Silencing of ghrelin receptor expression inhibits endometrial cancer cell growth in vitro and in vivo

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Am J Physiol Endocrinol Metab 305: E305–E313, 2013. First published June 4, 2013; doi:10.1152/ajpendo.00156.2013.—Ghrelin is a 28-amino-acid peptide hormone produced predominantly in the stomach but also in a range of normal cell types and tumors, where it has endocrine, paracrine, and autocrine roles. Previously, we have demonstrated that ghrelin has proliferative and antiapoptotic effects in endometrial cancer cell lines, suggesting a potential role in promoting tumor growth. In the present study, we investigated the effect of ghrelin receptor, GHSR, and gene silencing in vitro and in vivo and characterized ghrelin and GHSR1a protein expression in human endometrial tumors. GHSR gene silencing was achieved in the Ishikawa and KEI endometrial cancer cell lines, using a lentiviral short-hairpin RNA targeting GHSR. The effects of GHSR1a knockdown were further analyzed in vivo using the Ishikawa cell line in a NOD/SCID xenograft model. Cell proliferation was reduced in cultured GHSR1a knockdown Ishikawa and KEI cells compared with scrambled control tumors. Using immunohistochemistry, we demonstrated that ghrelin and GHSR1a are expressed in benign and cancerous glands in human endometrial tissue specimens, although there was no correlation between the intensity of staining and cancer grade. These data indicate that downregulation of GHSR expression significantly inhibits endometrial cancer cell line and mouse xenograft tumour growth. This is the first preclinical evidence that downregulation of GHSR may be therapeutic in endometrial cancer.

Ghrelin; growth hormone secretagogue receptor; endometrial cancer; mouse xenograft tumours; constitutive activity

Ghrelin is a 28-amino-acid peptide hormone that is synthesized primarily by endocrine X/A-like cells of the gastric mucosa (32) and is expressed in many other tissues and cell types (10). Previously, our group demonstrated that ghrelin and the cognate ghrelin receptor, the growth hormone secretagogue receptor (GHSR), are expressed in the human endometrium during the menstrual cycle (43), suggesting that they are involved in the extensive cyclical growth and development that occurs in endometrial tissues (42, 43). In addition to regulating normal growth and differentiation, ghrelin has been implicated in the maintenance and progression of several human cancers (10). Ghrelin and/or GHSR expression has been demonstrated in a number of hormone-dependent cancers (9, 10), including breast (29), testicular (20), ovarian (21), and prostate cancer (28, 46), suggesting a possible autocrine and/or paracrine role for ghrelin in cancer cell growth.

Endometrial carcinoma is a hormone-dependent malignancy. Tumors that are more advanced at diagnosis are associated with high levels of morbidity and mortality (2). Despite the fact that endometrial cancer is a common gynecological malignancy, molecular therapy remains largely unexplored and undeveloped (30). Previously, we demonstrated that ghrelin and GHSR1a mRNA are expressed in a range of endometrial cancer tissues and that ghrelin potently stimulates cell proliferation and inhibits apoptosis in endometrial cancer cell lines (19). These data suggest that ghrelin promotes the progression of endometrial cancer and may contribute to endometrial cancer pathogenesis.

The importance of GHSR1a in cancer cell progression remains unclear due to conflicting results and methodological differences between studies (10), and its role may be cell type specific. Studies in human pancreatic cancer cell lines (15), astrocytoma (14), and glioblastoma (8) suggest that GHSR1a is involved in cancer cell proliferation and/or cell migration. In contrast, GHSR1a may not be required for all ghrelin functions in adrenocortical tumors (4) and prostate cancer cell lines (6), suggesting the possibility of additional ghrelin receptor(s) in these cancers.

The unacylated form of ghrelin, desacyl or unacylated ghrelin (UAG), was originally thought to be an inactive form of ghrelin, since it does not activate GHSR1a. Recently, an increasing number of studies have reported that UAG has a range of biological effects, including cardiovascular actions (3), effects of food intake (45), and proliferative and antiapoptotic actions in normal and cancer cells (6, 18). Thus, it is highly likely that UAG signals through an alternative ghrelin receptor, which is distinct from the GHSR1a.

The GHSR1a has a very high level of constitutive activity and is active (≤50% activity) in the absence of the ligand acylated ghrelin (24). However, its effect on endometrial cancer cells is not known. The aim of this study was to further elucidate the effects of GHSR1a in endometrial cancer cell progression using short-hairpin (sh)RNA-mediated GHSR1a gene silencing in cell culture and in a mouse xenograft model.

MATERIALS AND METHODS

Bioinformatics. The ghrelin (GHR1) and GHSR genes and the well-established endometrial cancer oncogenes MYC and PIK3CA...
(12) were analyzed for gene amplification events using the eBio Cancer Genomics Portal (7). The Cancer Genome Atlas data set titled Uterine Corpus Endometrioid Carcinoma was interrogated.

**Endometrial tissue microarray.** Human endometrial cancer tissue microarrays were purchased from Biochain (Hayward, CA). This array consisted of pathologist-verified tissue samples from five normal endometrial tissues and 70 different endometrial tumours with grading data (tissues in duplicates, with 150 1.1-mm cores, 75 cases, and fixed in formalin fixation). Ages of tissue donors ranged from 30 to 78 yr.

**Cell culture.** The well-differentiated Ishikawa endometrial cancer cell line was obtained from Sigma-Aldrich (St. Louis, MO), and the poorly differentiated KLE endometrial cancer cell line was obtained from American Type Culture Collection (Rockville, MD), and they were cultivated in Dulbecco’s modified Eagle’s medium (DMEM): nutrient mixture F-12 (DMEM-F-12 medium (Sigma-Aldrich) containing 10% fetal bovine serum (FBS; Thermo Scientific HyClone), 50 U/ml penicillin, and 50 µg/ml streptomycin (Life Technologies, Carlsbad, CA). The human embryonic kidney (HEK)-293T cell line was a gift from Dr. Wengyi Gu (AIBN, Brisbane, Australia) and was maintained in DMEM, 10% FBS, 50 U/ml penicillin, and 50 µg/ml streptomycin. All cell lines were grown in a humidified atmosphere containing 5% CO₂ at 37°C and were tested to be free from Mycoplasma contamination.

**Quantitative real-time RT-PCR.** Total RNA was isolated from cell lines and xenografts using a TRizol Plus RNA purification kit (Life Technologies). The final concentration of RNA was determined spectrophotometrically using NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE), and the RNA quality was evaluated by calculating the ratio of optical density (260:280 nm). Total RNA (1 µg) was reverse transcribed in a 20-µl reaction, using an iScript cDNA synthesis kit (Bio-Rad Laboratories) according to the manufacturer’s instructions. Negative controls were prepared by omitting reverse transcriptase (no-RT control), or by the substitution of template by water.

Real-time RT-PCR for endometrial cancer cell lines was carried out using Applied Biosystems 2× SYBR green master mix (Life Technologies), using the Applied Biosystems StepOne Real-Time PCR System (Life Technologies) according to the manufacturer’s instructions. **GHSR1a** real-time RT-PCRs were performed using sense (5’-AGTGGGTTTGGGTCTCATCC-3’) and antisense primers (5’-TGATGGCAGCACTGAGGTAG-3’), as described previously (19). Ghrelin-O-acyltransferase (GOAT) real-time RT-PCR was performed using commercial GOAT (MBOAT4) gene primers from SABiosciences (Qiagen). The experiments were repeated three times, and each experiment was performed in triplicate and normalized against the reference primer set (18S ribosomal RNA): sense 5’-TCGGAACTGAGCCATGAT-3’ and antisense 5’-CGAACCTCCTCAGTTGCT-3’. Compared with scramble control Ishikawa or KLE endometrial cancer cells, the normalized fold change of mRNA expression was expressed as 2^ΔΔC_T, where ΔΔC_T = ΔC_T sample − ΔC_T control (37).

**Western immunoblotting.** Cultured cells (1 × 10⁷) were harvested and extracted in 500 µl of lysis buffer (150 mM sodium chloride, 50 mM Tris-HCl, 1% nonyl phenoxypolyethoxylethanol, and 1× complete protease inhibitor tablet; Roche Diagnostics) and phosphatase inhibitors (10 mM sodium fluoride, 1 mM sodium orthovanadate, and 10 mM tetrasodium pyrophosphate) and cleared by 12,000 g centrifugation at 4°C. Total protein concentration was measured using a BCA assay kit (Thermo Fisher Scientific, Rockford, IL), and absorbance was measured at 560 nm (Spectra Rainbow spectrophotometer, X-Read Plus Version 4.3; Tecan, Crailsheim, Germany). Cultured cell lysates (30 µg) were denatured at 70°C for 10 min in 2× sample buffer (250 mM Tris-Cl, pH 6.8, 2% sodium dodecyl sulfate, 10% glycerol, 20 mM dithiothreitol, and 0.01% bromophenol blue). Proteins were separated by 10% SDS-PAGE and electrottransferred onto nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany). The membranes were blocked for 1 h with 2.5% skim milk powder-Tris-buffered saline-Tween 20 (2.5% SMP-TBS-T) prior to incubation with a polyclonal goat anti-human GHSR1a primary antibody (1:1,000 in 2.5% SMP-TBS-T; Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. Membranes were washed 3 × 10 min in TBS-T and incubated for 1 h at room temperature with donkey anti-goat horseradish peroxidase (HRP) secondary antibody (1:2,000; Jackson ImmunoResearch, West Grove, PA) in 2.5% SMP-TBS-T, followed by detection with enhanced chemiluminescence (Thermo Fisher Scientific) according to the manufacturer’s instructions. After Western blotting for GHSR1a, membranes were stripped and reprobed with the β-tubulin loading control antibody (1:500 in 2.5% SMP-TBS-T; Abcam, Cambridge, UK) to ensure equal protein loading and to allow semiquantitative densitometry analysis on scanned films using Image J software (1).

**Immunohistochemistry.** Samples for immunohistochemistry were sectioned at 5 µm and deparaffinized in xylene, followed by dehydration in a series of graded alcohols. Antigen retrieval was performed in 10 mM citrate buffer (pH 6.0) heated to 96°C for 20 min (microwave heating at 750 W). Endogenous peroxidase activity was quenched using 3% hydrogen peroxide in PBS for 10 min. Blocking was performed by incubating sections in 10% milk diluent/ blocking solution (KPL, Gaithersburg, MD) for 20 min. Sections were incubated with the following primary antibodies: rabbit polyclonal anti-ghrerin (Phoenix), goat polyclonal anti-GHSR1a (Santa Cruz Biotechnology), or rabbit polyclonal anti-Ki67 (Abcam) at 4°C overnight. The sections were then stained using HRP goat or rabbit polymer conjugates (Biocare Medical, Concord, CA) for 15 min and incubated with the chromogen diaminobenzidine (DAB) for 5 min. All immunostained sections were lightly counterstained with hematoxylin.

**CyQUANT NF cell proliferation assay.** Cells were seeded in 96-well plates at a density of 2 × 10⁴ cells/well and cultured in the presence or absence of human n-Octanoylated (acylated) ghrelin (ProSpec; Tany TechnoGene, Rehovot, Israel) and unacylated ghrelin (Mimotopes, Victoria, Australia) over a range of concentrations between 10 and 1,000 nM. Medium was replaced every 24 h for both treated and untreated cells. After 4, 24, 48, or 72 h, cells were incubated with the CyQUANT NF Cell Proliferation assay reagent (Life Technologies) according to the manufacturer’s instructions. This assay measures cellular DNA content as a direct index of cell proliferation. After treatment, culture medium was removed, and a stock solution of the green fluorescent CyQUANT GR dye (prepared according to the manufacturer’s instructions) was added. Upon binding to DNA, the GR dye shows a measurable enhancement in the intensity of fluorescence. Cells were returned to the incubator (37°C) for 2 h, resulting in maximal and stable changes in fluorescence. Fluorescence was measured using an Envision Multilabel reader (excitation 480 nm, emission 530 nm; Perkin-Elmer, Waltham, MA) and expressed as percentage relative to untreated control. Each experiment was performed with six replicates, and the experiments were repeated three times.

**MTT cell viability assay.** Cells were seeded in 96-well plates at a density of 2 × 10⁴ cells/well and cultured in the presence or absence of the potent GHSR inverse agonist (D-Arg¹, D-Phe⁵, D-Trp⁷,⁹, Leu¹¹) substance P (Tocris Bioscience) (25) over a range of concentrations between 0.05 and 50 µM. Medium was replaced every 24 h for both treated and untreated cells. After 72 h, cells were incubated with MTT according to the manufacturer’s instructions. The optical density of samples was quantified by measuring absorbance at 490 nm (Spectra Rainbow Spectrophotometer, X-Read Plus Version 4.3) and expressed as percentage relative to untreated control. Each experiment provided 16 replicates per treatment, and the experiments were repeated a minimum of three times.

**Lentiviral silencing of GHSR1a in endometrial cancer cell lines** shRNA design and vector preparation. shRNAs specifically targeting human GHSR1a were designed to knockdown GHSR1a expression based on a published human GHSR1a sequence (NM_198407) and using a computer program (shRNA Explorer; Gene Link, Hawthorne, NY). One scrambled control was also designed to discount any changes in gene
profile via lentiviral delivery methods. The shRNA expression cassette contained 19 nucleotides (nt) of the target sequence, followed by the loop sequence (TTCAAGAGA), reverse complement to the 19 nt, stop codon for the U6 promoter, and an XhoI site. The shRNA sequences are GHSR1a (sense: 5’-TGAGAAGCTCTCCATCTGGAAAGAGAGCAGCCGTTACTAGTTTTTTCTCCTCGTCGGCAATGATCAAAAAAAAGAGCT-3’; antisense: 5’-ACTCTTTTGCAGACTGAGCAAACTGTCTGCTCTCTCTCTGAAAGAAAAAGACGT-3’), and scrambled (sense: 5’-TAACTAGTACCGGTACGCTTCAGAGGAGGACCGGCGTTTAAGTTTTTTTCC-3’; anti-sense: 5’-ATTGATCTATTGCAGGAGGAAAGTTCTCTCCTCTGCGAAATGATCAAAAAAGACGT-3’). The shRNA cassettes and their complementary sequences were synthesized commercially (Sigma-Aldrich) and annealed in buffer (100 mM K-acetate, 30 mM HEPES-KOH, pH 7.4, 2 mM Mg-acetate) by heating to 95°C for 5 min, followed by cooling to room temperature. The resulting double-stranded oligo DNA was cloned into plasmid pLentLox3.7 (pL3.7; a gift from Dr. Wengyi Gu; AIBN) at the HapI and XhoI sites.

The packaging plasmid and the pL3.7 were amplified in Escherichia coli and purified using W/Endo-free Invitrogen Maxi-Prep Kits (Life Technologies) according to the manufacturer’s instructions. The HEK-293T packaging cell line was transfected with pL3.7 (plus scrambled shRNA control or GHSR1a shRNA) and the lentiviral packaging plasmids pRSVRev, pMD.G (contains VSV.G gene), and pMD.LgpRRE (gifts from Dr. Wengyi Gu; AIBN). The next day, the medium was replaced with 10 ml of fresh DMEM (Life Technologies), and 48 h later, supernatant was collected and filtered (pore size 0.45 μm, Lutrol-F70, B.Braun Melsungen AG, Germany) by ultracentrifugation at 22,000 × g for 90 min, followed by cooling to room temperature. The resulting supernatant was concentrated 40–50 times using Vivaspin 220 ml concentrators (100 kDa MW; Sartorius, Goettingen, Germany) by heating to 80°C for RNA extraction. Tissue samples were also harvested for histological analysis, fixed in formalin, and embedded in paraffin wax. Sections (5 μm) were cut and immunostained for quantification of nuclei expressing the proliferation marker Ki67 (Abcam), as described above. Ki67-positive cells were counted in a minimum of 10 random fields with the highest density of staining (×400 magnification), and data were expressed as a proliferation index (Ki67-positive cells/total cells in field × 100). All slides were counted by an investigator blinded to the xenograft tumor groups.

Injection of target cell lines. Ishikawa and KLE cells were seeded into six-well plates (1 × 105 cells/well) and cultured overnight. Lentiviral stocks (GHSR1a shRNA lentivirus vector or scramble control shRNA vector) were diluted with DMEM-F-12 medium containing hexadimethrine bromide (final concentration 8 μg/ml) and added to the cells for incubation for 24 h at 37°C. After a 24-h infection, the medium was replaced with 10 ml of fresh DMEM-F-12 (Life Technologies) for another 48 h. Then cells were harvested for stably transfected cell line selection, and cells expressing green fluorescence were identified using a fluorescence microscope.

Fluorescence-activated cell sorting analysis. After successful transduction with eGFP-tagged vectors was confirmed, cells were trypsinized, collected in 15-ml tubes, and washed three times with phosphate-buffered saline (PBS) before being resuspended in a final volume of 500 μl PBS. Cells were sorted using an InFlux Cell Sorter (BD Biosciences, Franklin Lakes, NJ). All detectors were housed in a class II certified laminar flow cabinet. GFP-positive cells with GFP intensity >10^6 on a log scale were collected in 1-ml tubes containing 10 ml of complete DMEM-F-12 (10% FBS, 50 U/ml penicillin, and 50 μg/ml streptomycin) and pelleted by centrifugation at 1,500 g for 10 min. Cells were then resuspended in tissue culture medium and counted using a haemocytometer before being seeded into a tissue culture plate. Real-time RT-PCR and Western blotting were performed, as described previously (19), to determine the mRNA and protein levels of GHSR1a in Ishikawa cells transduced with GHSR1a-shRNA or scrambled shRNA and parental cell groups.

**Tumor xenografts.** Female 6-wk-old nonobese diabetes/severe combined immunodeficiency (NOD/SCID) mice (Monash University, Melbourne, Australia) were housed under specific pathogen-free conditions with a 12:12-h light-dark schedule and fed autoclaved standard chow and water ad libitum. Ishikawa cells infected with GHSR1a-shRNA, or scrambled-shRNA, were harvested from the culture flask with 0.25% trypsin and resuspended in 200 μl of growth factor-reduced Matrigel/PBS (1:1 vol/vol; Sigma-Aldrich). Ishikawa cells (1 × 10^7) stably infected with a lentivirus vector carrying GHSR1a-shRNA or scrambled control were injected subcutaneously into the left flank of mice (n = 6–7/group). Tumor volume was measured with digital calipers and calculated with the formula (width)^2 × length/2 and expressed as mm^3. All experiments were performed in accordance with the institutional and the National Health and Medical Research Council guidelines for the welfare of animals in experiments. The University of Queensland Institutional Animal Care and Use Committee reviewed the animal care experiments and gave full ethical approval.

Animals were euthanized at 5 wk postinoculation, and tumors were dissected. Tumor weight was measured, and tissue samples were snap-frozen at −80°C for RNA extraction. Tissue samples were also harvested for histological analysis, fixed in formalin, and embedded in paraffin wax. Sections (5 μm) were cut and immunostained for quantitation of nuclei expressing the proliferation marker Ki67 (Abcam), as described above. Ki67-positive cells were counted in a minimum of 10 random fields with the highest density of staining (×400 magnification), and data were expressed as a proliferation index (Ki67-positive cells/total cells in field × 100). All slides were counted by an investigator blinded to the xenograft tumor groups.

**Statistical analyses.** Data are expressed as means ± SE. Statistical significance was determined using one-way analysis of variance with Tukey’s post hoc analysis. Unpaired two-tailed Student t-tests were used for comparisons between two groups. A P value of <0.05 was considered to be statistically significant. Analyses were conducted using GraphPad Prism version 5 software (GraphPad Software, La Jolla, CA).

**RESULTS**

The expression of ghrelin and GHSR1a protein in normal human endometrial and endometrial cancer tissues. Using the cBio Cancer Genomics Portal (7), we found that GHSR is frequently amplified in high-grade serous (Fallopian tube-like) tumors but not in low-grade endometrioid tumors that make up

Fig. 1. OncoPrint showing the distribution of genomic alterations in endometrial cancer. A: growth hormone secretagoge receptor (GHSR) is amplified in 19/60 serous tumors. B: GHSR is not amplified in endometrioid tumors. The OncoPrint provides an overview of genomic alterations (legend) in particular genes (rows) affecting individual samples (columns). The endometrial cancer oncogenes Myc, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit-α (PIK3CA), and ghrelin (GHR1) are also shown.

**A**

<table>
<thead>
<tr>
<th>Gene</th>
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<td>GHSR</td>
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<tr>
<td>GHRL</td>
<td>0%</td>
</tr>
<tr>
<td>MYC</td>
<td>23%</td>
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<tr>
<td>PIK3CA</td>
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60 serous tumours

**B**

<table>
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<th>Gene</th>
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<tr>
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</tr>
<tr>
<td>GHRL</td>
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<td>3%</td>
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<tr>
<td>PIK3CA</td>
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90 endometrioid tumours

*Downloaded from http://ajpendo.physiology.org/ by 10.220.224.7 on August 28, 2017*
85% of endometrial carcinoma cases (Fig. 1) (35). For immunohistochemical detection of ghrelin and GHSR1α expression, we employed a tissue microarray with 70 endometrial adenocarcinoma tissues and five healthy endometrium controls. Positive immunohistochemical staining for ghrelin (Fig. 2) and the GHSR isoform GHSR1α (Fig. 3) was present in both the cytoplasmic stromal and glandular epithelial cells in all of the tissue samples; however, there was no correlation between the intensity of ghrelin or GHSR1α immunostaining and tumor grades.

Knockdown of GHSR1α inhibited cell proliferation in vitro. Lentivirus-mediated RNA interference was used to downregulate GHSR1α expression in Ishikawa and KLE cells. These experiments demonstrated that GHSR1α-shRNA transduction significantly reduced expression of GHSR1α mRNA by 60–70% (Fig. 4, A and B) and protein by 70–80% compared with parental Ishikawa and KLE cells and the scrambled control groups (Fig. 4, C–F). The basal cell proliferation rate for GHSR1α-shRNA and scrambled shRNA-transduced Ishikawa and KLE cells was monitored for 3 days. In the absence of exogenous ghrelin treatment, Ishikawa cells transduced with GHSR1α-shRNA showed 25.3 ± 3.2% less cell proliferation in vitro over 72 h compared with the scrambled controls (P < 0.05; Fig. 5 A). Similarly, the growth of KLE cells transduced with GHSR1α-shRNA was markedly inhibited by 36.7 ± 3.1% compared with the scrambled controls (P < 0.05; Fig. 5 B).

To elucidate the importance of GHSR1α in ghrelin-stimulated cell proliferation in endometrial cancer cells, Ishikawa and KLE cells transduced with either GHSR1α-shRNA or scrambled shRNA were cultured in vitro and exposed to a range of concentrations of exogenous acylated ghrelin (10–1,000 nM) for 72 h, and cell proliferation was examined using a CyQUANT NF cell proliferation assay. A significant increase in cell proliferation was observed after ghrelin treatment in both Ishikawa and KLE cell lines transduced with scrambled shRNA. Ghrelin (1,000 nM) increased cell proliferation significantly by 13.7% in the Ishikawa cell line (0 nM ghrelin: 100 ± 0.8% of control; 1,000 nM ghrelin: 113.7 ± 4.3% of control; P < 0.05; Fig. 5 C) and by 13.3% in the KLE endometrial cancer cell line (0 nM ghrelin: 100 ± 0.1% of control, 1,000 nM ghrelin: 113.3 ± 2.4% of control; P < 0.001; Fig. 5 D) compared with no ghrelin treatment (0 nM) in scrambled
controls. There was no increase in proliferation in GHSR1a-shRNA-transduced cells regardless of acylated ghrelin dose and time point (Fig. 5, C and D).

Since unacylated ghrelin has been shown to be functional and has the same effect as acylated ghrelin in cancer cells (6, 44), we investigated its effect on endometrial cancer cell proliferation in vitro. A range of concentrations of UAG had no effect on endometrial cancer cell proliferation in either scrambled control or GHSR1a-knockdown (KD) cells compared with untreated controls (data not shown).

We also have demonstrated that the inverse agonist [D-Arg1, D-Phe5, D-Trp7,9, Leu11] substance P, which potently and specifically inhibits the ligand-independent activity of GHSR1a (24), statistically significantly reduced Ishikawa cell viability by 17.0 ± 1.8% (P = 0.016) of control at 50 μM (data not shown).

Knockdown of GHSR1a inhibited tumor growth in vivo. We next examined the effects of GHSR1a KD in Ishikawa cells in a NOD/SCID Ishikawa cell line mouse xenograft model. Two weeks after the subcutaneous injection of Ishikawa cells, all mice developed palpable tumors that grew at the site of injection. Tumor size was measured twice/wk for 5 wk. The average volume of the tumors in the GHSR1a-KD group was significantly smaller than in the scrambled control group at weeks 3 (P = 0.0001; Fig. 6A) and 4 (P = 0.0001; Fig. 6A). The inhibition of tumor growth in the GHSR1a-KD group remained significant until the end of study (week 5). After 5 wk, dissected tumor volumes of GHSR1a-KD xenograft tumors (333 ± 173 mm³) were reduced significantly compared with scrambled control tumors (1,217 ± 227 mm³, P = 0.0012; Fig. 6B).

Immunohistochemical staining for the cell proliferation marker Ki67 was performed to determine whether tumor cell proliferation plays a role in the reduced tumor size of xenograft tumors in vivo. Positive immunohistochemical staining for Ki67 was present in all of the xenograft tumors (Fig. 6C). The percentage of Ki67-labeled cells was significantly lower in GHSR1a-KD xenograft tumors (75.0 ± 2.07%) compared with scrambled control tumors (96.3 ± 1.07%, P = 0.0079; Fig. 6D).

DISCUSSION

This is the first study to demonstrate that downregulation of GHSR1a expression significantly inhibits endometrial cancer cell line proliferation and xenograft tumor growth. The expression of ghrelin and its cognate receptor, the growth hormone secretagogue receptor GHSR, was examined in endometrial cancer tissue specimens in this study. We have confirmed that ghrelin and GHSR1a are expressed in normal human endometrium, as reported previously by our laboratory (43). We also provide further evidence that the ghrelin axis is expressed in human endometrial cancer tissues, suggesting that ghrelin and GHSR1a may be involved in endometrial cancer cell progression.

Analysis using the cBio Cancer Genomics Portal suggests that GHSR is amplified in high-grade serous endometrial cancer but not in endometrioid tumors, which make up 85% of endometrial carcinomas (35). Serous endometrial carcinoma shares many
features of ovarian tumors and is indeed often treated in the same manner as ovarian carcinoma (13). Although it is difficult to predict whether the GHSR amplification events result in increased gene expression, it has been shown that the region harboring the GHSR locus is amplified in prostate cancer (39) and non-small cell lung cancer (31), and GHSR1a and/or GHSR1b isoform expression is upregulated in these and other cancers (10). Further studies on the GHRL locus in serous endometrial carcinoma, as well as ovarian carcinoma, may yield interesting insights into these cancer subtypes. Taken together, these data suggest that mechanisms other than increased GHSR1a expression and gene amplification contribute to the altered ghrelin axis function in endometrial cancer observed here and in our previous study (19). We have demonstrated previously that ghrelin increases cell proliferation and protects against doxorubicin-induced apoptosis in the endometrial cell lines examined (19). Ghrelin and GHSR1a are expressed in endometrial cancer (19), and recently, we have detected mRNA expression of GOAT in all of the xenograft tumor samples collected from the in vivo experiments (data not shown). Ghrelin may act through GHSR1a as an autocrine/paracrine growth factor, promoting the progression of endometrial cancer cells, and may contribute to endometrial cancer pathogenesis (19). Because ghrelin may also stimulate cell proliferation by acting through the unknown alternative ghrelin receptor (10), we aimed to determine whether ghrelin stimulates endometrial cancer growth through GHSR1a in endometrial cancer. We used a lentivirus-based system to silence GHSR1a expression in Ishikawa and KLE endometrial cancer cells. Exogenous acylated ghrelin treatment increased proliferation in vitro in the scrambled control cell line, whereas no significant cell proliferation was observed in GHSR1a KD cells. This suggests that ghrelin acts through GHSR1a to increase cell proliferation in endometrial cancer cell lines. It is possible, however, that ghrelin may have effects in the endometrium that are mediated by the alternative ghrelin receptor.

UAG is believed to act via an alternative ghrelin receptor, as it does not activate GHSR1a at physiological concentrations (5). Endometrial cancer cell lines were treated with UAG to determine whether it may also have effects on cell proliferation in endometrial cancer cell lines, acting through an alternative ghrelin receptor. However, it did not stimulate cell proliferation in the scrambled control cell lines or in the GHSR1a KD cells (data not shown). Although UAG and ghrelin have similar actions in many cell types (3, 44), this does not appear to be the case in endometrial cancer.

GHSR1a possesses a high level of ligand-independent constitutive activity, and this is reported to be up to 50% of its maximal activity (11, 16, 24). Cell proliferation was reduced in the GHSR1a-KD cells in the absence of ghrelin treatment compared with the scrambled controls. It is currently unclear whether this is due to inhibition of an autocrine ghrelin pathway or whether GHSR1a constitutive activity also contributes to endometrial cancer cell growth. We have demonstrated that the inverse agonist [D-Arg1, D-Phe5, D-Trp7,9, Leu11] substance P, which potently and specifically inhibits the ligand-independent activity of GHSR1a (24), reduced Ishikawa cell viability (data not shown), suggesting that the constitutive activity of the receptor plays a role in cell proliferation in endometrial cancer. [D-Arg1, D-Phe5, D-Trp7,9, Leu11] substance P and newer-generations of inverse agonists, including inverse agonists, based on its active core (17), or spirocyclic piperidine-azetidine inverse agonists (34) could be useful therapeutics for reducing ligand-independent GHSR1a activity in endometrial cancer. [D-Arg1, D-Phe5, D-Trp7,9, Leu11] subst...

Fig. 5. Effect of GHSR1a knockdown (KD) on Ishikawa and KLE human endometrial cancer cell proliferation, measured using the CyQUANT NF cell proliferation assay. Ishikawa (A) and KLE cells (B) transduced with scrambled shRNA (control) or GHSR1a-shRNA (GHSR-1a-KD) were monitored for 4, 24, 48, and 72 h. Absorbance readings for each time point were converted to percentages of scrambled control at 4 h. Data are derived from 3 experiments with 6 replicates and represented as means ± SE. Effect of exogenous ghrelin on scrambled control and GHSR1a-KD Ishikawa (C) and KLE cell proliferation (D), measured using the CyQUANT NF cell proliferation assay. Cells were incubated with ghrelin treatment (0–1,000 nM) for 72 h. Absorbance readings for each treatment were converted to percentages of no treatment control. Data are derived from 3 experiments with 6 replicates/condition and represented as means ± SE. ***P < 0.001; *P < 0.05.
stance P has also been shown to inhibit cell proliferation in lung cancer cells in vitro (38). Interestingly, a recent study has shown that GHSR1a mediates constitutive antiapoptotic activity and increased cell survival when stably expressed in HEK-293 cells but has no effect on cell proliferation (36). The GHSR1a inverse agonists [d-Arg¹, d-Phe⁵, d-Trp⁷,⁹, Leu¹¹] substance P and d-Lys³-GHRP-6 and newer-generation, more potent, and specific inverse agonists are likely to be useful for blocking the constitutive activity of the GHSR1a in vitro and in vivo (17, 24, 25, 27, 33, 38), and GHSR1a antagonists may be useful in inhibiting ghrelin-stimulated cell proliferation in endometrial cancer.

Our in vitro findings indicate that ghrelin and GHSR1a appear to be important in endometrial cancer cell growth. To investigate whether the effects of GHSR1a silencing on endometrial cancer cell growth inhibition are sustained in vivo, we established stable silencing of GHSR1a in Ishikawa xenografts in NOD/SCID mice. This study demonstrated that downregulation of GHSR1a in endometrial cancer cells inhibited cell proliferation, leading to a marked reduction in tumor size compared with scrambled control cells (where GHSR1a had not been silenced). This in vivo study indicates a critical role for GHSR1a as a regulator of cell proliferation and suggests that it may provide a future therapeutic target for endometrial cancer. GHSR antagonists and inverse agonists may be useful in inhibiting ghrelin-stimulated cell proliferation in endometrial cancer.

In conclusion, our study indicates that decreasing the expression of GHSR1a by RNAi inhibits endometrial cancer cell line and xenograft tumor growth significantly. This work provides further evidence for the role of the ghrelin axis and particularly the ghrelin receptor GHSR1a in the functional regulation of the human endometrial cancer. Demonstration of a functional role for ghrelin in endometrial growth and the detection of its receptor in endometrial cancers suggests that blocking GHSR1a activity may be a potential therapeutic approach for this cancer.

ACKNOWLEDGMENTS

We thank Dr. Wengyi Gu for lentiviral vectors, Jie Wang for technical support (AIBN), and Drs. Peta Fairweather and Nick Musgrave from Sullivan and Nicolaides Pathology for assistance in tissue collection.

GRANTS

This work was supported by grants from National Health and Medical Research Council (to L. K. Chopin and C. Chen), the Cancer Council Queensland (to C. Chen and L. K. Chopin), the University of Queensland (to C. Chen), the Queensland University of Technology (to L. K. Chopin), a Queensland Government Smart state fellowship (to P. L. Jeffery), and a Queensland University of Technology Early Career Researcher grant (to I. Seim).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Ghrelin receptor gene silencing in endometrial cancer

manuscript; J.N.F., P.L.J., I.S., A.O., L.K.C., and C.C. approved the final version of the manuscript.

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AJP-Endocrinol Metab • doi:10.1152/ajpendo.00156.2013 • www.ajpendo.org


