵) in serum-free medium, with or without GnRHa, were seeded in triplicate in the upper chamber. Serum-free medium was placed in the lower wells. The chambers were incubated for 24 hours at 37°C in a 5% CO₂ atmosphere. Cells that had not penetrated the filter were wiped out, and invaded cells on the lower surface of the filter were fixed with ice-cold methanol and stained with 0.5% crystal violet. Results are presented as the mean number of invaded cells of five fields ± SD of three independent experiments. To assess cellular migration potential, the protocol described above was used, except that Matrigel was omitted and ST3 fibroblast conditioned medium was placed in the lower chamber as a chemoattractant.

**Statistical analysis.** Statistical analysis was carried out using ANOVA followed by Tukey’s post hoc test (GraphPad Software, San Diego, CA). P < 0.05 was considered statistically significant.

**Results**

GnRH stimulates ovarian tumor cell migration and invasion. During metastasis, invasion of basement membrane by tumor cells is thought to be a critical event (21). To study whether expression of GnRH receptor is associated with metastasis of ovarian cancer cells, the effect of GnRH on cellular motility and invasive activity was evaluated. Because native GnRH has a relatively short half-life both in vivo and in vitro, GnRHa, in

**Figure 2.** GnRH induces production and synthesis of MMP-2 and MMP-9. A, cells were grown for 24 hours in the presence or absence of increasing doses of GnRHa (0.1 nmol/L to 1 µmol/L). Equal amounts of protein (50 µg) were then analyzed by Western blot using specific antibodies recognizing MMP-2 and MMP-9. Immunoblotting for β-actin was included as loading controls. The signal intensity was determined by densitometry, and the levels of MMP-2 and MMP-9 protein were normalized against that of β-actin. B, conditioned medium from treated cells was also collected and analyzed for MMP activities by gelatin zymography. Arrows, gelatinase activities corresponding to pro-MMP-2, active MMP-2, pro-MMP-9, and active MMP-9. Lane 1, a human MMP-2 and MMP-9 standard was used as a positive control. C, cells were transiently transfected with vector expressing short hairpin RNA targeted against GnRH receptor or nonspecific siRNA as control. Left, aliquots of conditioned medium normalized for same total protein concentration were subjected to gelatin zymography; right, cell lysates (50 µg) were analyzed by Western blot for the presence of MMP-2 and MMP-9 proteins. Immunoblotting for β-actin was included as loading controls. D, cells were grown in the presence or absence of 0.1 nmol/L GnRHa for 24 hours and total protein was harvested for Western blot analysis using specific antibodies against TIMP-1, TIMP-2, and β-actin. Experiments were repeated thrice. Columns, mean; bars, SD. * P < 0.05, statistically significant difference when treated samples were compared with untreated controls.
Numerous studies have shown that alteration of GnRH receptor mRNA levels is involved in the regulation of ovarian responsiveness to GnRH (19, 25, 26). We therefore examined whether the effect of GnRH on ovarian cancer invasion was associated with altered GnRH receptor gene expression. As shown in Fig. 1A, treatment with the GnRHa induced a biphasic regulation pattern for GnRH receptor mRNA in both Caov-3 and OVCAR-3. Low doses of GnRHa (0.1-10 nmol/L) increased GnRH receptor mRNA levels, with a maximal effective dose of 0.1 nmol/L (80.5% increase); thereafter, the levels decreased in cells treated with GnRHa at 100 nmol/L to 1 μmol/L. Cotreatment of cells with the GnRH antagonist antide prevented the biphasic effects of GnRHa, whereas antide alone had no effect, indicating the specificity of the response (data not shown).

To further confirm the role of GnRH receptor on the invasive properties of ovarian cancer cells, we inhibited the expression of GnRH receptor using vector-based RNA interference assay. The effectiveness of this siRNA to deplete GnRH receptor expression was confirmed by semiquantitative reverse transcription-PCR (RT-PCR) and Western blot analysis (data not shown) and was consistent with the previous study (18). Importantly, siRNA-mediated depletion of GnRH receptor, but not nonspecific siRNA, reduced invasion to near basal levels (80.8% inhibition; Fig. 1D, left). Similarly, we showed that depletion of GnRH receptor inhibited the cell migration by 85.2% (Fig. 1D, right), confirming a direct involvement of GnRH receptor in the acquisition of a migratory and invasive phenotype in ovarian cancer cells.

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concentrations (0.1-10 nmol/L) of GnRHa resulted in an up-regulation of MMP-2/MMP-9 expression \((P < 0.05)\), whereas high concentration (100 nmol/L to 1 μmol/L) had minimal response (Fig. 2A). Consistently, gelatin zymography showed stronger lytic zones at the molecular mass corresponding to latent and active forms of MMP-2 (72-kDa and 66-kDa forms) and MMP-9 (92-kDa and 86-kDa forms) in cells treated with 0.1 nmol/L GnRHa with respect to untreated cells, showing their elevated secretion and enzyme activities by GnRHa (Fig. 2B). These results matched well with those observed in cellular invasion (Fig. 1), suggesting that GnRH-mediated induction of MMP-2 and MMP-9 may play a role in GnRH-enhanced cell invasion/migration. To ascertain whether GnRH plays a critical role in the induction of MMP-2 and MMP-9, we monitored the regulation of these proteinases after transfection of a GnRH receptor-targeted siRNA. As shown in Fig. 2C, both the activity and expression of MMP-2 and MMP-9 were clearly reduced in the presence of the GnRH receptor siRNA. In contrast, control experiments with nonspecific siRNA showed negligible effect.

MMP expression is often coordinately regulated with production of their endogenous inhibitors, TIMPs (21). However, there was no change in the expression of TIMP-1 and TIMP-2 irrespective of the presence of different concentration of GnRHa as shown by Western blot analysis (Fig. 2D).

**GnRH modulates transcription activities of MMP-2 and MMP-9.** To determine whether the observed alteration in MMP-2 and MMP-9 was the result of changes in gene expression, RT-PCR was done. In accordance with changes in protein expression, MMP-2 and MMP-9 mRNA expression were found to be significantly enhanced with 0.1 nmol/L GnRHa (5.3-fold for MMP-2 and 2.5-fold for MMP-9 compared with untreated control cells; \(P < 0.05\); Fig. 3A).

To test whether the effect of GnRH on MMP-2 and MMP-9 mRNA expression was the result of increased mRNA stability, we did actinomycin D (ActD) chase experiments to determine the half-life of MMP-2 and MMP-9 mRNA. Cells were preincubated with 0.1 nmol/L GnRHa for 3 hours. Then, ActD (4 μg/mL) was added to stop transcription and RNA was isolated after 0, 1, 2, 4, and 6 hours. The amount of gelatinase mRNA compared with β-actin was determined by RT-PCR. As shown in Fig. 3B, GnRHa did not affect the decay rate of the MMP-2 and MMP-9 mRNA.

To assess whether GnRHa causes transcriptional activation at the MMP-2 and MMP-9 promoters, human MMP-2 or MMP-9 promoter-driven luciferase constructs were transiently transfected into Caov-3 and OVCAR-3 cells in either the presence or the absence of 0.1 nmol/L GnRHa for 24 hours. The results showed that exposure of cells to GnRHa increased the activities of MMP-2 and MMP-9 promoters by 10- and 3-fold, respectively \((P < 0.005\); Fig. 3C). Moreover, these increases in promoter activities were completely abolished by GnRH receptor siRNA treatment but not the nonspecific siRNA (Fig. 3C). These results collectively show a role for GnRH in the transcriptional activation of MMP-2 and MMP-9.

**Figure 4.** Inhibition of MMP-2 and MMP-9 suppresses cell invasion and migration in response to GnRH. Cells were left untreated (control) or pretreated with OA-Hy (15 μmol/L), SB-3CT (10 μmol/L), or neutralizing MMP-9 antibody (anti-MMP-9; 15 μg/mL) for 30 minutes. A, pretreated cells (1.5 × 10^5) were seeded on Matrigel-coated (50 μg/mL) Transwell chambers (8-μm pore) in the presence or absence of 0.1 nmol/L GnRHa. After 24 hours, cells on the upper surface were removed; the invaded cells were stained by crystal violet and counted. **, \(P < 0.005\), compared with untreated controls. **, \(P < 0.005\), compared with untreated controls.
Inhibition of MMP blocks GnRH-enhanced invasion and migration in ovarian cancer cells.

To evaluate the potential contribution of MMP-2 and MMP-9 in the GnRH-induced invasion of ovarian cancer cells, we blocked the gelatinase activities using potent MMP-2 (OA-Hy, 15 μmol/L) or MMP-9 (SB-3CT, 10 μmol/L) specific inhibitors or MMP-9 neutralizing antibody (15 μg/mL) in the presence or absence of 0.1 nmol/L GnRHa. Specific inhibition of MMP-2 by OA-Hy reversed the stimulatory effect of GnRH on cell invasion (by 81.8% in Caov-3 and 82.9% in OVCAR-3), whereas concomitant treatment with MMP-9 inhibitor SB-3CT or anti-MMP-9 significantly blocked the increase in invaded cells after GnRHa treatment (by 75.6% and 72.7%, respectively, in Caov-3 and 70.3% and 73.1%, respectively, in OVCAR-3; Fig. 4A). Similar results were obtained in migration assays as shown in Fig. 4B. Together, these experiments show a crucial role for both MMP-2 and MMP-9 in the GnRH-dependent invasion and migration.

Prolonged activation of JNK by GnRH. Because studies have shown that the MAPK pathway is critical for the activation of gene expression by GnRH in some cell types (28, 29), we asked if they also play a role in GnRH-induced human ovarian cancer cell invasion and/or migration. We first examined the levels of phosphorylated (active) forms of MAPK family members (ERK1/2, JNK, and p38 MAPK) in Caov-3 and OVCAR-3 cells treated with 0.1 nmol/L GnRHa for increasing time course of 0, 5, 15, 30, 60, and 120 minutes. As shown, addition of GnRHa to the cells significantly increased the phosphorylation of ERK1/2, JNK, and p38 MAPK (Fig. 5). Interestingly, whereas prominent (15-fold) and sustained activation (60 minutes) of JNK was shown in GnRH-activated cells (Fig. 5), activation (60 minutes) of JNK was shown in GnRH-activated cells (Fig. 5). Interestingly, whereas prominent (15-fold) and sustained activation (60 minutes) of JNK was shown in GnRH-activated cells (Fig. 5). Interestingly, whereas prominent (15-fold) and sustained activation (60 minutes) of JNK was shown in GnRH-activated cells (Fig. 5). Interestingly, whereas prominent (15-fold) and sustained activation (60 minutes) of JNK was shown in GnRH-activated cells (Fig. 5). Interestingly, whereas prominent (15-fold) and sustained activation (60 minutes) of JNK was shown in GnRH-activated cells (Fig. 5). Interestingly, whereas prominent (15-fold) and sustained activation (60 minutes) of JNK was shown in GnRH-activated cells (Fig. 5). Interestingly, whereas prominent (15-fold) and sustained activation (60 minutes) of JNK was shown in GnRH-activated cells (Fig. 5). Interestingly, whereas prominent (15-fold) and sustained activation (60 minutes) of JNK was shown in GnRH-activated cells (Fig. 5). Interestingly, whereas prominent (15-fold) and sustained activation (60 minutes) of JNK was shown in GnRH-activated cells (Fig. 5). Interestingly, whereas prominent (15-fold) and sustained activation (60 minutes) of JNK was shown in GnRH-activated cells (Fig. 5). Interestingly, whereas prominent (15-fold) and sustained activation (60 minutes) of JNK was shown in GnRH-activated cells (Fig. 5). Interestingly, whereas prominent (15-fold) and sustained activation (60 minutes) of JNK was shown in GnRH-activated cells (Fig. 5). Interestingly, whereas prominent (15-fold) and sustained activation (60 minutes) of JNK was shown in GnRH-activated cells (Fig. 5). Interestingly, whereas prominent (15-fold) and sustained activation (60 minutes) of JNK was shown in GnRH-activated cells (Fig. 5). Interestingly, whereas prominent (15-fold) and sustained activation (60 minutes) of JNK was shown in GnRH-activated cells (Fig. 5). Interestingly, whereas prominent (15-fold) and sustained activation (60 minutes) of JNK was shown in GnRH-activated cells (Fig. 5). Interestingly, whereas prominent (15-fold) and sustained activation (60 minutes) of JNK was shown in GnRH-activated cells (Fig. 5). Interestingly, whereas prominent (15-fold) and sustained activation (60 minutes) of JNK was shown in GnRH-activated cells (Fig. 5).

GnRH-induced invasion and motility are JNK dependent. To assess the functional significance of GnRH-activated ERK1/2, JNK, and p38 MAPK in invasion and motility, we asked if interfering with their activation by specific inhibitors diminishes GnRH-mediated in vitro invasiveness and motility. As shown, treatment of 50 μmol/L SP600125, a specific JNK inhibitor, significantly reduced the number of both invaded (Fig. 6A) and migrated (Fig. 6B) cells to near basal levels. In contrast, inhibition of GnRH-stimulated ERK1/2 and p38 MAPK activation by 50 μmol/L PD98059 or 10 μmol/L SB203580, respectively, had no effect (Fig. 6A and B). These results indicate a critical role for JNK, but not ERK1/2 or p38 MAPK, in GnRH-induced invasive phenotype and migration in ovarian cancer cells.

To additionally investigate the role for JNK in cellular invasion and migration, we transfected Caov-3 and OVCAR-3 cells with a dominant-negative JNK (DN-JNK) construct in which the dual phosphorylation motif Thr-Pro-Tyr was mutated to Ala-Pro-Phe (30). Specific inactivation of JNK in DN-JNK-transfected cells was confirmed by Western blot analysis (Fig. 6A, inset). Both the invasive phenotype and cell migration were clearly reduced in DN-JNK transfectants as shown in Fig. 6A and B, showing the significance of the JNK pathway in GnRH-induced invasion and migration in Caov-3 and OVCAR-3 cells.

GnRH-mediated up-regulation of MMP-2 and MMP-9 involves JNK. Next, we asked whether JNK mediates GnRH induction of MMP-2 and MMP-9 activities. As shown in Fig. 6C, both basal and GnRH-stimulated proform and active form of the MMP-2 and MMP-9 production were inhibited with 50 μmol/L SP600125. GnRH-induced MMP-2 and MMP-9 promoter activities were also inhibited by targeted inhibition of JNK using SP600125 or DN-JNK (Fig. 6D). Consistent with the effects on invasion and cell migration, pretreatment with either PD98059 or SB203580 showed...
no inhibition on GnRH-mediated MMP induction (data not shown). These results suggest that activation of JNK is required for enhanced MMP-2 and MMP-9 expression.

Discussion

There is much evidence that implicates an important role for GnRH in regulating the proliferation for a wide range of hormone-dependent malignancies, including ovarian cancer (1). However, little is known about its role in tumor invasion, metastasis, and other aspects of cancer progression. In this study, we report for the first time that GnRH may contribute to the metastatic dissemination of ovarian cancer cells by promoting the expression and/or processing of metastasis-related MMP, with subsequent downstream changes in migratory and invasive behavior, providing an insight into the prospect of developing targeted therapy for ovarian carcinoma.

The present study showed a biphasic effect of GnRH on cellular migration and invasion. Whereas lower concentrations of the GnRHa stimulated cellular migration and invasion in a dose-dependent manner, high concentrations were not as efficient. Alteration of GnRH receptor numbers or gene expression is involved in mechanisms underlying the biphasic effect of GnRH in the pituitary and ovary (19, 25, 26, 31). In ovarian carcinoma cells, we showed previously (25) and in this study that low doses of GnRH up-regulated its receptor, whereas high doses decreased it. This also argues for a role for GnRH receptor to activate intracellular signals necessary for cellular responses. Notably, the effects of GnRH on JNK and MMP-2/MMP-9 activation were consistent with changes in the GnRH receptor mRNA levels. When GnRH receptor mRNA was up-regulated, GnRH enhanced JNK and MMP-2/MMP-9 activities; however, when GnRH receptor mRNA was unaffected or down-regulated, GnRH had no effect on JNK3 and MMP-2/MMP-9. Our finding that siRNA-mediated down-regulation of the GnRH receptor inhibited MMP-dependent invasion and cellular migration further upholds this view.

3 Unpublished data.
Metastasis is a complex phenomenon that requires several specific steps, such as decreased adhesion, increased motility, and proteolysis. In prostate cancer, GnRH has been shown to regulate cell motility through its interaction with the small GTPases Rac1, Cdc42, and RhoA, which are involved in the regulation of actin polymerization (32). Our data show that another mechanism by which GnRH may promote tumor cell migration and invasion is through increased expression and proteolytic activities of MMP-2 and MMP-9 that specifically degrade the basement membrane. MMP-2 and MMP-9 have been suggested to play critical roles in ovarian cancer metastasis, and up-regulation of MMP-2 and MMP-9 is associated with increased invasion and poor prognosis in late-stage or invasive ovarian cancer patients (8–11). In addition to their enzymatic activities, MMP-2 and MMP-9 can also promote tumor cell migration by influencing cytoskeletal organization through their association with different families of adhesion receptors (33). They have also been reported to promote ascites formation in ovarian cancer cells via vascular endothelial growth factor production (34). Surprisingly, activation of MMP-2 and MMP-9 was not related to a decrease in the specific inhibitor of TIMP-1 and TIMP-2 in the GnRH-induced ovarian cancer cell invasion and motility. There was, however, an increase in MMP/TIMP ratios, which also in favor of ECM degradation.

One of the novel findings of the present study is the demonstration that GnRH is a direct transcriptional activator of MMP-2 and MMP-9 synthesis. MMP-2 and MMP-9 expression are reported to be regulated at many levels, including gene activation, mRNA stability, proenzyme activation, and inactivation by endogenous inhibitors in a complex fashion by numerous oncogene and tumor suppressor pathways and conditions of hypoxia (35–38). In this study, we showed that GnRH increased MMP-2 and MMP-9 mRNA expression, and such expression was not due to changes in mRNA stabilities. We further showed that GnRH directly induced MMP-2 and MMP-9 promoter activities. This is the first demonstration that MMP-2 and MMP-9 are target genes of GnRH receptor activation and adding GnRH as a new member of MMP-2 and MMP-9 transcriptional modulators.

Our results identify a JNK signaling pathway that mediates GnRH-stimulated MMP expression, secretion, and cell invasion.

The kinetic profile revealed differential regulation of ERK1/2, p38 MAPK, and JNK by GnRH with a sustained signaling through the JNK pathway. The duration of kinase activation is a major determinant for signal outcome in different cellular contexts (39), and its influence may have included invasiveness. In support of this hypothesis, GnRH-stimulated MMP expression and cell invasion were attenuated by specific inhibition of JNK but not ERK1/2 and p38 MAPK. Consistently, a recent report shows that activated JNK levels are much higher in the malignant pleural and peritoneal effusions of patients with advanced-stage ovarian carcinomas, suggesting that activation of JNK may contribute to a more invasive phenotype (40). However, unlike ERK1/2 and p38 MAPK, a role for JNK in tumor invasion or migration has not been widely reported. JNK signaling was shown recently to regulate MMP-2 production and invasiveness in human gliomas (41). The JNK pathway targets multiple transcription factors, including c-Jun and c-Fos (42), ATF (43), and PEA (44), and putative binding sites for these DNA-binding proteins are present in the MMP promoters (45). Whether these putative regulatory elements participate in the GnRH-dependent activation of the MMP-2 and MMP-9 genes remains to be determined.

In summary, our findings emphasize the potential role of GnRH in promoting cellular migration and metastasis-related proteinase activities in GnRH receptor-expressing ovarian tumors. This information provides a mechanistic rationale for the observed GnRH receptor overexpression in advanced-stage ovarian carcinomas. The promising role for GnRH in invasion of ovarian cancer cells in vitro merits further investigation in vivo, which may provide additional insight into its potential as a therapeutic molecular target to decrease metastasis.

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