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ORIGINAL PAPER

Differential role of gonadotropin-releasing hormone on human ovarian epithelial cancer cell invasion

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Abstract Ovarian cancer is the most lethal of all gynecological cancers. Most deaths from ovarian cancer are due to widespread intraperitoneal metastases and malignant ascites. However, mechanisms of invasion in ovarian cancer remain poorly understood. In this study, we examined the effects of gonadotropin-releasing hormone (GnRH)-I (the classical mammalian GnRH), GnRH-II (a second form of GnRH), and GnRH receptor on invasion using two human ovarian carcinoma cell lines, OVCAR-3 and SKOV-3. Here we demonstrated that in OVCAR-3, GnRH-I and GnRH-II promoted cell invasion, whereas in SKOV-3, GnRH-I and GnRH-II inhibited cell invasion. Transfection of small interfering RNA to abrogate the gene expression of GnRH receptor reversed GnRH-I and GnRH-II-mediated invasion activities, suggesting that the same receptor, type I GnRH receptor, is essential for the effects of GnRH-I and GnRH-II in both OVCAR-3 and SKOV-3. Treatment of SKOV-3 cells with GnRH-I or GnRH-II resulted in a decrease in matrix metalloproteinase 2 but an increase in tissue inhibitor of metalloproteinase 2 secretions. In addition, we found that GnRH-I and GnRH-II interfered with activation of the phosphatidylinositol-3-kinase/AKT pathway that is well documented to stimulate proteolysis and invasion of ovarian cancer cells. Taken together, these observations suggest that GnRH-I and GnRH-II play key regulatory roles in ovarian tumor cell invasion and extracellular matrix degradation.

Keywords GnRH · Matrix metalloproteinases · PI3K · Invasion · Ovarian cancer

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Introduction

Ovarian cancer is the sixth most common cancer and the fifth leading cause of cancer-related deaths among women in developed countries [1]. In the United States, about 23,000 new cases were diagnosed while approximately 14,000 women died from the disease in the same year [2]. Due to the absence of specific symptoms and the lack of a trustworthy screening system, most patients (almost 70%) are diagnosed with advanced-stage disease. The five-year survival rates of this group of patients (FIGO stage III or IV) are only 16–28%. In contrast, the 5-year survival rate is up to 80% for patients in stage I [3, 4]. However, the factors that regulate the metastastic process of ovarian cancer are poorly understood.

The hypothalamic decapeptide GnRH is a key neuroendocrine regulator in the mammalian reproductive system. It is released in a pulsatile manner from hypothalamic

GnRH neurons and regulates the biosynthesis and secretion of gonadotropins from pituitary gonadotropes. To date, approximately 12 isoforms of GnRH have been identified in vertebrates. The classical mammalian GnRH (now referred to as GnRH-I) as well as a second form of GnRH (GnRH-II) that is identical to chicken GnRH-II are expressed in humans [5, 6]. In addition to the well-established function of GnRH in the control of gonadotropin secretion from the pituitary, both GnRH-I and GnRH-II have been shown to exert autocrine and/or paracrine effects in extrapituitary tissues, including the ovary [7, 8]. GnRH-I and its receptor are expressed in 80% of human ovarian epithelial tumors, ovarian surface epithelial (OSE) cells, and ovarian cancer cell lines [9, 10], suggesting that this decapeptide hormone may be an autocrine and/or paracrine regulator of the OSE and play a role in the pathophysiology of ovarian cancer [11–15]. Native GnRH-I and its synthetic analogs (GnRH-I-a) inhibit the growth of numerous GnRH receptor-bearing ovarian cancer cell lines in vitro [9, 16–19]. The growth inhibitory effects of GnRH-I-a have also been observed in normal OSE cells [20]. Moreover, several reports suggest that GnRH-I-a may modulate cell growth through the induction of apoptosis in ovarian cancers [21–25]. Like GnRH-I, GnRH-II may have an anti-proliferative effect in immortalized OSE cells and ovarian cancer cells [13, 26–29]. Besides its effect in the regulation of growth, a role of GnRH in tumor invasion and metastasis has also been suggested. 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 [30]. GnRH-I has been shown to stimulate ovarian epithelial cancer cell migration and invasion [31]. However, it is not known if GnRH-II, like GnRH-I, have an effect on the invasive properties of ovarian cancer cells. Nor is it clear if these effects are similar in cell lines with different invasion abilities. In fact, several studies have demonstrated that the effects of GnRH on cell growth, cell migration, and cell attachment vary depending on the cellular context [32].

In the present study, we investigated whether the two forms of GnRH, GnRH-I and GnRH-II, differ in their effects on invasiveness, metastasis-related proteases, and the regulation of the phosphatidylinositol-3-kinase (PI3K)/AKT pathway in two human ovarian cancer cell lines, OVCAR-3 and SKOV-3. These two cell lines differ in their intrinsic invasiveness in vitro. Although the OVCAR-3 cells were previously found to adhere to basement membrane matrix, these cells did not invade Matrigel, whereas SKOV-3 cells displayed a highly invasive phenotype [33]. Moreover, we examined the contribution of the type I GnRH receptor, which is known to bind both forms of GnRH, to the effects of GnRH-I and GnRH-II on cellular invasion.

Materials and methods

Reagents and cell culture

GnRH-I analog (D-Trp6-GnRH) and GnRH-II analog (D-Arg6-Azagly10-GnRH-II) were purchased from Bachem (Belmont, CA). The human ovarian cancer cell lines, OVCAR-3 and SKOV-3, were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and their use was approved by the University of British Columbia Clinical Screening Committee for Research and Other Studies Involving Human Subjects. Cells were cultured in medium 199:MCDB 105 (1:1; Sigma-Aldrich Corp., St. Louis, MO) containing 10% fetal bovine serum (FBS; Hyclone Laboratories Ltd., Logan, UT), 100 U/ml penicillin G, and 100 g/ml streptomycin (Life Technologies, Inc., Rockville, MD) in a humidified atmosphere of 5% CO_2–95% air at 37°C. The cells were passaged with 0.06% trypsin (1:250)/0.01% EDTA in Mg^{2+}/Ca^{2+}-free HBSS at confluence.

Small interfering RNA transfection

The pSUPER vector (OligoEngine, Seattle, WA) encoding 19-mer hairpin small interfering RNA (siRNA) duplex specific to GnRH receptor sequence 5′-AGATCCGAGTGACGGTTAC-3′ (nucleotides 107–125 downstream of the start codon) has been described elsewhere [34]. As a nonspecific siRNA control, scrambled siRNA duplex was used. Transfection was performed using LipofectAMINE 2000 reagent (Invitrogen, San Diego, CA) following manufacturer's instruction. Twenty-four hour later, the transfected cells were collected for invasion assay.

Invasion assay

The invasion assay was performed in Boyden chambers essentially as reported previously [31]. Twenty-four-well Transwell inserts with 8-μm pore coated with 1 mg/ml Matrigel (75 μl/well; BD sciences, Mississauga, ON, Canada) were used to assess cell invasion. Trypsinized cells (1×10^5) in serum-free medium, with or without GnRH-I and -II, were seeded in triplicate in the upper chamber. Serum-free medium was placed in the lower wells. The chambers were incubated for 24 h at 37°C in a 5% CO_2 atmosphere. Cells that had not penetrated the filter were wiped off, 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 (at 100× magnification) ±SEM of three independent experiments.

MTT assay

Cell viability was assessed colorimetrically using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] assay (Sigma, St. Louis, MO). SKOV-3 cells were seeded in 24-well plates. On the day of collection, the cells were incubated with 500 µl MTT solution (2 mg/ml in phosphate-buffered saline solution) at 37°C for 4 h. The medium was removed and the dye was eluted with 200 µl DMSO. The optical density at 570 nm was determined using a microplate spectrophotometer (Fisher Scientific Ltd., Ottawa, ON, Canada).

Antibodies

Matrix metalloproteinase (MMP) 2, MMP9, and tissue inhibitor of metalloproteinase (TIMP) 1 antibodies were purchased from Neomarkers (Fremont, CA), and GnRH receptor, TIMP2, urokinase plasminogen activator (uPA) and plasminogen activator inhibitor-1 (PAI-1) antibodies were acquired from Santa Cruz Biotechnology Ltd. (Santa Cruz, CA). Phospho-AKT (Ser 473 and Thr 308), phospho-GSK-3α/β (glycogen synthase kinase-3α/β), phospho-FKHR (Forkhead in rhabdomyosarcoma), (phosphatidylinositol-dependent kinase), phospho-SGK (serum- and glucocorticoid-induced protein kinase), and pan-AKT antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA).

Western blot assay

Thirty microliter of conditioned medium was electrophoresed under reducing conditions on an 8% SDS-PAGE to determine the secretion of MMPs, TIMPs, uPA, and PAI-1 from ovarian cancer cells. To examine the activation of PI3K signaling pathway, the cells were washed once with medium, and serum starved for 4 h prior to treatments with GnRH-I or GnRH-II for 15 min in dose-dependent manner (1 nM and 100 nM). Forty microgram of extracted protein was electrophoresed on 10% SDS-PAGE, transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech., Oakville, ON, Canada), and reacted with specific primary antibodies at 4°C overnight. The signals were detected with horseradish peroxidase-conjugated secondary antibody for 1 h, and visualized using the ECL chemiluminescent system (Amersham Pharmacia Biotech.). Densitometric analysis was performed using Scion Image software (Scion Corporation, Maryland, MD).

Real-time PCR

Total RNA was prepared using TRIzol reagent (Invitrogen Canada, Burlington, ON, Canada), according to manufacturer's instructions. Total RNA (2.5 µg) was reverse transcribed into first-strand cDNA (Amersham Pharmacia Biotech.) following manufacturer's procedure. Briefly, the RNA solution was incubated at 65°C for 10 min and then chilled on ice. A total quantity of 5 µl of the bulk first-strand cDNA reaction mix, 1 µl of 200 mM DTT, 1 µl of 0.2 µM Not I-d(T)18 primer, and the heat-denatured RNA were mixed, and incubated at 37°C for 1 h. The primers used for SYBR Green real-time RT-PCR were as follows: for human GnRH receptor, sense primer, 5′-ACCGCTCCCTGGCTATCAC-3′ and antisense primer, 5′-ACTGTCCGACTTTGCTGTTGCT-3′; for MMP2, sense primer, 5′-CCGCA GTGAC GGAAA GATGT-3′ and antisense primer, 5′-CACTT GCGGT CGTCA TCGTA-3′; for TIMP2, sense primer, 5′-AGCAT TTGAC CCAGA GTGGA A-3′ and antisense primer, 5′-CCAAA GGAAA GACCT GAAGG A-3′; for GAPDH, sense primer, 5′-ATGGA AATCC CATCA CCATC TT-3′ and antisense primer, 5′-CGCCC CACTT GATTT TGG-3′. Real-time PCR was performed using the ABI prism 7300 Sequence Detection System (Perkin-Elmer Applied Biosystems, CA) equipped with a 96-well optical reaction plate. The relative expression of mRNA was calculated using the comparative CT method according to manufacturer's literature (Applied Biosystems). The steady-state concentration of mRNA for GnRH receptor was normalized to the amount of GAPDH mRNA.

Data analysis

All the experiments were performed at least twice. All values are expressed as means ± SEM. Data were analyzed by Student's *t*-test or one-way ANOVA followed by Dunnett's test (for comparing treatment groups with the control group) using GraphPad Prism 4 for Windows (GraphPad Software, San Diego, CA). $P < 0.05$ was considered statistically significant.

Results

Effect of GnRH-I and GnRH-II on ovarian cancer invasion

To examine the effect of GnRH on the invasive capacity of ovarian cancer cells, Boyden chamber assay using a Matrigel-coated inserts was performed. Our result showed that GnRH-I and GnRH-II differentially modulated the invasive phenotype of OVCAR-3 and SKOV-3 cells. Cell invasion of OVCAR-3 was significantly stimulated by both GnRH-I and GnRH-II treatment at low doses of 0.1 nM to 10 nM (Fig. 1A), which was consistent with the recent findings [31]. In contrast, cell invasion of SKOV-3 cells was

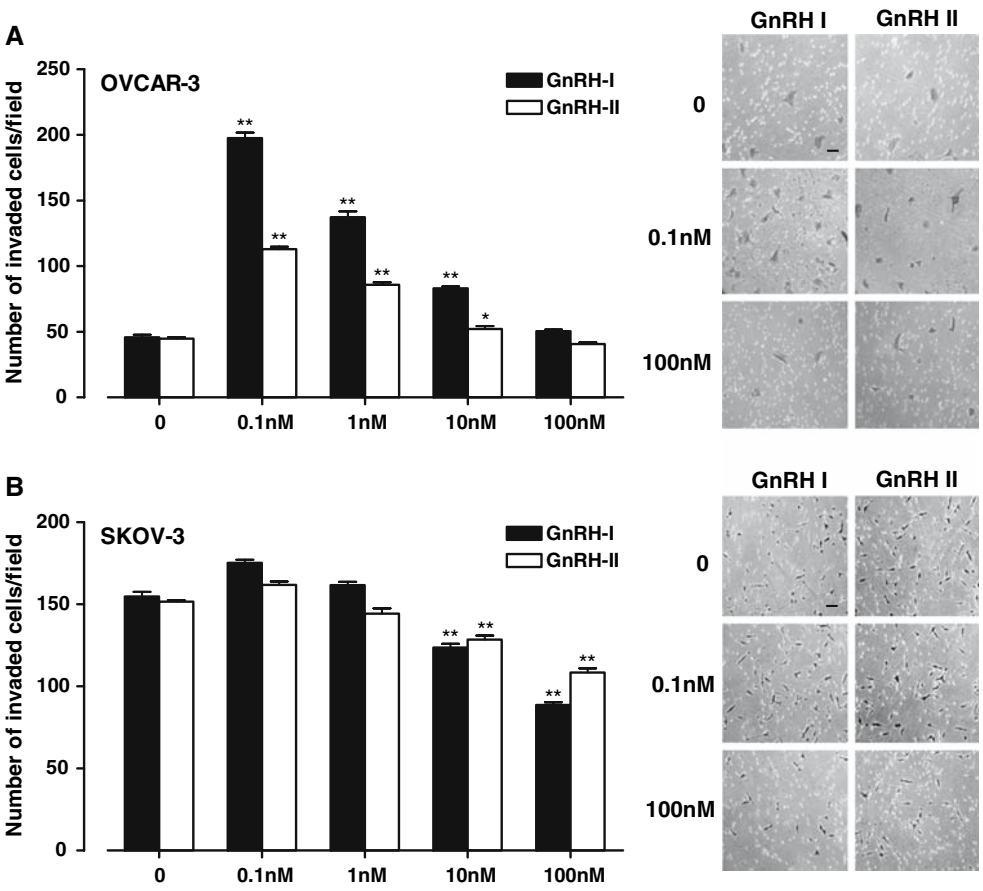

Fig. 1 Effect of GnRH-I and GnRH-II on invasion of OVCAR-3 and SKOV-3 cells. (**A**) OVCAR-3 and (**B**) SKOV-3 cells were seeded onto Matrigel-coated invasion chamber in the presence or absence of GnRH-I or -II (0.1, 1, 10, and 100 nM) as indicated. After 24 h, filters were stained and invading cells were quantified using inverted microscope. Each value represents the mean ± SEM of three replicates. Right panel, representative pictures. $^{*}P < 0.05$; $^{**}P < 0.001$ vs. control. Bar, 20 μm

unaffected at low doses but was suppressed at high doses of 10–100 nM (Fig. 1B).

Effect of RNAi-mediated GnRH receptor silencing on ovarian cancer invasion

Previous findings in the literature regarding the expression of GnRH receptor in SKOV-3 cells have been inconsistent. Whereas the presence of this receptor has been observed in some studies [35], it was not detected in other cases [36]. We therefore re-examined the expression of GnRH receptor in SKOV-3 using real-time PCR with gene-specific primers. The results show that GnRH receptor mRNA was present in this cell line (Fig. 2A) as well as in the positive control cell line, OVCAR-3, which is known to express the GnRH receptor [31]. To address the role of GnRH receptor on the invasive properties of ovarian cancer cells, we asked whether expression of siRNA known to deplete GnRH receptor [34] could affect ovarian cancer invasion. The effectiveness of this siRNA to knockdown GnRH receptor expression in both cell lines was confirmed by Western blot analysis (Fig. 2B). In OVCAR-3 cells, siRNA-mediated depletion of GnRH receptor significantly inhibited the stimulatory effects of GnRH-I and GnRH-II on cell invasion (Fig. 3A, B). Similarly, depletion of GnRH receptor abrogated the inhibitory effects of GnRH-I and GnRH-II in SKOV-3 cells (Fig. 3C, D). These data suggest that GnRH receptor is directly involved in mediating the opposite effects of GnRH on ovarian cancer cell invasion in these two cell lines.

Effect of GnRH-I and GnRH-II on proteolysis in SKOV-3 cells

Among various cancer-related protineases, MMP2/9 and uPA were found at high concentrations in ovarian ascites and ovarian carcinomas and their levels correlated inversely with prognosis of ovarian cancer patients [37–41]. In this regard, we investigated whether GnRH-I and GnRH-II could modulate the secretion of MMP2/9 and uPA from SKOV-3 cells by Western blot analysis. Treatment with GnRH-I and GnRH-II for 24 h resulted in a significant down-regulation of MMP2 but not MMP9 levels in SKOV-3 cells (Fig. 4A). TIMPs, the endogenous inhibitor of MMPs, bind to the catalytic site of MMPs in 1:1 ratio, and control MMP-mediated proteolysis [42]. In this study, secretion of TIMP2, but not TIMP1, was significantly increased by treatment with GnRH-I or GnRH-II

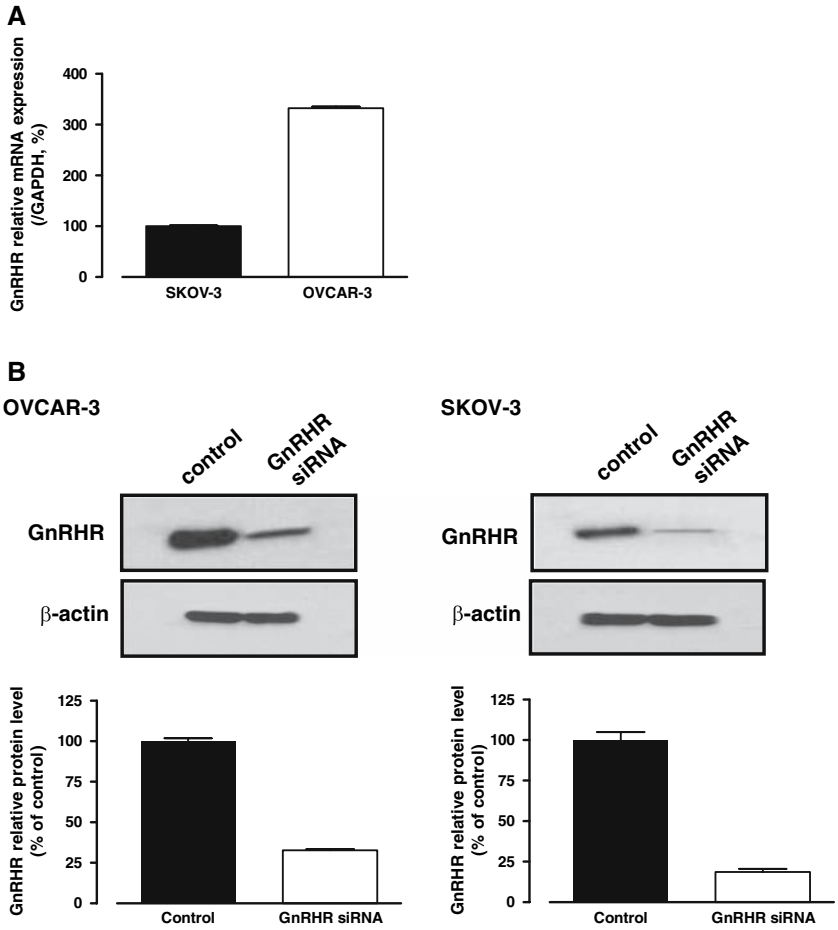

Fig. 2 Effect of GnRH receptor siRNA on GnRH receptor expression in OVCAR-3 and SKOV-3 cells. (**A**) mRNA expression of the human GnRH receptors in OVCAR-3 and SKOV-3 cells. Cells were lysed with TRIzol reagent and for real-time PCR with GnRH receptor specific primers as described in Materials and methods. GAPDH served as an internal control. (**B**) Cells were transiently transfected with vector expressing short hairpin RNA directed against GnRH receptor or nonspecific siRNA as control. Transfected cells were collected for Western blotting with anti-GnRH receptor antibody. Each value represents the mean ± SEM of three replicates

in a dose-dependent manner, as shown by immunoblot analysis (Fig. 5A). These data suggest that GnRH-I and GnRH-II may inhibit the proteolytic potential of SKOV-3 ovarian cancer cells by regulating the net balance of MMP2/TIMP2. Neither uPA nor its inhibitor PAI-1 was altered by treatment with GnRH (Fig. 6).

To elucidate whether gonadotropin-regulated MMP2 and TIMP2 secretion was transcriptionally controlled, mRNA expression levels of MMP2 and TIMP2 were examined by real-time PCR following GnRH stimulation. Treatment with 100 nM, but not 1 nM and 10 nM, GnRH-I and GnRH-II only moderately decreased mRNA levels of MMP2 and had no effect on TIMP2 mRNA (Figs. 4B, 5B), suggesting that the changes in protein expression may not result from mRNA synthesis.

Interference of PI3K/AKT pathway by GnRH-I and GnRH-II in SKOV-3 cells

It is well documented that PI3K/AKT is frequently amplified and serves as a survival pathway in ovarian carcinomas [43, 44]. In addition, growing evidence suggests that the PI3K/AKT pathway plays a critical role in cell migration, invasion, and metastasis of ovarian cancer cells [45–50]. To address the involvement of GnRH in the PI3K/AKT pathway, we examined the phosphorylation status of their downstream molecules upon treatment with 100 nM GnRH for increasing time course of 5, 15, and 30 min. Whole-cell lysates were analyzed for phosphorylated (active) forms of AKT, GSK-3α/β, FHKR, PDK, and SGK by Western blotting with phospho-specific antibodies. The membranes were then re-probed to evaluate total AKT. As shown, treatment with GnRH-I and GnRH-II decreased the phosphorylation of AKT (Ser 473), PDK, and SGK in a time-dependent manner (Fig. 7). In contrast, they had no effect on the phosphorylation of AKT at Thr 308, GSK-3α/β, and FKHR (data not shown). These results suggest that GnRH-I and GnRH-II inhibit the activation of the PI3K-PDK-AKT/SGK pathway, which may play a role in regulating ovarian tumor cell invasion.

Discussion

GnRH was originally identified as a hypothalamic decapeptide that promotes gonadotropin release from pituitary

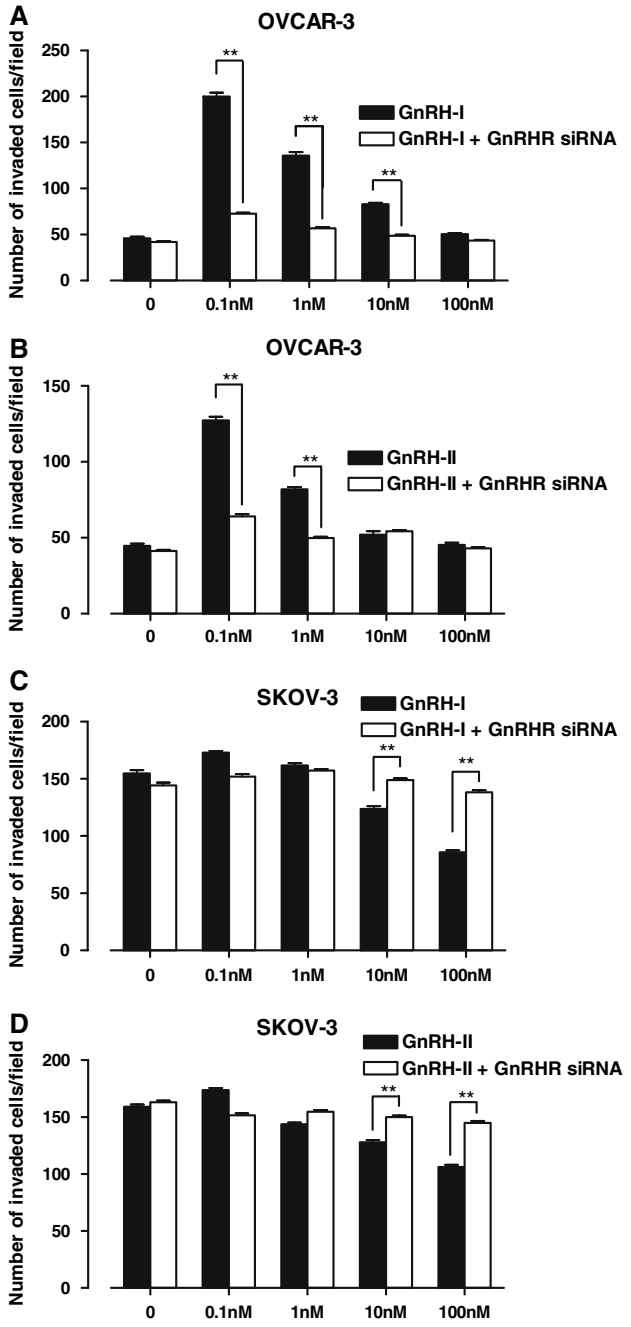

Fig. 3 Effect of RNAi-mediated GnRH receptor silencing on invasion of OVCAR-3 and SKOV-3 cells. (**A** and **B**) OVCAR-3 and (**C** and **D**) SKOV-3 cells were transiently transfected with vector expressing short hairpin RNA directed against GnRH receptor or nonspecific siRNA as control. Transfected cells were collected for Matrigel invasion assay in the presence or absence of GnRH-I or GnRH-II (0.1, 1, 10, and 100 nM) as indicated. After 24 h, filters were stained and invading cells were quantified using inverted microscope. Each value represents the mean ± SEM of three replicates. $^{**}P < 0.001$ vs. control

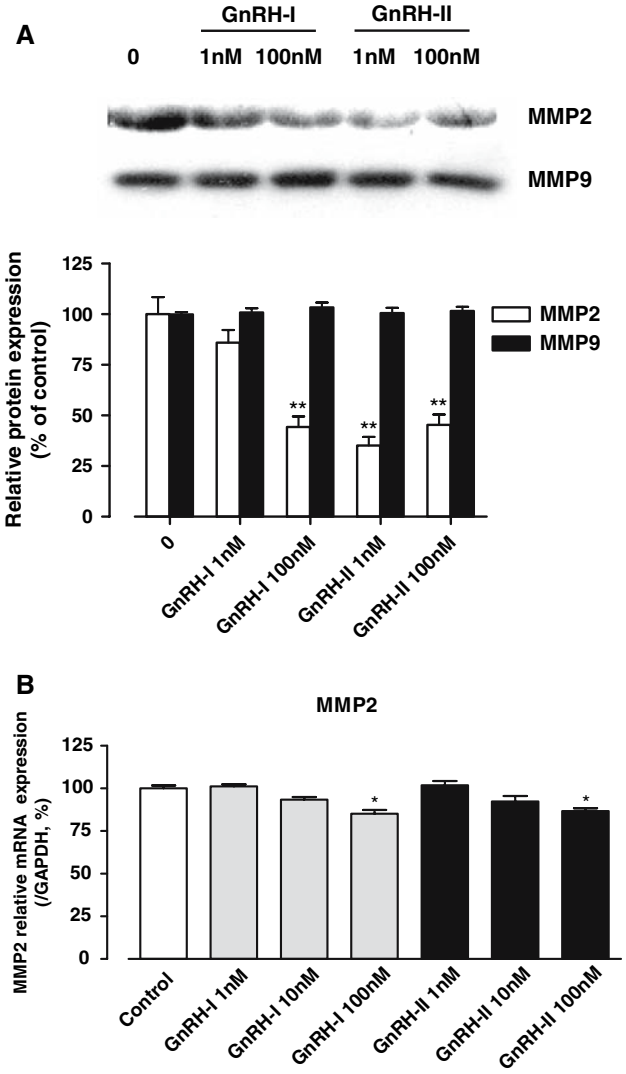

Fig. 4 Effect of GnRH-I and GnRH-II on expression of MMP2 and MMP9 in SKOV-3 cells. (**A**) Conditioned medium was collected from SKOV-3 cells after 24 h incubation with GnRH-I or GnRH-II (1 and 100 nM). The supernatants were normalized based on the viable cell number using MTT assay. Immunoblot assay was performed for MMP2 and MMP9 (72- and 92-kDa) as described in the Materials and methods. *Lower panel*, the signal intensity was determined by densitometry, and the fold changes in MMP2 and MMP9 release were shown. (**B**) Following 24 h incubation with GnRH-I or GnRH-II (1, 10, and 100 nM), expression of mRNA transcripts for MMP2 was detected by real-time PCR. The level of MMP2 mRNA was normalized against that of GAPDH. $^{*}P < 0.05$; $^{**}P < 0.001$ vs. control

gonadotropes. Although there is increasing evidence that GnRH also affects the proliferation of a number of human malignant tumors, including ovarian cancer [13], 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 the stimulatory and inhibitory effects of the two forms of GnRH, GnRH-I and GnRH-II, on the invasion of human ovarian cancer cells through a common type I GnRH receptor. Moreover, the apparent divergent effects of both GnRH-I and GnRH-II on cell invasion in OVCAR-3 and SKOV-3 have been observed at different

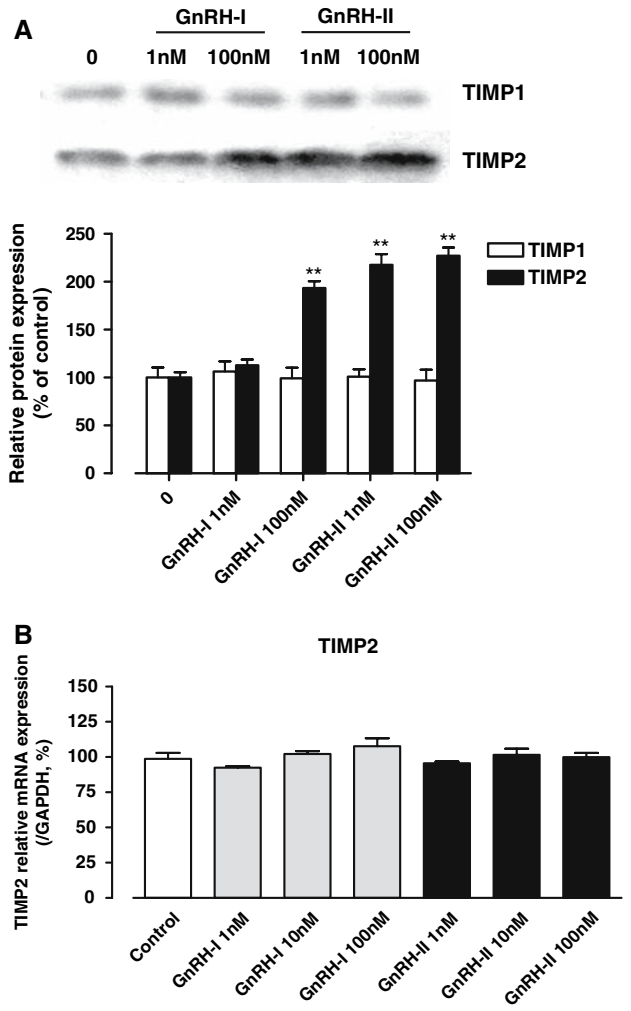

Fig. 5 Effect of GnRH-I and GnRH-II on expression of TIMP1 and TIMP2 in SKOV-3 cells. (**A**) Conditioned medium was collected from SKOV-3 cells after 24 h-incubation with GnRH-I or GnRH-II (1 and 100 nM). The supernatants were normalized based on the measurement of viable cell number using MTT assay. Immunoblot assay was performed for TIMP1 and TIMP2 (28- and 21-kDa) as described in the Materials and methods. *Lower panel*, the signal intensity was determined by densitometry, and the fold changes in TIMP1 and TIMP2 release were shown. (**B**) Following 24 h incubation with GnRH-I or GnRH-II (1, 10, and 100 nM), expression of mRNA transcripts for TIMP2 was detected by real-time PCR. The level of TIMP2 mRNA was normalized against that of GAPDH. $^{**}P < 0.001$ vs. control

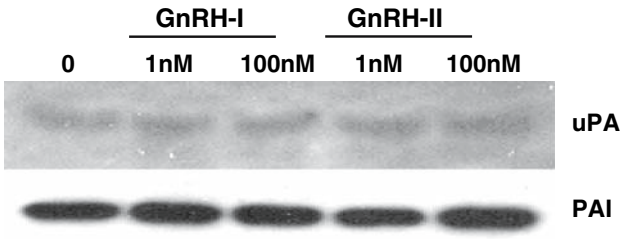

Fig. 6 Effect of GnRH-I and GnRH-II on expression of uPA and PAI-1 in SKOV-3 cells. Conditioned medium was collected from SKOV-3 cells after 24 h incubation with GnRH-I or GnRH-II (1 and 100 nM). The supernatants were normalized based on the measurement of viable cell number using MTT assay. Immunoblotting was performed for uPA and PAI-1 (54- and 45-kDa) as described in the Materials and methods

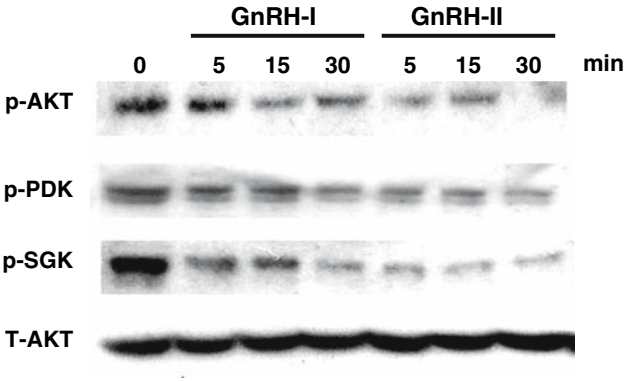

Fig. 7 Effect of GnRH-I and GnRH-II on the activation of PI3K signaling pathway in SKOV-3 cells. Cells were treated with GnRH-I or GnRH-II (100 nM) for indicated time. Immunoblot analysis was performed to detect phosphorylated AKT (Ser 473) (p-AKT), PDK (p-PDK), and SGK (p-SGK). The membranes were then reprobed to evaluate total AKT (T-AKT) expression for normalization

GnRH-I and GnRH-II doses. Cell invasion was enhanced by GnRH-I and GnRH-II treatment in OVCAR-3 at low doses (0.1–10 nM), whereas in contrast, that of SKOV-3 cells was attenuated at high doses (10–100 nM).

It is well established that GnRH-I exerts its action through the type I GnRH receptor. In humans, a full-length functional type II GnRH receptor has not been found. However, a ''putative'' GnRH-II receptor with only five transmembrane domains has been suggested and specific GnRH-II responses have been observed in some cell types in which the expression of type I GnRH receptor was inhibited by the antisense oligonucleotides [12]. OVCAR-3 expresses functional type I GnRH receptor and responds to GnRH agonist stimulation [17, 31]. However, the findings regarding the expression of type I GnRH receptor in SKOV-3 are inconsistent, i.e. the presence of this receptor was reported in some studies [35] but was not detected in others [36]. In this study, both mRNA and protein levels of the type I GnRH receptor were found in the SKOV-3 cell line (Fig. 2A, B). Our finding that siRNA-mediated down-regulation of the type I GnRH receptor completely reversed the effects of both GnRH-I and GnRH-II on cell invasion further supports this view. It is possible that the use of different subclones of the cell line and/or variation in culture or experimental condition may account for the conflicting reports of type I GnRH receptor expression in SKOV-3 cells.

One major finding of this study is that OVCAR-3 and SKOV-3, which are derived from human ovarian carcinoma, exhibited completely opposite response to GnRH stimulation. Similar difference was observed in the effects of GnRH on cell proliferation and cell migration in prostate carcinoma cell lines, TSU-Pr1 and DU145. While GnRH-I and GnRH-II stimulated cell proliferation, induced actin cytoskeleton re-modeling and promoted cell migration in TSU-Pr1, they were inhibitory in DU145 [32]. Importantly, our results also demonstrated that the type I GnRH receptor is indispensable for both the stimulatory and inhibitory effects of GnRH-I and GnRH-II. It is not yet known how a common receptor mediates opposite responses to the same ligand activation. However, several possibilities can be envisaged. First, the receptor expression level is known to be a determinant for different signal outcomes [20, 51–53]. While low doses of GnRH upregulate its receptor, high doses decrease it. We have previously reported the antiproliferative actions of high dose (100 nM) GnRH-I and GnRH-II in both OVCAR-3 and SKOV-3 lines [52, 54]. These results seem to confirm the negative effects of high dose of GnRH-I and GnRH-II on tumor progression in these cell lines. The present study also showed that low doses GnRH-I and GnRH-II induced invasive response only in OVCAR-3 cells which express high levels of the GnRH receptor, but had no effect in SKOV-3 that express low levels of the GnRH receptor. This difference in regulation suggests that high levels of GnRH receptor expression may enhance cellular response to GnRH stimulation, presumably due to more efficient signal amplification or altered signaling. Alternatively, ligand selectivity has been proposed to explain the opposite (stimulatory and inhibitory) effects of GnRH in prostate carcinoma cell lines. In positively responding cell lines, GnRH-I was more effective than GnRH-II. On the other hand, in negatively responding cell lines, GnRH-II was much more effective than GnRH-I [55]. Finally, it is also possible that differences in response may be ascribed to the intrinsic invasive properties of the two cell lines in vitro. The number of invaded cells is consistent with previous findings that in contrast to SKOV-3, OVCAR-3 cells have little or no effect on invasion [31, 33, 56, 57]. Thus, whereas low doses of GnRH can exert a significant invasive effect in the OVCAR-3 cells, they failed to stimulate SKOV-3 maximally. GnRH only exerted inhibitory effects on SKOV-3 at high doses.

In contrast to most other cancers, ovarian carcinomas rarely metastasize through the bloodstream and usually spread only locally into the peritoneal fluid [58]. The dissemination of ovarian cancer occurs via shedding of cells from the primary tumor, followed by seeding of the peritoneal cavity, leading to invasion and growth at a secondary site. This unique biological behavior of ovarian cancers in vivo seems to correlate with our in vitro findings for a low invasive ability, which very few cells degrade or invade the Matrigel, as compared to some other cell types [58].

Proteolytic degradation of the extracellular matrix is thought to be a prerequisite for successful invasion, intravasation, and extravasation of tumors [59, 60]. Proteolysis also has an important role in intraperitoneal metastasis, such as during extension of the implanted tumor through the submesothelial basement membrane into the peritoneal organ stroma [61, 62]. Of various tumor proteases, gelatinases (MMP2 and MMP9), which can degrade collagen IV, the major component of the basement membranes, and uPA has been suggested to be crucial for the invasive and metastatic potential in ovarian carcinoma. Increased expression of MMP2, MMP9, and uPA has been observed in several ovarian cancer cell lines and detected in ascitic fluid from patients with advanced ovarian cancer, correlating with the invasiveness of the ovarian cancer cells [37–39, 41]. Recently, we have shown that GnRH-I agonist induces invasion of OVCAR-3 cells and that this requires the activation of MMP2 and MMP9 [31]. In this study, we showed that MMP2 was involved in the effects of both GnRH-I and GnRH-II on invasiveness of SKOV-3 cells. The inhibition of MMP2 was related to an increase in the specific inhibitor of TIMP2 in these cells. These results indicate that GnRH may regulate the proteolytic potential of ovarian cancer cells by modulating the net MMP/TIMP ratio. On the other hand, we did not observe any significant change in uPA secretion [63]. Interestingly, the changes in MMP2 and TIMP2 expression did not appear to be linked to an alteration in synthesis and/or stability of these transcripts, suggesting a translational or post-translational mechanism.

The diverse signaling pathways underlying the antiproliferative effects of GnRH have been well studied. Particularly, ERK1/2 has been demonstrated to play a critical role in the effects of GnRH on cell proliferation of ovarian cancer cells [28, 29]. The PI3K/AKT signaling pathway is now accepted as being at least as important as the MAPK pathway to promote survival and proliferation of these cells PI3K/AKT is also a well-known key pathway for invasion in normal and neoplastic tissues including ovarian cancer [64, 65]. For example, epidermal growth factor induced MMP9-dependent invasion via PI3K signaling in ovarian cancer cells [47]. Up-regulation of AKT2 stimulated invasion by overexpression of integrin $\beta 1$ [48]. Analysis of upstream regulators has shown that growth factors, estrogen, gonadotropins, and lysophosphatidic acid contribute to activation of PI3K/AKT in ovarian cancer cells [66–70]. Here we show for the first time that the PI3K/AKT cascade can be activated by both GnRH-I and GnRH-II in ovarian cancer cells. Activation of PI3K/AKT is also implicated in gonadotropes and prostate cancer

cells. In prostate cancer DU145 cells, GnRH-I reduces the activity of the PI3K-AKT pathway, which results in the activation of JNK and induction of apoptosis [71]. In the gonadotrope cells, it is of interest that GnRH did not affect basal levels of PI3K/AKT activation; however, co-treatment of GnRH-I with insulin growth factor resulted in a significant decrease in IGF-induced AKT activation via protein kinase C [72], suggesting cross-talk between growth factor and GnRH receptor signaling pathways.

Taken together, this study provides the first evidence that both GnRH-I and GnRH-II exert a direct effect on cellular invasion and metastasis-related protease activities in ovarian cancer cells and that this regulation is mediated via a common type I GnRH receptor. The difference in the responses to GnRH-I and GnRH-II by two ovarian carcinoma lines is important to better understand the molecular basis of metastasis in ovarian cancer. Further investigation of the signaling pathways in the invasion and metastatic process influenced by GnRH will provide novel insights into the progression of ovarian cancer and the development of new therapeutic strategies.

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