GnRH-(1-5) Activates Matrix Metalloproteinase-9 to release Epidermal Growth Factor and Promote Cellular Invasion.

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ABSTRACT

In the extracellular space, the gonadotropin-releasing hormone (GnRH) is metabolized by the zinc metalloendopeptidase EC3.4.24.15 (EP24.15) to form the pentapeptide, GnRH-(1-5). GnRH-(1-5) diverges in function and mechanism of action from GnRH in the brain and periphery. GnRH-(1-5) acts on the orphan G protein-coupled receptor 101 (GPR101) to sequentially stimulate epidermal growth factor (EGF) release, phosphorylate the EGF receptor (EGFR), and facilitate cellular migration. These GnRH-(1-5) actions are dependent on matrix metalloproteinase (MMP) activity. Here, we demonstrated that these GnRH-(1-5) effects are dependent on increased MMP-9 enzymatic activity in the Ishikawa and ECC-1 cell lines. Furthermore, the effects of GnRH-(1-5) mediated by GPR101 and the subsequent increase in MMP-9 enzymatic activity lead to an increase in cellular invasion. These results suggest that GnRH-(1-5) and GPR101 regulation of MMP-9 may have physiological relevance in the metastatic potential of endometrial cancer cells.

1. Introduction

Although the decapeptide gonadotropin-releasing hormone (GnRH) is commonly known as a central regulator of the hypothalamic-pituitary-gonadal (HPG) axis, GnRH and its cognate receptor, GnRHR, are also expressed in peripheral tissues and can regulate processes independent of reproduction (1-5). For example, GnRH and its analogues may have direct effects on gynecologic tumors expressing the GnRHR by inhibiting cell proliferation, migration and metastasis through a G protein-dependent pathway (6-11). Generally, the anti-tumorigenic activity of the GnRHR signals through the $G\alpha_i$ pathway. Stimulating this pathway results in increased activity of phosphotyrosine-phosphatase, inhibition of MAPK signaling, cell-cycle arrest, and activation of apoptotic pathways (12-17). Yet, in other tumors the ability of GnRH and GnRH-II to stimulate proliferation and invasion is mediated by the GnRHR coupling to the $G\alpha_q$ subunit (18, 19). These cell-type dependent mechanisms underscore the complexity of the mechanism of GnRHR action via multiple $G\alpha$ proteins.

In the extracellular space, the full-length GnRH peptide undergoes cleavage by the zinc metalloendopeptidase EC 3.4.24.15 (EP24.15) between the Tyr⁵-Gly⁶ bond to generate the bioactive pentapeptide, GnRH-(1-5) (20, 21). Our previous studies have shown that the receptor and mechanism of GnRH-(1-5) is distinct from that of its parent peptide both in the brain and periphery (22-27). In the Ishikawa endometrial cell line, GnRH-(1-5) increases GnRH-II expression and the activation of oncogenic pathways to promote cell proliferation with GnRH-(1-5) treatment whereas GnRH has opposing or no effects (18, 27, 28). We have recently demonstrated that the actions of GnRH-(1-5) are mediated by the orphan G protein-coupled receptor 101 (GPR101). GnRH-(1-5) activation of GPR101 results in the release of epidermal growth factor (EGF), transactivation of the epidermal growth factor receptor (EGFR), and cellular migration (27). The EGFR signaling pathway has attracted much attention due to its adverse role in cancer prognosis and outcome (29, 30). Endometrial carcinomas with dysregulated EGFR signaling can lead to aberrant ECM remodeling, which can increase the cancer's invasive and metastatic phenotype (31-35). Thus, the interaction of GnRH-(1-5) with GPR101 to activate EGFR offers a novel pathway with therapeutic potential, which warrants further investigation.

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that are implicated in the promotion of tumor invasion and metastasis. The majority of these studies show that MMPs are involved in the release of growth factors and the digestion of the extracellular matrix (ECM) to promote growth, differentiation, cytoskeletal rearrangement, and cell motility (36, 37). Elevated levels of MMP expression and its activation were also detected in various cancer tissues and cell lines. Furthermore, the inhibition of MMP activity reduced cellular invasion *in vitro* and metastatic progression *in vivo* (38-40). MMPs expressed as inactive zymogen forms (proMMPs) are generally secreted and localized to the cell surface through its interactions with cell surface receptors and basement membrane proteins collagen type IV with the exception of membrane-type MMPs (MT-MMPs), which have transmembrane domains with short cytoplasmic tails. The MMP-mediated digestion of collagen IV and growth factor release are mechanisms implicated in tumor invasiveness and metastatic potential (41-51).

We have previously demonstrated that a global MMP inhibitor blocks the GnRH-(1-5)-induced EGF release and phosphorylation of EGFR indicating that this mechanism is MMP-dependent. In order to implicate GnRH-(1-5) and GPR101 in tumor metastasis, we determined their potential role in cellular invasion. In the current study, we have extended these observations by identifying the MMPs involved in the GPR101-mediated GnRH-(1-5) regulation of cell migration and invasion in two cell models of endometrial cancer, the Ishikawa and ECC-1 cell lines. We focused on type IV collagenases, MMP-2 and MMP-9, known to be involved in tumor growth and metastasis (18, 19, 37, 41, 52-56). MMP-2 and MMP-9 are secreted as inactive proMMP-2 and proMMP-9 and are activated by the extracellular cleavage of the pro-peptide domain from the catalytic domain (36, 57, 58). We present evidence suggesting that GnRH-(1-5) binding to GPR101 leads to increased activation of MMP-9 with a subsequent increase in EGFR phosphorylation, migration, and invasion in both endometrial cancer cell models.

2. Materials and Methods

2.1 Reagents

GnRH-(1-5) was purchased from Bachem (Torrance, CA) and reconstituted to 1mM in distilled water and stored in 20µL aliquots at -80°C. The MMP-2 Inhibitor I (cis-9-Octadecenoyl-N-hydroxylamide, Oleoyl-N-hydroxylamide, OA-Hy) and MMP-9 Inhibitor I (2-(N-benzyl-4-methoxyphenylsulfonamido)-5-((diethylamino)methyl)-N-hydroxy-3-methylbenzamide) were purchased from Millipore (Billerica, MA) and reconstituted to 20mM and 1mM in DMSO, respectively, and stored in 20µL aliquots at -80°C. Another MMP-2 inhibitor, ARP100 (2-(((1,1'-Biphenyl)-4-ylsulfonyl)-(1-methylethoxy)amino]-N-hydroxyacetamide), was purchased from Tocris (Bristol, UK) and reconstituted to 100mM in DMSO and stored in 20µL aliquots at -80°C.

2.2 Cell Culture and Peptide Treatment

To determine whether GnRH-(1-5)-mediated effects is MMP-2 or MMP-9 dependent, inhibitor studies were conducted on well-established and characterized human endometrial cancer cell lines. The Ishikawa (59) cells were obtained from American Type Culture Collection (Manassas, VA) and the ECC-1 cells were obtained from NCI (Dr. John Farley)(60, 61). The range of cell passage numbers is p243-250 and p11-21 respectively. The cells were grown and maintained as previously described (27). In brief, cells were grown in phenol red free-DMEM ([Cellgro-Mediatech, Inc.](#), Manassas, VA) supplemented with 10% FBS (Atlanta Biologicals, Lawrenceville, GA) and 2mM L-Glutamine (Quality Biological Inc., Gaithersburg, MD). Cells were maintained at 37°C with humidified atmosphere of 5% CO₂. At 90-100% confluence, cells were passaged (1:5 ratio) into 10-cm dishes (Costar, Corning, NY).

For each experiment, cells were passaged (1:5 ratio) into 6-well plates (Costar), grown to ~90% confluence, and maintained for 48h in serum-free DMEM before treatment. The effective treatment concentration of GnRH-(1-5) used in this study (100nM) was based on a dose-response curve from our previous studies (24, 27, 28). We have demonstrated that treatment with 100nM GnRH-(1-5) consistently elicited transcription of GnRH-II, increased cell proliferation, upregulated phosphorylation of EGFR, and

enhanced cell migration in the Ishikawa cell line. In the MMP inhibition studies, cells were treated with 100nM GnRH-(1-5) in presence or absence of 200nM-20 μ M MMP-2 Inhibitor I, 1-100nM ARP100, or the 500pM-50nM MMP-9 Inhibitor I (58, 62-70). Inhibitor concentrations utilized for our studies were based on published K_i values in conjunction with a dose-response curve in the Ishikawa and ECC-1 cell line (18, 71-73). Vehicle (VEH) contained less than 0.01% DMSO.

2.3 Western Blot Analysis

Western blot analyses were conducted using previously established methods (27). Cells were lysed at 4°C in lysis buffer, centrifuged and protein concentrations of the supernatant were quantitated using BCA Protein Assay Kit (Pierce Biochemicals (Rockford, IL). Samples were resolved in 4–15% SDS-PAGE Tris-glycine (TG) gels (Biorad, Hercules, CA) and transferred onto Immobilon Polyvinylidene fluoride (PVDF) membranes purchased from Sigma-Aldrich (St. Louis, MO). Blots were blocked for 1 h in 5% non-fat dry milk in 1x Tris-buffered saline(TBS)/0.1% Tween and immunostained overnight in primary antibodies phospho-EGFR Y1068 (1:1000) or total EGFR (1:2000) containing 5% BSA in 1x TBS/0.1% Tween. Subsequently, blots were incubated in goat anti-rabbit IgG (1:3000) horseradish peroxidase-conjugated secondary antibodies in 5% non-fat dry milk/TBST 0.1% for 2 h at room temperature. Immunoblots were visualized using the Immobilon Western Chemiluminescence Kit (Millipore), digitized using LAS-3000 Imager, and quantified with Fuji Multi-Gauge Software (Fujifilm; Stamford, CT). Western blots were normalized to total EGFR and expressed as ratio of treatment relative to VEH. All primary antibodies were purchased from Cell Signaling (Beverly, MA) whereas goat anti-rabbit IgG horseradish peroxidase-conjugated secondary antibodies were purchased from Bio-Rad (Hercules, CA).

2.4 Enzyme-linked immunosorbent Assay (ELISA) for EGF

The EGF concentration in conditioned media was performed as previously described (27). In brief, cells were treated with GnRH-(1-5) with or without the presence of 200nM- 20 μ M MMP-2, 1-

100nM ARP100, or 500pM-50nM MMP-9 inhibitors in DMEM. After 5 minutes, 100μL of media were removed and assessed for EGF using the Human EGF ELISA Kit (Invitrogen) according to manufacturer's instructions. The optical density was detected using a microplate reader at wavelength of 450 nm (Victor X5; PerkinElmer). Concentrations of EGF were reported as pg/mL based on a standard curve provided by the manufacturer. The intra- and inter-assay coefficients of variance were $2.5 \pm 0.3\%$ and 11% (based on five assays), respectively.

2.5 Gelatin Zymography

The effect of GnRH-(1-5) on MMP-9 activity in conditioned medium was determined by zymography (18, 68, 74-76). Serum-starved Ishikawa or ECC-1 cells in 6-well plates were treated with 200μL of serum-free DMEM containing 100nM GnRH-(1-5) with or without the presence of 5nM MMP-9 Inhibitor I. After 5 minutes, the surrounding media was diluted 1:1 with sample buffer (Biorad) and subjected to electrophoresis in 10% gelatin polyacrylamide gels (Biorad) under non-reducing conditions. Proteins within gels were renatured by washing SDS from gels for 30min in 2.5% Triton X-100. Gels were developed in collagenase buffer (50 mM Tris-HCl (pH 7.5), 200mM NaCl, 5mM CaCl₂, and 0.02% Brij-35) for 72 h and stained with 0.5% Coomassie blue R-250. After destaining gels in 30% (v/v) methanol and 10% acetic acid, areas of digestion were digitized using LAS-3000 Imager. Clear bands representing proteolytic activity were quantified with Fuji Multi-Gauge Software.

2.6 In vitro Scratch Wound and Invasion Assay

The *in vitro* scratch wound assay was conducted as previously described to determine if prolonged GnRH-(1-5) treatment had an effect on migration in the Ishikawa and ECC-1 cell lines (27, 77-79). Briefly, cells were grown in 6 well plates (Corning) to 95% confluence and scratched across the cell monolayer. Cells were treated with serum-free DMEM containing GnRH-(1-5) with and without the presence of 5nM MMP-9 Inhibitor I. The scratch wound area at t=0 was demarcated and imaged with an inverted microscope (Leica AF6000, Wetzlar, Germany). The wound area was measured using the Multi

Gauge software (Fujifilm, Valhalla, NY). The calculation for % wound area remaining = $(\text{area}_{t=24 \text{ or } 48\text{h}})/(\text{area}_{t=0\text{h}}) \times 100$.

The invasion assay was conducted using the Matrigel-coated Transwell inserts with 8 μ M pores (BD Biosciences, Palo Alto, CA). The Matrigel coat on the transwell inserts is a mixture of various compounds such as collagen IV, laminin, enactin, and proteoglycan that resemble the constituents in the extracellular matrix. Cells with higher phenotype for invasion can migrate through the porous membrane and traverse throughout the matrix by digestion. In brief, cells were seeded onto inserts at 20% confluence in serum-free DMEM with or without GnRH-(1-5). Co-treatments with 5nM MMP-9 Inhibitor I or transfections with GPR101 antisense or sense oligonucleotides were added directly to the inserts while feeding media was placed into the lower wells as chemo-attractant. After 48-72h, cells that had not traversed through the matrigel matrix were gently washed off and the invading cells on the underside of the membrane were fixed and stained with 0.5% crystal violet in 10% buffered paraformaldehyde. Stained cells in the center of the membrane were imaged at 5x with the Leica AF6000 microscope and counted using Image J (NIH, Bethesda, MD). The calculation for fold change in cell invasion = (# of invading cells in treatments/# of invading cells in VEH) (18, 80, 81).

2.7 GPR101 Knockdown and Quantitative Real-Time PCR (qRT-PCR)

GPR101 knockdown was accomplished using oligonucleotides with a phosphorothioate backbone (Integrated DNA Technologies, Inc, Dallas, TX). The GPR101 antisense oligonucleotides were designed to target sequences around the translational initiation region of human GPR101 mRNA (82). The gene knockdown method, total RNA isolation, and cDNA synthesis has been previously described (27). In brief, cells were grown to 50% confluence and transfected with 200 nmol antisense or sense oligonucleotides using oligofectamine (Invitrogen, Grand Island, NY) as the transfection reagent. After 72h, cells were extracted for total RNA. Primer sequences for GPR101 and Cyclophilin D and sequences for antisense and sense oligonucleotides have been described in our earlier publication (27). The qRT-PCR reactions for each sample were sequentially denatured for 10 s at 95°C, annealed for 30s at 60°C,

and extended for 30s at 72°C for 40 cycles using the CFX Connect Real-Time System (BioRad). Melt curve analysis was performed at the end of run to confirm single amplicon production. Reactions were performed in triplicate and normalized to Cyclophilin D. The Delta Delta Ct ($2^{-\Delta\Delta C_T}$) Method was utilized to determine fold changes between VEH and experimental samples (83). Primer efficiency was determined from slopes of serially diluted cDNA and calculated to be greater than 95%.

2.8 Statistical Analysis

All data presented in this study was obtained at a minimum of 3 independent experiments at different passages of the Ishikawa or ECC-1 cell line. Differences between groups were determined by ANOVA models that included main effects of treatment and passage, followed by *post hoc* comparison using Holm-Sidak test for all pairwise comparisons among treatments after adjusting for passage. Differences at $p < 0.05$ was considered significant. All statistical analyses and graphs were performed using SigmaStat 10.0 and SigmaPlot 10.0, respectively.

3. Results

3.1 The effect of MMP-2 and MMP-9 inhibitors on GnRH-(1-5)-mediated EGFR phosphorylation

We have previously demonstrated that GnRH-(1-5) transactivation of EGFR is MMP dependent (27). In order to determine whether type IV collagenases MMP-2 and MMP-9 is involved the activation of EGFR, lysates from Ishikawa and ECC-1 cells treated with GnRH-(1-5) in presence of MMP-2 or MMP-9 inhibitors were examined using immunoblot analysis. GnRH-(1-5) rapidly increased ($p<0.05$) EGFR phosphorylation in both Ishikawa and ECC-1 cell lines (Figure 1A). The presence of MMP-2 inhibitors, MMP-2 Inhibitor I or ARP100, at varying doses during GnRH-(1-5) treatment did not inhibit GnRH-(1-5) effects on EGFR phosphorylation ($p>0.10$) (Figure 1A, 1B). Conversely, co-treatment of GnRH-(1-5) with MMP-9 Inhibitor I demonstrated reduction ($p<0.05$) in EGFR phosphorylation a dosage-dependent manner (Figure 1C).

Figure 1. Effect of MMP-2 and MMP-9 inhibitors on GnRH-(1-5)-mediated EGFR phosphorylation in Ishikawa (left column) and ECC-1 (right column) cells. Relative levels of phospho-EGFR and EGFR protein expression were determined using Western blot analysis for cells treated for 5 min with 100nM GnRH-(1-5) in presence of MMP-2 and MMP-9 inhibitors. Signal intensity was determined by densitometry and pEGFR was normalized to total EGFR. (A) MMP-2 Inhibitor I (200nM-20µM) did not affect ($p > 0.10$) the GnRH-(1-5) transactivation of EGFR in both cell lines. (B) A second MMP-2 inhibitor, ARP100, also did not affect ($p > 0.10$) the GnRH-(1-5) transactivation of EGFR in both cell lines. (C) MMP-9 Inhibitor I (500pM-50nM) decreased ($p < 0.05$) GnRH-(1-5) transactivation of pEGFR in a dose dependent manner. Each bar shows the mean $\pm$ SEM for each treatment and represent four to six independent experiments. * $p < 0.05$ relative to VEH.

3.2 *GnRH-(1-5) stimulated EGF release is MMP-9 dependent*

The mechanism for GnRH-(1-5) activation of EGFR signaling through EGF release was described previously to be MMP-dependent (27). To further determine whether collagenases MMP-2 or MMP-9 facilitates the release of EGF in both cell lines, conditioned media from cells treated with GnRH-(1-5) in presence of MMP-2 or MMP-9 inhibitors were studied using photometric immunoassay. GnRH-(1-5) treatment increased ($p<0.05$) the release of EGF in the Ishikawa and ECC-1 cell lines (Figure 2). Both the MMP-2 inhibitors, MMP-2 Inhibitor I and ARP100 were unable to deter GnRH-(1-5) elicited EGF release ($p>0.10$) (Figure 2A-B); however, the presence of MMP-9 Inhibitor I during GnRH-(1-5) treatment resulted in reduced ($p<0.05$) EGF release in a dosage-dependent manner (Figure 2C). These results indicate that MMP-9 has a role in facilitating the release of EGF to phosphorylate EGFR.

Figure 2. Effect of MMP-2 and MMP-9 inhibitors on GnRH-(1-5)-mediated EGF release in Ishikawa (left column) and ECC-1 (right column) cells. The release of EGF in media was analyzed using an immunoassay. Treatment with 100nM GnRH-(1-5) for 5 min rapidly stimulated the release of EGF in both Ishikawa and ECC-1 cells. (A) MMP-2 Inhibitor I (200nM-20μM) did not affect ($p>0.10$) the GnRH-(1-5)-mediated EGF release in both cell lines. (B) A second MMP-2 inhibitor, ARP100, likewise had no effect ($p>0.10$) on GnRH-(1-5) stimulated EGF release in both cell lines. (C) The MMP-9 Inhibitor I (500pM-50nM) decreased ($p<0.05$) GnRH-(1-5)-mediated EGF release in a dosage dependent manner in both cell lines. The results are the means $\pm$ SEM of three to four independent experiments. * $p < 0.05$ relative to VEH.

3.3 The effect of GnRH-(1-5) on MMP-9 enzymatic activity

To determine whether GnRH-(1-5) has an effect on MMP-9 enzymatic activity, conditioned media from cells treated with GnRH-(1-5) were analyzed using gelatin zymography. GnRH-(1-5) treatment rapidly increased ($p<0.05$) the proteolytic activity of MMP-9 compared to VEH in both

Ishikawa and ECC-1 cells. This increase in proteolytic activity of MMP-9 was inhibited ($p<0.05$) by MMP-9 Inhibitor I, demonstrating the specificity of MMP-9. MMP-9 Inhibitor I by itself had no effect ($p>0.10$) on MMP-9 activity compared to VEH (Figure 3). These results demonstrate that GnRH-(1-5) enhances the proteolytic activity of MMP-9 in both endometrial cancer cells.

Figure 3. Effect of GnRH-(1-5) on MMP-9 enzymatic activity in Ishikawa (left column) and ECC-1 (right column) cells. Changes in MMP-9 activity in conditioned media of Ishikawa (left column) and ECC-1 cells (right column) were determined using zymography analysis. The clear bands shown in the 10% gelatin polyacrylamide zymogram gels stained with 0.5% Commasie blue R-250 represents areas of digestion due to the proteolytic activity of MMP-9. Treatment with 100nM GnRH-(1-5) for 5 min rapidly increased the proteolytic activity of MMP-9 in both Ishikawa and ECC-1 cell lines. In the presence of 5nM MMP-9 Inhibitor I, the effects of GnRH-(1-5) were reduced whereas MMP-9 Inhibitor I alone had no effect compared to VEH. Bars indicate SEM (n=4). Treatments were compared using ANOVA followed by Holm-Sidak *post hoc* test, adjusting for differences among passages. * $p < 0.05$ relative to VEH.

3.4 The effect of MMP-9 inhibitor on GnRH-(1-5) stimulated increase on cellular migration and invasion

In our previous study, GnRH-(1-5) increased proliferation and migration in the Ishikawa cell line (24, 27). To determine whether MMP-9 has a role in mediating GnRH-(1-5) effects on migration and invasion in the Ishikawa and ECC-1 cell lines, cells were co-incubated with GnRH-(1-5) and MMP-9 Inhibitor I. Treatment with GnRH-(1-5) for 48h increased ($p<0.05$) the migration of cells into the wound area for both cells *in vitro* scratch wound assay (Figure 4 A-B). Co-incubation of GnRH-(1-5) with MMP-9 Inhibitor I reduced this effect (Figure 4 A-B). Similar results were observed in the invasion

studies where treatment with GnRH-(1-5) for 48h demonstrated increased ($p<0.05$) cellular invasion into the matrigel matrix; however co-incubation of GnRH-(1-5) with MMP-9 Inhibitor I diminished ($p<0.05$) this effect (Figure 5 A-B). In both *in vitro* scratch wound and matrigel studies, MMP-9 Inhibitor alone had no effect ($p>0.10$) on migration and invasion compared to VEH in both the Ishikawa and ECC-1 cells. These studies demonstrate that MMP-9 is involved in the pro-migratory and invasion effects of GnRH-(1-5).

Figure 4. Effect of MMP-9 Inhibitor on GnRH-(1-5)-mediated cellular migration in Ishikawa (Figure A) and ECC-1 (Figure B) cells. Migration changes in Ishikawa and ECC-1 cells were determined by using the *in vitro* scratch wound assay. (A) Ishikawa cells treated with 100nM GnRH-(1-5) for 48h increased ($p<0.05$) the migration of cells into the wound area compared to VEH; whereas co-incubation with 5nM MMP-9 Inhibitor I attenuated this effect. (B) Likewise in the ECC-1 cells, increases ($p<0.05$) in migration by GnRH-(1-5) were reduced ($p<0.05$) in presence of MMP-9 Inhibitor I. The representative photomicrographs of each treatment in the wound closure assay are shown in the left panel. The quantification of the wound closure assay is shown in the right panel. The results are the means $\pm$ SEM of four to seven independent experiments. * $p < 0.05$ relative to VEH.

Figure 5. Effect of MMP-9 inhibitor on GnRH-(1-5)-mediated cellular invasion in Ishikawa (Figure A) and ECC-1 (Figure B) cells. The ability of Ishikawa and ECC-1 cells to invade the extracellular matrix was determined using the Matrigel invasion assay. (A) Ishikawa cells treated with GnRH-(1-5) for 48h significantly increased ($p < 0.05$) the number of cells invading into the matrigel matrix; whereas co-incubation with MMP-9 Inhibitor I impeded this effect. (B) Similarly in the ECC-1 cells, the increase in the number of cells that invaded the matrigel matrix by GnRH-(1-5) was inhibited by MMP-9 Inhibitor I. The representative images of each treatment in the wound closure assay (left panel). The quantification of the invasion assay (right panel). The results are the means $\pm$ SEM of four independent experiments ($n=4$). Treatments were compared using ANOVA followed by Holm-Sidak *post hoc* test, adjusting for differences among passages. * $p < 0.05$ relative to VEH.

3.5 *GPR101 Knockdown reverses GnRH-(1-5) effects on MMP-9 proteolytic activity and invasion*

To determine whether GPR101 regulates GnRH-(1-5) effects in the Ishikawa and ECC-1 cell lines, the antisense oligonucleotide approach was utilized in the zymography and invasion studies. In both cell-lines, treatment with GnRH-(1-5) alone or prior transfection with sense oligonucleotide demonstrated an increase ($p<0.05$) in MMP-9 proteolytic activity measured in the conditioned media compared to VEH (Figure 6A). Cells transfected with GPR101 antisense and treated with GnRH-(1-5) showed decreased MMP-9 proteolytic activity ($p<0.05$) compared to cells treated with GnRH-(1-5) (Figure 6A). Transfection with GPR101 antisense or sense oligonucleotide alone exhibited no changes ($p>0.10$) in enzyme activity compared to VEH. RNA extracted from respective cells demonstrated that cells transfected with GPR101 antisense oligonucleotides showed decreases ($p<0.05$) in relative GPR101 mRNA expression compared to VEH (Figure 6B).

For invasion studies, treatment with GnRH-(1-5) in both cell lines increased ($p<0.05$) invasion into the matrigel compared to VEH, whereas knockdown of GPR101 reduced these effects (Figure 6C). Transfection of the Ishikawa and ECC-1 cells with sense oligonucleotides and subsequent treatment with GnRH-(1-5) demonstrated similar increases ($p<0.05$) in invasion into the matrigel matrix. Transfection with GPR101 antisense and sense alone had no effect ($p>0.10$) on invasion compared to VEH.

Figure 6. Effect of down-regulating GPR101 expression on the GnRH-(1-5)-mediated MMP-9 activation and cellular invasion. Zymography and invasion experiments performed earlier in this study were repeated in Ishikawa cells with GPR101 knockdown using the antisense oligonucleotide approach. (A) To determine MMP-9 enzymatic activity, Ishikawa cells were transfected with antisense or sense oligonucleotides for 72h then treated with DMEM containing 100nM GnRH-(1-5) for 5min. Conditioned media was removed for zymogram analysis. Treatment with GnRH-(1-5) alone (lane 2) or transfection with sense oligonucleotide prior to treatment with GnRH-(1-5) (lane 4) increased ($p<0.05$) the presence of active MMP-9 in the surrounding media compared to VEH (lane 1). Cells transfected with GPR101 antisense and treated with GnRH-(1-5) (lane 3) showed no changes ($p>0.10$) in MMP-9 presence and activation in media compared to VEH. (B) qRT-PCR analysis of respective cells transfected with GPR101 antisense oligonucleotides demonstrated a decrease in GPR101 mRNA expression; however, GPR101 sense oligonucleotides exhibited no effect on GPR101 mRNA expression. (C) Invasion studies utilizing the matrigel-coated transwell inserts demonstrated that cells treated with 100nM GnRH-(1-5) for 72h in serum-free DMEM increased ($p<0.05$) cellular invasion into the matrigel compared to VEH. Similar increase ($p<0.05$) in invasion was also exhibited by cells transfected with sense oligonucleotide and treated with GnRH-(1-5), whereas transfection with GPR101 antisense decreased ($p<0.05$) GnRH-(1-5) effects. GPR101 antisense and sense alone had no effect ($p>0.10$) in invasion compared to VEH. Bars indicate SEM ($n=3$). * $p < 0.05$ relative to VEH.

4. Discussion

We have previously shown that GnRH-(1-5) enhances cell migration in Ishikawa cells by transactivating EGFR. This mechanism required GnRH-(1-5) binding to GPR101 to initiate the release of EGF, which was contingent upon MMP activation (27). In the present study, we further demonstrate that GnRH-(1-5) transactivation of EGFR results in cellular invasion in a GPR101-dependent via the activation of MMP-9 in Ishikawa and ECC-1 endometrial cancer cell lines. These cancer cell lines are established models of stage 2 endometrial adenocarcinomas with distinct genetic pathologies. Our results demonstrate that the mechanism of GnRH-(1-5) binding GPR101 and subsequent MMP-9 activation is conserved across our endometrial cancer cell models.

MMP-2 and MMP-9 are a class of zinc-dependent peptidases known for its ability to digest the ECM and cleave growth factor precursors. The release of these growth factors results in the activation of receptors implicated in promoting cell proliferation, migration, and invasion. To determine whether GnRH-(1-5) transactivation of EGFR is MMP-2 and MMP-9 dependent, we measured EGF release and EGFR phosphorylation in the presence of well-established MMP-2 and MMP-9 inhibitors (62-66, 86-88). In both Ishikawa and ECC-1 cell lines, GnRH-(1-5)-induced EGF release and EGFR phosphorylation was specifically blocked by MMP-9 Inhibitor I in a dose-dependent manner. Varying doses of MMP-2 Inhibitor I, further verified by another MMP-2 inhibitor, ARP100, did not alter the effects of GnRH-(1-5) in either cell line. These results suggest that GnRH-(1-5)-mediated stimulation of EGFR phosphorylation is dependent on MMP-9 and not MMP-2. Although our studies focused primarily on the release of EGF, future experiments should examine the release of other growth factors such as TGF- α and TGF- β . TGF- α is a growth factor known to activate EGFR signaling while TGF- β isoforms can also stimulate signaling pathways associated with increased invasiveness in certain cancer types. Growth factor-dependent increases in MMP-9 expression and activity have been correlated to tumor growth and metastasis (41, 70). The down-regulation of MMP-9 expression in various types of tumor cells resulted in cell cycle arrest, increased apoptosis, and the inhibition of cell proliferation (89-92). Furthermore, treatment of

KLE endometrial cancer cells with isoform TGF- β 3 resulted in PKC-dependent transient increase in MMP-9 mRNA levels, resulting in increased invasiveness (70).

In our initial studies with the Ishikawa cell line, we determined that GnRH-(1-5) had divergent effects on cell proliferation and migration from its parent peptide and addressed its physiological relevance in cancer (27, 93, 94). In the present study, we implicate GPR101 in mediating GnRH-(1-5) effects through the activation of MMP-9. Down-regulation of GPR101 expression using the GPR101 antisense oligonucleotides with subsequent treatment with GnRH-(1-5) blocked MMP-9 activation and cell invasion. These results suggest that GPR101 has a role in the activation of MMP-9 to promote invasion. Studies have shown that GPCRs can activate MMPs to enhance secretion and increase cell migration; however, associating GPCRs and G-proteins to distinct members of the MMP family vary by cell-type (95). For instance, GnRHR transactivation of EGFR in α T3-1 pituitary cells is facilitated by $G\alpha_q$ -mediated MMP-2 and MMP-9 activation (96). In contrast, EGFR transactivation by luteinizing hormone receptor (LHR) in ovarian follicles is regulated by $G\alpha_s$ isoform to activate MMP-2 and MMP-9 activation (67). Although a G protein subunit coupled to GPR101 has yet to be identified, our previous study with a G protein antagonist 2 (GPAnt-2) demonstrates that GnRH-(1-5) stimulation of GPR101 to release EGF, phosphorylate EGFR, and increase migration is G protein-dependent. Our future studies will identify the G protein complex coupling to GPR101 to further elucidate the mechanism of MMP-9 recruitment and activation by GnRH-(1-5).

Our previous study demonstrated that GnRH-(1-5) increased the migration of Ishikawa cells via GPR101 using an *in vitro* scratch assay (27). Here we also show that GnRH-(1-5) transactivation of EGFR increases wound closure in a second model of endometrial cancer, the ECC-1 cell line. EGF and its receptor have been extensively studied to show that they can play a prominent role in both the migratory and mitogenic potentials of many cancers (84, 85). In our studies we have previously demonstrated that GnRH-(1-5) can also stimulate the proliferation of Ishikawa cells (24); and thus, it is unclear whether the effects of GnRH-(1-5) are due to cell division or migration using an *in vitro* scratch assay. In addition, the proliferative and migratory properties of cancer cells in their native environment can drastically differ

due to presence of ECM proteins and factors not present in an *in vitro* scratch assay. Therefore, in these studies we implemented an invasion assay to examine the specific invasive migratory phenotypes of both endometrial cancer cell models in response to GnRH-(1-5). This migratory assay consists of porous transwells coated with a gelatinous mixture composed of collagen IV, laminin, enactin, and proteoglycans. The presence of these proteins reflects the composition of the ECM environment where cancer cells reside. Furthermore, the synthetic ECM acts as a barrier in which cells displaying a more invasive phenotype will digest the matrix to migrate through the pores. In the current study, we demonstrate that GnRH-(1-5) increases the invasive potential in both Ishikawa and ECC-1 cells. The GnRH-(1-5)-mediated increase in cell invasion was also dependent on GPR101 and MMP-9 activation. This observation corresponds with our zymogram studies demonstrating that GnRH-(1-5) increases MMP-9 activity in a GPR101-dependent mechanism. Collectively, these data indicate that GnRH-(1-5) can contribute to the regulation of ECM remodeling via an autocrine mechanism in tissues under GnRH regulation. Physiologically, this mechanism may play a role in the development of gynecologic tissues or in female reproductive cycle but this remains to be determined. Under a pathogenic state, our current findings and previous work strongly suggest that GnRH-(1-5) likely stimulates both mitogenic and migratory pathways via GPR101 increasing MMP-9 activity. This cascade subsequently enhances the invasive and metastatic potential of tumor cells. Further investigation is necessary to delineate the mechanism of GnRH-(1-5) to increase the pathogenicity of endometrial cancer in regards to cell migration and invasion in other cancer models using an animal approach. Pharmacologic manipulation of GPR101 and MMP-9 may offer novel insights into potential therapeutics to treat endometrial cancer.

Proteolytic cleavage of GnRH to generate GnRH-(1-5) has been observed in central and peripheral tissues (93, 102, 103). A kinetic study using ovarian tumor tissues demonstrated that GnRH treatment can enhance its own processing; and that the GnRH-(1-5) half-life is longer than that of its parent (28, 93). These results indicate that the metabolite GnRH-(1-5) may achieve optimal levels to indeed induce an autocrine and/or paracrine effect in tissues under GnRH regulation. In the case for our endometrial cancer cell models, we have previously demonstrated that Ishikawa cells also express GnRH

and the endopeptidase, EP24.15, the enzyme responsible for cleaving GnRH to GnRH-(1-5) (28). Furthermore, in agreement with previous studies, Ishikawa cells treated with GnRH stimulated its own expression, suggesting a positive feedback mechanism to potentially drive the formation of GnRH-(1-5) (28, 93). While the endogenous levels of GnRH-(1-5) in our cell models are unknown, our previous dose and time response studies demonstrated that 1 nM GnRH-(1-5) was sufficient to induce an effect (28, unpublished data). In the current study, 100 nM treatments were used as it gave us the most consistent responses across multiple cell lines of various origins (25, 27). This dose has also been widely reported in studies investigating the mechanism of GnRH (or GnRH-like peptides) (14, 18). Lastly, alterations in the expression and activity of the GnRH-cleaving enzyme, EP24.15, may promote the synthesis of GnRH-(1-5) as seen in prostate cancer (97); and in our lab using various endometrial cancer cell lines representative of different stages (unpublished data). Furthermore, EP24.15 cleavage of GnRH to GnRH-(1-5) is enhanced 10-fold upon prolyl endopeptidase-mediated removal of the 10th amino acid (104). Therefore, EP24.15 changes in expression and/or activity along with its ability to associate with lipid rafts, which serve to centralize signaling complexes, are efficient mechanisms to potentially regulate peptide/receptor interactions (105). In our model, EP24.15 in close proximity to GPR101 and GnRH may facilitate the synthesis of GnRH-(1-5) so that the local concentration of this metabolite reaches optimal levels to induce an effect. Further investigation is warranted into kinetics of GnRH processing in endometrial cancer.

In this study, we demonstrate utilizing different endometrial cancer cell lines that the metabolite GnRH-(1-5) increases MMP-9 activity by binding to GPR101. Of the collagenases examined, dysregulated MMP-9 activity and not MMP-2 is mostly associated with increased invasion and advanced stages in endometrial cancer (98-101). In agreement, our findings that show that GnRH-(1-5) activating GPR101 increases MMP-9 activity to increase cellular migration and invasion in both Ishikawa and ECC-1 cells. The increase in MMP-9 activity resulted in rapid EGF release, EGFR phosphorylation, and the subsequent promotion of cell invasion. Future studies will entail investigating whether GnRH-(1-5) binding GPR101 regulates the release of other growth factors and whether MMP-9 is also involved. MMP-9 can exert direct effects on cell motility, migration, and invasion through cytoskeletal

440 rearrangement and the digestion of basement membrane. Furthermore, MMP-9 recruitment and
441 activation in cancer cells can lead ECM remodeling, which also contributes to increased cellular invasion.
442 The additive effects in the dysregulation of EGFR, MMP-9, and other factors increases the aggressiveness
443 of gynecologic cancers, such as those found in endometrial cancer. In conclusion, our results indicate that
444 GnRH-(1-5) exerts distinct mechanistic and physiological effects on endometrial cancer cells compared to
445 its parent peptide. These studies highlight the complexities of GnRH signaling in peripheral tissues.

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Gonadotrophin Releasing Hormone
Metabolite GnRH-(1-5)

pGlu-His-Trp-Ser-Tyr

MMP-9 Inhibitor I

Antisense