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

Jenny N. T. Fung,1 Penny L. Jeffery,2 John D. Lee,1 Inge Seim,3 Deborah Roche,2 Andreas Obermair,4 Lisa K. Chopin,3 and Chen Chen1

1School of Biomedical Sciences, University of Queensland, St. Lucia, Queensland, Australia; 2Mater Medical Research Institute, Mater Health Services, South Brisbane, Queensland, Australia; 3Ghrelin Research Group, Translational Research Institute and Institute of Health and Biomedical Innovation, and Australian Prostate Cancer Research Center-Queensland, Queensland University of Technology, Brisbane, Queensland, Australia; and 4University Queensland Central Clinical Division, Queensland Centre for Gynaecological Cancer, Herston, Queensland, Australia

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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 antiproliferative actions in normal and cancer cells (6, 18). Thus, it is highly likely that UAG signals through an alternative ghrelin receptor (UAG), was originally thought to be an inactive form of ghrelin, since it does not activate GHSR1a.

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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 (GHRL) 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 Endometrial 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, 73 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 with 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. GHSR1α real-time RT-PCR were performed using sense (5'-AGTGCGTTTGGCCTCATCC-3') and antisense primers (5'-GATGCGACAGCCTGAGTAG-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’-TGGAACTGAGGCCATG-3’ and antisense 5’-CGAACCTCTCGATTTGG-3’. Compared with scramble control Ishikawa or KLE endometrial cancer cells, the normalized fold change of mRNA expression was expressed as 2ΔΔCt, where ΔΔCt = ΔCt sample – ΔCt 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 10,000 g centrifugation at 4°C. Total protein concentration was measured using a bicinchoninic acid (BCA) assay kit (Thermo Fisher Scientific, Rockford, IL), and absorption was measured at 560 nm (Spectra Rainbow spectrophotometer, X-Read Plus version 4.3; Tecxan, 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 electrotroduced 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 GHSR1α 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 GHSR1α, 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 was 3% hydrogen peroxide in PBS for 10 min. Blocking was performed by incubating sections in 10% milk diluted/ blocking solution (KPL, Gaithersburg, MD) for 20 min. Sections were incubated with the following primary antibodies: rabbit polyclonal anti-ghrelin (Phoenix), goat polyclonal anti-GHSR1α (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 chromagen 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 (Toocris 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 GHSR1α in endometrial cancer cell lines**

shRNA design and vector preparation. shRNAs specifically targeting human GHSR1α were designed to knockdown GHSR1α expression based on a published human GHSR1α 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 nucleotide (nt) of the target sequence, followed by the loop sequence (TTCAGAGAG), reverse complement to the 19 nt, stop codon for the U6 promoter, and an XhoI site. The shRNA sequences are GHSL1a (sense: 5′-TCTCCCTTTCAAGAGAGGAGCAGCCGTTACTAGTT-3′; antisense: 5′-ACTTTCCCTTTCAAGAGGAGCAGCCGTTACTAGTT-3′) and scrambled (sense: 5′-TAACTAATGTAACGGCTGCTCCTTCAAGAGAGGAGCAGCCGTTACTAGTT-3′; anti-sense: 5′-ATTGATCATTTGCGAGACGGGAGTTTCCTTCTGTCGGAATGATCAAAAAAAAGAGCT-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 pLentiLox3.7 (pL3.7; a gift from Dr. Wengyi Gu; AIBN) at the HapI and XhoI sites. The pL3.7 plasmid also expresses enhanced green fluorescent protein (EGFP). The insert was confirmed by both restriction enzyme digestion and DNA sequencing by the Australian Genome Research Facility (Brisbane, Australia), using the ABI PRISM BigDye Terminator Cycle Sequencing Kit version 3.1 protocol (Life Technologies).

Lentiviral packaging. 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 GHSL1a shRNA) and the lentiviral packaging plasmids pRSRev, pMD.G (contains VSV.G gene), and pMDLgpRRE (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 harvested for infection. The HEK-293T cell line was transduced with pLentiLox3.7 (pL3.7; a gift from Dr. Wengyi Gu; AIBN) at 8 g/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 GHSL1a in Ishikawa cells transduced with GHSL1a-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 GHSL1a-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 × 10⁷) stably infected with a lentivirus vector carrying GHSL1a-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 mm³. All experiments were performed in accordance with the institutional and 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 GHSL1a protein in normal human endometrial and endometrial cancer tissues. Using the cBio Cancer Genomics Portal (7), we found that GHSL1 is frequently amplified in high-grade serous (Fallopian tube-like) tumors but not in low-grade endometrioid tumors that make up...
85% of endometrial carcinoma cases (Fig. 1) (35). For immu-
nohistochemical detection of ghrelin and GHSR1a expression, we
employed a tissue microarray with 70 endometrial adenocarci-
noma tissues and five healthy endometrium controls. Positive
immunohistochemical staining for ghrelin (Fig. 2) and the GHSR
isoform GHSR1a (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 GHSR1a immunostaining and tumor grades.

Knockdown of GHSR1a inhibited cell proliferation in vitro.
Lentivirus-mediated RNA interference was used to downregu-
late GHSR1a expression in Ishikawa and KLE cells. These
experiments demonstrated that GHSR1a-shRNA transduction
significantly reduced expression of GHSR1a 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
GHSR1a-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
GHSR1a-shRNA showed 25.3 ± 3.2% less cell proliferation in
vitro over 72 h compared with the scrambled controls (P <
0.05; Fig. 5A). Similarly, the growth of KLE cells transduced
with GHSR1a-shRNA was markedly inhibited by 36.7 ± 3.1%
compared with the scrambled controls (P < 0.05; Fig. 5B).

To elucidate the importance of GHSR1a in ghrelin-stimulated

cell proliferation in endometrial cancer cells, Ishikawa and KLE
cells transduced with either GHSR1a-shRNA or scrambled
shRNA were cultured in vitro and exposed to a range of concen-
trations 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. 5C) 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. 5D) 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 \pm 1.8\% (P = 0.016) of control at 50 \mu M (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 \pm 173mm^3) were reduced significantly compared with scrambled control tumors (1,217 \pm 227 mm^3, 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 \pm 2.07\%) compared with scrambled control tumors (96.3 \pm 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 isofrom 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-generation, more potent, and specific 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] sub-
stance P has also been shown to inhibit cell proliferation in lung cancer cells in vitro (38). Interestingly, a recent study has shown that GHSR1α mediates constitutive antiapoptotic activity and increased cell survival when stably expressed in HEK-293 cells but has no effect on cell proliferation (36). The GHSR1α 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 GHSR1α in vitro and in vivo (17, 24, 25, 27, 33, 38), and GHSR1α antagonists may be useful in inhibiting ghrelin-stimulated cell proliferation in endometrial cancer.

Our in vitro findings indicate that ghrelin and GHSR1α appear to be important in endometrial cancer cell growth. To investigate whether the effects of GHSR1α silencing on endometrial cancer cell growth inhibition are sustained in vivo, we established stable silencing of GHSR1α in Ishikawa xenografts in NOD/SCID mice. This study demonstrated that downregulation of GHSR1α in endometrial cancer cells inhibited cell proliferation, leading to a marked reduction in tumor size compared with scrambled control cells (where GHSR1α had not been silenced). This in vivo study indicates a critical role for GHSR1α 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 GHSR1α 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 GHSR1α 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 GHSR1α activity may be a potential therapeutic approach for this cancer.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


Fig. 6. Effects of GHSR1α gene silencing on tumor growth in a mouse xenograft model. Ishikawa cells infected with GHSR1α-shRNA (GHSR1α-KD) or scrambled shRNA (scrambled control) were implanted subcutaneously into the left flank of female immunocompromised mice. A: the tumor volume was measured twice/wk from 1 to 5 wk after implantation and plotted. Data are represented as means ± SE (n = 6 in GHSR1α-KD group; n = 7 in scrambled control group). B: the tumor volume was measured on the day of euthanasia and plotted. C: representative images of Ki67 staining of scrambled control and GHSR1α-KD Ishikawa xenograft tumors. Ki67 immunoreactivity appears as brown-black granules (DAB staining) in the nuclei of positive cells. Scale bars, 50 μm. D: Ki67-positive nuclei were counted, and data are represented as means ± SE (n = 6 in GHSR1α-KD group; n = 7 in scrambled control group). ***P < 0.001; **P < 0.01.

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