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

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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 were 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(s). UAG activates the possibility of additional ghrelin receptor(s) in these cancers.

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...
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profile via lentiviral delivery methods. The shRNA expression cassette contained 19 nucleotides (nt) of the target sequence, followed by the loop sequence (TTCAGAGAAGAGCACCATCTGCCTGAGAGAAGCAGCCGTTACTAGTT), amplified in 3′-GTTCTCTCCTCGTCGGCAATGATCAAATAAAAGAGCT-5′. The shRNA sequences are GHSR1a (sense: 5′-TGAGAAGACCTCCTACCTGTTCAGAGAGCAGAGAGCAGCCGTTACTAGTT-3′; antisense: 5′-ACTCTTTTCGAGGATGGACAGAGAGCAGCCGTTACTAGTT-3′) and scrambled (sense: 5′-TAACATGTAACGGCTGCTCCTTCAAGAGAGGAGCAGCCGTTACTAGTT-3′; anti-sense: 5′-ATTGATCATTGGCAGCGAGGAGAAGACGGCAGCAAGAGCTTTTTTTT-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 Xhol 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 GHSR1a shRNA) and the lentiviral packaging plasmids pRSVRev, pMD.G (contains VSV.G gene), and pMDLgpRRE (gifts from Dr. Wengyi Gu; AIBN). The next day, the medium was replaced with 10% fresh DMEM (Life Technologies), and 48 h later, supernatant was collected and filtered (pore size 0.45 μm, Sigma-Aldrich). The viral supernatant was concentrated by centrifugation at 22,000 g for 90 min at 4°C. The lentiviral stocks were stored in small aliquots at −80°C for titration and cell infection.

**Infection 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 >10² 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 × 10⁵) 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 (width³ × 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 histologic 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 secretagogue 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 (GHRRL) are also shown.
85% of endometrial carcinoma cases (Fig. 1) (35). For immunohistochemical detection of ghrelin and GHSR1a 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 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 downregulate 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 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. 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.
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
through GHSR1a to increase cell proliferation in endometrial cancer cells. This suggests that ghrelin acts through the unknown alternative ghrelin receptor (10). 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 detected in the GHSR1a KD cells (data not shown), suggesting that the constitutive activity of the GHSR1a receptor plays a role in cell proliferation in endometrial cancer. 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. ghrelin receptor.

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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 \([D-\text{Arg}^1, D-\text{Phe}^5, D-\text{Trp}^7,9, \text{Leu}^{11}]\) substance P and \(D-\text{Lys}^3\)-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.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

REFERENCES


