GPR54 is a target for suppression of metastasis in endometrial cancer

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Abstract

Invasion into deep myometrium and/or lymphovascular space is a well-known risk factor for endometrial cancer metastasis, resulting in poor prognosis. It is therefore clinically important to identify novel molecules that suppress tumor invasion. Reduced expression of the metastasis suppressor, KISS1 (kisspeptin), and its endogenous receptor, GPR54, has been reported in several cancers, but the significance of the KISS1/GPR54 axis in endometrial cancer metastasis has not been clarified. Metastin-10 is the minimal bioactive sequence of genetic products of KISS1. Clinicopathological analysis of 92 endometrial cancers revealed overall survival is improved in cancers with high expression of GPR54 (p<0.05) and that GPR54 expression is associated with known prognostic factors including FIGO stage, grade, and deep myometrial invasion. Through RNAi and microarray analyses, metastin-10 was predicted to suppress metastasis of GPR54-expressing endometrial cancers in vivo. Methylation analysis revealed GPR54 is epigenetically regulated. Metastin-GPR54 axis function was restored following treatment with the DNA hypomethylating agent 5-aza-DC. These data suggest that metastin-10 may be effective at inhibiting metastatic spread of endometrial cancers in combination with demethylating agents to induce GPR54 expression.
Introduction

Uterine corpus cancer is the leading cause of malignant gynecological disease with more than 42,000 cases diagnosed per year in the United States and disease incidence has been steadily increasing (1). Extra-uterine spread of cancer cells profoundly impacts patient prognosis. A multi-centered retrospective study with more than 7,500 patients revealed hazard ratios of 4.9 and 15.0 for stage III and stage IV, respectively, compared with stage I disease (2). Multivariate analysis in the same study also revealed that lymphovascular space involvement and deep myometrial invasion as well as histological grade are life-threatening risk factors for stage I cases with hazard ratios greater than 2.0. Thus, cancer cell invasion followed by metastasis is an important issue to consider for improving prognosis for women diagnosed with endometrial cancer.

Tumor metastasis involves multiple complex steps such as loss of intracellular adhesion, stromal invasion, intravasation, dissemination and attachment at distant sites. As such, each step has been extensively investigated with the goal of establishing targeted anti-metastatic agents. The rate of lymph node metastasis in endometrial cancers approaches 26% in cases with invasion that extends through more than half of the myometrium while it is only 6% in those cases with invasion extending to less than half the thickness of the myometrium (2). Biological blockades for the early steps in this
process are a crucial need for development of effective metastasis suppressive therapy for endometrial
cancer. Among more than 20 metastasis suppressor proteins previously reported, NM23, KAI1, 
RHOGD12, and KISS1 prevent outgrowth of isolated cell clusters, and several drugs that upregulate 
NM23 are already in use clinically for acute promyelocytic leukemia and are undergoing phase II-III 
clinical studies in breast, prostate, and esophageal cancers (3, 4). KISS1 was originally described as a 
metastasis suppressor in melanoma. The KISS1 gene encodes a number of peptides via alternative 
splicing that function as endogenous ligands for the G-protein coupled receptor, GPR54. There are two 
reports showing that direct administration of the Kiss1 peptide suppresses pulmonary metastasis of 
melanoma cells in mice (5). Metastin is a 54-amino acid splice variant of KISS1. Binding of metastin 
to GPR54 induces a series of intracellular signals such as activation of ERK and Rho or suppression of 
AKT, resulting in inhibition of chemotaxis and invasion (6, 7). Expression of metastin or GPR54 has 
been associated with favorable prognosis in gastric, esophageal, pancreatic, thyroid, and bladder cancers. 
On the other hand, metastin is highly expressed in breast and liver cancers with poor prognosis (6). Thus, 
the functional role of metastin/GPR54 expression appears to be organ specific. In endometrial cancer, 
one report analyzed 32 cases and described low KISS1 expression in aggressive tumors (6); however the 
role of metastin/GPR54 expression in endometrial cancer remains to be clarified in a sufficient number 
of cases to determine the significance.
Through endocrinological studies, the role of metastin/GPR54 signaling in non-cancer tissue is at least partially understood. Continuous metastin stimulation desensitizes GPR54 signaling, resulting in the loss of downstream GnRH release in neurons (8). The expression of GPR54 in female rat pituitary glands is positively regulated by GnRH and negatively controlled by chronic exposure to estradiol (9). The G-protein coupled receptor serine/threonine kinase-2 (GRK2) mediates GPR54 desensitization in HEK293 cells, probably by interacting with the intracellular loop or cytoplasmic tail of GPR54 (10). However, little is known about the regulation of GPR54 expression in cancer cells in spite of the demonstrated clinical impact and important roles in cancer biology that have been revealed. In this study, we demonstrate the clinical significance and epigenetic regulation of GPR54 expression in endometrial cancer and describe a potential role for the metastin peptide as a metastasis suppressor for clinical use.

Material and Methods

Tissue collection
Specimens and relevant clinicopathological information were obtained with written consent from each patient and used under protocols approved by the Kyoto University Institutional Review Board. Ninety two adenocarcinomas (79 endometrioid and 13 serous) were obtained at the time of initial surgery, fixed in 10% buffered formalin, embedded in paraffin, and sectioned.

Immunohistochemistry

Immunohistochemical staining was performed using the streptavidin-biotin peroxidase complex method as previously reported (11). An endogenous peroxidase block was followed by nonspecific background blocking and incubation with a 1:100 dilution of anti-human KISS1 monoclonal antibody H00003814-M05 (Abnova, Taipei, Taiwan) or a 1:50 dilution of anti-human GPR54 polyclonal antibody LS-A1927 (MBL, Woburn, MA). The primary antibody was omitted for negative controls.

Three independent gynecologic pathologists examined the immunohistochemical slides while blinded to the clinical history of the patients. Discrepancies in interpretation were resolved using a multi-head microscope to reach consensus. The expression of metastin and GPR54 was evaluated by integrating the intensity and the area of the staining. The intensity was graded on a 0-2 scale: 0, none-to-weak (almost negative); 1, intermediate (less positive than control, cytotrophoblasts of chorionic...
villi (12)); 2, strong (as positive as control). The expression of metastin and GPR54 was scored as follows, with % referring to the percentage of cells on a slide exhibiting each category of staining intensity: \( \{(0\times\%\text{weak}) + (1\times\%\text{intermediate}) + (2\times\%\text{strong})\}/100. \)

**Cell lines and culture**

Human endometrial cancer cell lines AN3CA, HEC1A, HEC1B, HHUA, Ishikawa, and TEN (ATCC; Rockville, MD) were maintained in RPMI1640 (Nikken, Kyoto, Japan) supplemented with 10% FBS. KLE and RL95-2 (ATCC) were maintained in DMEM/Ham’s F12 (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (KLE) and with 10% FBS and 0.005mg/ml bovine insulin (RL95-2). All the cell lines were passaged for less than 6 month after receipt. Immortalized human endometrial glandular cells (hEM cell) were kindly provided by Dr. Kyo and maintained as previously described (13).

**Quantitative RT-PCR analysis of GPR54**

Total RNA was extracted from cell lines using the RNeasy® Mini Kit (QIAGEN, Valencia,
CA). To monitor gene expression, quantitative RT-PCR amplification of human GPR54 and GAPDH mRNAs was performed using SYBR® Premix Ex Taq™ (Takara, Otsu, Japan) and analyzed on a Model 7000 Sequence Detector (Applied Biosystems, Foster City, CA, USA). The following primers were used for analysis: GPR54, 5’-CGA CTT CAT CTG CAA GTT CGT C-3’ (forward), 5’-CAC ACT CAT CAT GCG GTC AGA G-3’ (reverse); GAPDH, 5’-GAA GGT GAA GGT CGG AGT-3’ (forward) and 5’-CTT CTA CCA CTA CCC TAA AG-3’ (reverse). Cycling parameters were 95°C for 10sec and 40 cycles of 95°C for 5sec and 60°C for 31sec, followed by a dissociation cycle of 95°C for 15sec, 60°C for 20sec, and 95°C for 15sec. The expression of human GPR54 mRNAs was estimated by dividing the GPR54 threshold cycle (C_T) values by the GAPDH C_T values.

**Methylation analysis**

To analyse GPR54 promoter methylation, genomic DNA was extracted from cell lines using the QIAamp® DNA Mini Kit (QIAGEN) followed by treatment with sodium bisulfite using the EpiTect® BiSulfite kit (QIAGEN). Methylation-specific PCR (MS-PCR) was performed as previously reported (14). For positive and negative controls, EpiTect® PCR Control DNA (QIAGEN) was used. Epigenetic reactivation of GPR54 was examined by treating cells with 5µM 5-aza-DC (Sigma-Aldrich,
St Louis, MO) for 72h. *GPR54* expression was subsequently analyzed by quantitative PCR as described above.

**GPR54 knockdown**

*GPR54* specific siRNA (Gene Solution siRNA Cat. No. 1027416) and negative control siRNA (AllStars Negative Control siRNA) were obtained from QIAGEN. These siRNAs were transfected into Ishikawa cells using HiPerFect Transfection Reagent (QIAGEN) as previously described (15).

Ishikawa cells were seeded and incubated for 48h. The medium was aspirated and the cells were gently washed with RPMI1640 followed by transfection with the siRNAs. To confirm down-regulation of *GPR54* expression, quantitative RT-PCR was performed 48h post-transfection as described above.

**Proliferation assay**

Ishikawa or HEC1A cells were seeded into 96-well (2×10³ cells/well) tissue culture plates and incubated for 24h. The cell culture medium was replaced with fresh medium containing 10μM metastin-10 peptide or control peptide (16) and incubated for 24h. The resulting cell number was

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measured using a WST-1 assay kit (a modified MTT assay using a water-soluble tetrazolium salts, Premix WST-1, Takara, Otsu, Japan) as described previously (17) and was compared between groups. The experiment was carried out in sextuplicate.

Motility tracking assay

Ishikawa or HEC1A cells were placed into 6cm dish and incubated until they were 100% confluent. Experimental ‘wounds’ were introduced by dragging a 1 ml plastic pipette tip across the cell monolayer. After wounding, the cultured cells were rinsed with PBS and media was added with or without 10μM metastin-10. The cells were then incubated for 24h to track cell motility. A total of four wounds were sampled for each specimen. Migration rate was evaluated by measuring the gap between the cells most closely spaced on each leading edge at 0h and 24h post-wounding, expressed as the percentage of the distance filled. The motility of Ishikawa cells treated with or without metastin-10 was also tracked under a microscope equipped with a temperature-regulated stage and digital camera (IX71/AP71, Olympus, Tokyo, Japan). Cell images were captured every two minutes for 6h and movies were assembled using Metamorph (Molecular Devices, Sunnyvale, CA) as previously described (7).
**Invasion assay**

Invasive potential into a reconstituted basement membrane was assayed on 24-well companion plates (Becton Dickinson Labware, Franklin Lakes, NJ) with an 8-μm pore polycarbonate filter coated with Matrigel® (Becton Dickinson Labware) as described previously (17). The lower chamber contained 0.8ml medium with 10% FBS as a chemoattractant. In the upper compartment, 1-2×10⁶ cells in 0.2ml medium were seeded and incubated with or without metastin-10 peptide at 37°C for 24h to allow for migration to the lower chamber. Student’s t-tests were used to analyze the differences in invasion rates between groups. FTM080 (16), a peptide smaller than metastin-10, was also assessed in the same manner.

**Microarray analysis**

Gene expression microarray data (Affymetrix U133 Plus 2.0) for Ishikawa cells treated with or without 10μM metastin-10 and/or siRNAs were generated in triplicate and RMA-normalized as described previously (18, 19). SAM was employed to detect the genes specifically expressed in metastin-10 treated cells with a false discovery rate (FDR) of less than 0.1 as described previously (18,
19). A metastasis gene signature was developed from two independent published microarray datasets, GSE11683 and GSE14405 (20), after removing batch effects using ComBat as previously described (21). Expression microarray data of endometrial cancers (GSE2109) (20) was also obtained from the website as above.

Orthotopic mouse model of endometrial cancer

Five to six-week CD1 Foxn1nu female mice were obtained from Oriental BioService, Inc. (Kyoto, Japan). Animal care and experimental procedures were performed in accordance with the guidelines of the Institute of Laboratory Animals Graduate School of Medicine, Kyoto University.

Subcutaneous xenografts were established by inoculating $1 \times 10^7$ Ishikawa cells into the flanks. Four weeks after inoculation, subcutaneous tumors were removed and cut into $\sim 2$mm$^3$ blocks for orthotopic implantation. One tumor block was implanted onto the uterine posterior wall in each of 21 mice, and these mice were divided into two groups. Each group was treated for 15 days with intraperitoneal administration of 50nmol metasin-10 or control, respectively, beginning four days after implantation.

Six weeks later, paraaortic lymph node metastasis was examined as previously described (22) and tissues
were preserved in RNAlater (Ambion, Austin, TX) for RNA extraction. A portion (~1/2) of bulky lymph node and the uterine tumor was fixed in 10% buffered formalin for IHC using anti-human cytokeratin monoclonal antibody (1:20, Abcam Inc., Cambridge, MA) and anti ER monoclonal antibody (1:20, AbD Serotec, Raleigh, NC). The presence of metastatic tumor cells in lymph nodes was examined through detection of the presence of human β2-microglobulin (β2-M) mRNAs by quantitative PCR as previously described (17). The β2-M specific primers were as follows: 5’-CCA TCC GAC ATT GAA GTT GA-3’ (forward), 5’-TGG AGC AAC CTG CTC AGA TA-3’ (reverse). Cycling parameters were 95°C for 10 min, 45 cycles of 95°C for 10 sec, 60°C for 30 sec, and 72°C for 1 sec, followed by a final 30 sec extension at 40°C. Those lymph nodes with human β2-M C_T < 33 were assessed as positive.

Statistical analysis

Comparison of the groups was performed using Mann-Whitney U tests, while categorical variables were compared using χ² tests. Overall survival was analyzed using the Kaplan-Meier method. Statistical analyses were performed using GraphPad Prism 4 software. Probability values below 0.05 were considered significant.
Results

GPR54 expression in endometrial cancers and its significance for prognosis

The expression of metastin and GPR54 in 92 endometrial cancer patients (mean age, 57.3 years) was assessed by immunohistochemistry. Grade 1 endometrioid adenocarcinoma was observed in 36 patients, grade 2 in 12 patients, grade 3 in 31 patients, and serous adenocarcinoma in 13 patients. Fifty-five patients were at FIGO stage I, 5 patients at stage II, 24 patients at stage III and 8 patients at stage IV. GPR54 expression is observed in chorionic villi at early gestation and was used as a positive control. In grade 1 endometrioid adenocarcinoma, strong GPR54 expression was observed at the cell membrane of cancer cells (Figure 1A), while it was weaker in grade 3 and serous adenocarcinoma (Figure 1B, p=0.0003 and 0.0128, respectively). On the other hand, metastin expression showed no significant difference among histologic grades. The overall survival (OS) of patients bearing GPR54-positive (GPR54+) cancers was significantly longer than that of patients bearing GPR54-negative (GPR54-) cancers (Figure 1C, p=0.0398) while no significant difference in OS was observed based on metastin expression (p=0.1773). Clinicopathological analysis revealed that each
known prognostic factor including FIGO stage, histological grade, lymphovascular space invasion, and lymph node metastasis, showed significant differences in OS (p=0.022, 0.0071, 0.0004, and 0.0073, respectively). Moreover, as shown in Table 1, decreased GPR54 expression was associated with the following clinicopathological factors: age [relative risk (RR), 1.566; 95% confidence interval (C.I.), 1.211-2.026; p=0.0113], FIGO stage (RR, 1.529; 95% C.I., 1.023-2.285; p=0.0190), histological type (RR, 1.742; 95% C.I., 1.221-2.483; p=0.0008), myometrial invasion (RR, 1.436; 95% C.I., 1.025-2.012; p=0.0270), and lymph node metastasis (RR, 1.610; 95% C.I., 0.959-2.703; p=0.0291). In contrast, metastin expression was not significantly associated with these factors.

Effects of exogenous metastin on the phenotype of endometrial cancer

To examine the effect of exogenous metastin-10 on the phenotype of endometrial cancers with or without GPR54 expression, changes in proliferation, migration, and invasion were examined, effects that had previously been reported in several cancers (5, 23, 24). GPR54 expression in eight endometrial cancer cell lines was assessed by quantitative PCR (Figure 2). Ishikawa cells and KLE cells were used for further analysis as representative GPR54+ endometrial cancer cells while HEC1A cells were used as representative GPR54- cells. There were no changes in cell proliferation for both GPR54+ Ishikawa cells
and GPR54- HEC1A cells (p=0.9768 and 0.9170, respectively, supplementary figure 1). However, migration of GPR54+ Ishikawa cells was significantly inhibited by metastin-10 (p=0.0423), while GPR54- HEC1A cells were not affected (p=0.2162, Figure 3A). Cell motility of Ishikawa cells with or without metastin-10 treatment was tracked using time-lapse microscopy over six hours. In the supplementary movie file, there are two images presented (left and right frames) with the right frame showing cell migration in the presence of metastin-10 and the left frame showing the control cells, and active migration was hardly observed in the right reference frame to demonstrate the migration inhibitory effect of metastin-10 (Supplementary movie). Matrigel invasion of GPR54+ Ishikawa cells was also significantly inhibited by metastin-10 (p<0.0001, Figure 3B). We further confirmed this invasion-inhibitory effect of metastin-10 using GPR54+ KLE cells (p=0.0187), which was not evident in GPR54- HEC1A cells (p=0.8995). FTM080, a GPR54 agonistic smaller peptide, also showed similar effects on Ishikawa and KLE cells (p=0.0141 and 0.0009, respectively, Figure 3B).

To determine whether metastin has an invasion-inhibitory role through interaction with its receptor GPR54, we examined the functional consequence of GPR54 knockdown using RNAi. We introduced four different GPR54 siRNAs into Ishikawa cells, each of which targeted independent sequences within GPR54 mRNA, and examined the efficiency of GPR54 knockdown using quantitative RT-PCR. Among these siRNAs, the knockdown efficiency of siRNA4 was most effective.
(48.29+/−2.98%) (supplementary figure 2), and thus was selected for further analysis. There was no significant difference in matrigel-invasion following GPR54 suppression (p=0.3516, Figure 3C).

Metastin-10 significantly inhibited invasion of cells that received the negative control siRNA (p<0.0001), but not that of GPR54 suppressed cells (p=0.4169).

Methylation-dependent regulation of GPR54 expression and its targeting

GPR54 is reported to contain many CpG dinucleotides in the promoter region that are targeted by DNA methylation (14). To assess potential epigenetic regulation of GPR54 expression in endometrial cancer cells, expression and methylation status was examined in 8 endometrial cancer cell lines using quantitative PCR and MS-PCR (Figure 4A). GPR54 expression was low and methylation was absent in non-malignant hEM cells. Methylation was present in seven cell lines and five of these showed evidence of complete methylation. Intriguingly, the expression of GPR54 was suppressed in these five cell lines relative to the other three cell lines (p=0.036). Promoter methylation was also observed in six of ten clinical samples (supplementary figure 3). Moreover, GPR54 expression in all five GPR54- cell lines with methylation was restored following treatment with the DNA methyltransferase inhibitor 5-aza-2’-deoxycytidine (5-aza-DC) (p=0.048, Figure 4B, C). We then investigated the possibility of
re-sensitizing GPR54- endometrial cancers cells to metastin treatment using 5-aza-DC. Following 5-aza-DC pretreatment, the ability to invade matrigel in both GPR54- cell lines, HEC1A and AN3CA, was significantly inhibited after metastin-10 addition (Figure 4D, p=0.0034 and 0.0022, respectively), while this was not observed in cells pretreated with 5-aza-DC but without the addition of metastin-10 (p=0.1797 and 0.6991, respectively).

*Usefulness of metastin as a treatment to suppress GPR54+ endometrial cancer metastasis*

Although there are several reports showing metastin inhibits migration and invasion of cancer cells *in vitro*, it is not clear whether cancer cells treated with metastin *in vivo* will become less metastatic. Genome-wide analysis of gene expression changes following metastin-10 treatment, using the web software named of GATHER (25), revealed that 318 probes (285 genes) were downregulated in Ishikawa cells, and “cell motility” (GO:0006928) as well as “regulation of cellular physiological process” (GO:0051244) were Gene Ontology (GO) terms enriched among these genes. Next, a metastasis gene signature was developed using the Bayesian binary method (26) from available microarray datasets that distinguished highly metastatic subclones from their parental cell lines, including PC-3 (GSE14405) and MDA-MB-468 (GSE11683). The accuracy of leave-one-out validation in the ability of the gene
signature to accurately assign the status of metastatic and non-metastatic cells was 100%. The propriety of this signature was externally validated in GSE2109, in which the metastasis signature probability was significantly higher in grade 3 endometrial cancers at stage III-IV than those at stage I-II (p=0.028, supplementary figure 4). The metastasis signature consisted of 172 upregulated and 28 downregulated probes in highly metastatic subclones, and protein transport GO terms such as “intracellular protein transport” (GO:0006886) and “establishment of protein localization” (GO:0045184) as well as “cellular physiological process” (GO:0050875) were enriched among the upregulated probes. This metastasis signature was applied to the microarray data from Ishikawa cells with/without metastin-10 treatment to assign a metastatic probability to each group (microarray data are available at GEO repository with the accession No. GSE25458). The metastasis signature probability was significantly suppressed by metastin-10 treatment (p=0.0395, Figure 5A), while the probability was not changed following GPR54 knockdown (p=0.5250). Intriguingly, metastatic suppression with metastin-10 treatment was not observed in GPR54 knockdown cells (p=0.8757).

Using a lymph node metastasis model (22), the suppressive effect of metastin-10 on metastasis was assessed in vivo. Six weeks after inoculation of a 2mm³ section of subcutaneous tumor, solid tumor growth was confirmed on the uterus as previously reported (Figure 5B). On the cut surface, solid tumor grew from the uterine serosa toward the endometrium. The estrogen receptor was uniformly expressed in
the nucleus of both Ishikawa tumor cells and mouse endometrial glands, while human cytokeratin was expressed only in the cytoplasm of tumor cells (Figure 5C). Tumor cell infiltration that resembled the primary site was observed in swollen paraaortic lymph nodes beneath the left renal vein, and they were positive for both human cytokeratin and the estrogen receptor (Figure 5C). Although there was no difference in the diameter of uterine tumors treated with/without metastin-10, paraaortic nodes in the group treated with metastin-10 were smaller (2.45+/−0.55mm) than those without metastin-10 (4.1+/−1.25mm, p=0.1686). Since all the lymph nodes positive for metastasis in microscopic examination was also positive for the expression of human β2-microglobulin in RT-PCR, the presence of tumor cells in lymph nodes was confirmed by the expression of human β2-microglobulin, which revealed that paraaortic metastasis was less frequent in the group treated with metastin-10 (p=0.029, Figure 5D). Together, the results obtained from the microarray analysis and the orthotopic endometrial cancer metastasis model demonstrates the ability of metastin-10 to suppress metastasis of GPR54+ endometrial cancers in vivo.

Discussion
Metastasis is a critical factor that determines the prognosis of patients with endometrial cancer.

To confirm the genetic propensity toward a metastatic phenotype in high grade histology tumors, we analyzed microarray data of 146 endometrial cancers for expression of metastasis suppressor genes.

Hierarchical clustering based on the expression of seven representative metastasis suppressor genes divided the 146 endometrial cancers into two clusters, with high grade cancers enriched in cluster 1 (p=0.0045, supplementary figure 5). This result supports the notion that tumors with a high risk of metastasis possess an inherent genetic propensity with similar expression profiles of metastasis suppressor genes.

To elucidate if metastin-GPR54 interaction plays a role in the clinical and biological behavior of endometrial carcinoma, we first evaluated expression of these proteins in 92 clinical samples using immunohistochemistry. The expression of GPR54 was lower in serous and grade 3 endometrioid adenocarcinomas compared with grade 1 cases. Analysis of microarray data from 57 endometrial cancers (27) also revealed that low expression of GPR54 mRNA (expression value <-0.1) is more frequently observed in serous and clear cell adenocarcinoma (p=0.0394), suggesting that GPR54 expression is suppressed in high grade endometrial carcinomas. Furthermore, loss of immunohistochemical expression of GPR54 was significantly associated with invasion into lymphovascular space and deep myometrium, well-known poor prognostic factors that are associated with distant metastasis (2). More importantly,
prognosis of patients with endometrial carcinomas negative for GPR54 expression was significantly poorer than those positive for GPR54. These results strongly suggest that decreased expression of GPR54 is a poor prognostic factor and is relevant to both invasive and metastatic capacity of endometrial cancers. On the other hand, metasin is ubiquitously expressed both in cancer and stromal cells, irrespective of histological grade, and expression is not relevant to patient prognosis. The weak expression of metasin in stromal cells might prevent invasion of cancer cells, which was not clarified in this study.

Migration and invasion of cancer cells into the stroma or vascular space is regarded as the initial step of distant metastasis. We next examined if the metasin-GPR54 axis is relevant to the migration and invasion of endometrial cancer cells in vitro. Previous reports showed that metasin prevented migration or invasion in several cancers (23, 28-30) and HUVEC (31). In this study, we used metasin-10, a short peptide of 10 amino acids which is proteolytically processed from metasin. Metasin-10 is ten times as active as metasin and regarded as a candidate for clinical use (16). Migration of the GPR54+ Ishikawa endometrial cancer cell line was significantly inhibited by metasin-10 in the wound healing assay, while proliferation was not affected. These findings were confirmed using time-lapse cell imaging, in which active migration of Ishikawa cells was suppressed by metasin-10 treatment while cell division of treated cells appeared similar to that of non-treated cells. Invasion of
these cells was also inhibited by metastin-10 as evaluated using the Boyden chamber assay. In contrast, these inhibitory effects of metastin-10 on migration and invasion were not observed in GPR54-HEC1A cells, and siRNA-mediated knockdown of GPR54 resulted in lack of response of Ishikawa cells to metastin-10 treatment. These findings suggest that the effect of metastin is exclusively dependent on its receptor, GPR54.

To evaluate if metastin treatment alters gene expression patterns, we conducted gene expression microarray analysis of Ishikawa cells that were treated with or without metastin-10. By comparing gene expression of highly metastatic cancer subclones with their parental cells, a metastatic probability score was generated using the Bayesian binary method (26). The calculated metastatic probability score was significantly decreased after metastin-10 treatment, suggesting that metastin converted Ishikawa cells to a less metastatic phenotype. This was not observed when GPR54 was suppressed by siRNA treatment, again indicating that the effect of metastin is receptor-dependent. These results are in agreement with the previously reported anti-metastatic effects of metastin on intracellular signaling pathways (7, 32).

Thus far, little is known how GPR54 expression is regulated in cancer cells. Epigenetic events contribute to the initiation and progression of human cancers. Acquisition of methylation at gene promoters in cancer is often linked with transcriptional repression. In endometrial cancers, inactivation
by promoter hypermethylation is frequent for a number of genes such as *PTEN*, *p16*, and *RASSF1A* (33, 34). Chemical genomic screening of gastric cancer cell lines showed several metastasis suppressor genes including *GPR54*, *BMP7*, *IGFBP3*, and *MTSS1* are silenced by DNA methylation (14). In this study, promoter methylation was present in seven endometrial cell lines and five of these showed complete methylation, while methylation was not observed in hEM cells derived from normal endometrium.

Notably, the expression of *GPR54* was suppressed in the five completely methylated cell lines relative to the other three cell lines (p<0.05). These findings indicate that expression of *GPR54* is epigenetically regulated in endometrial cancers. Considering that GPR54- endometrial cancers have a poor prognosis and a highly metastatic phenotype, combined with the fact that metastin-10 does not inhibit migration and invasion of endometrial cancer cells lacking GPR54, restoration of GPR54 is crucial to bring about the efficacy of metastin treatment on GPR54- endometrial cancers in preventing metastasis. In this regard, we tried to restore GPR54 expression in five GPR54- endometrial cancer cell lines by alleviating *GPR54* promoter hypermethylation using 5-aza-DC. Following 5-aza-DC pretreatment, matrigel invasion of both GPR54- cell lines, HEC1A and AN3CA, was significantly inhibited with metastin-10.

Thus, epigenetically-mediated restoration of GPR54 using hypomethylating agents might provide a means to resensitize cells to the antimitastatic effects of metastin-10 in GPR54- endometrial cancers. Since 5-aza-DC is already used in clinical settings (35), combined use of 5-aza-DC with metastin-10
may be effective in inhibiting metastatic spread of endometrial cancers without GPR54 expression.

Finally, the in vivo effect of metastin-10 was confirmed using a recently established orthotopic model in which metastatic ability of human endometrial cancer cells can be evaluated by assessing paraaortic lymph node metastasis from uterine transplants in nude mice (22). The frequency of lymph node metastases of GPR54+ Ishikawa cells was significantly diminished in metastin-10-treated mice. The fact that tumor size at the primary site was not significantly different between the metastin-treated and non-treated groups indicates that metastin predominantly affects metastatic potential rather than tumor growth. This is the first animal model that demonstrates the efficacy of peptide administration on the prevention of nodal metastasis from endometrial cancer with deep myometrial invasion. In clinical settings, the rate of nodal metastases approaches 26% in cases with deep myometrial invasion, and the 5-year survival rate of such cases is 30% less than those without nodal metastasis (2). In this regard, metastin-10 treatment may be effective in preventing nodal metastasis in cases with deep myometrial invasion and provide a safer alternative to lymphadenectomy.

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Table 1

Clinicopathological analysis of expression of GPR54 or metastin in 92 endometrial cancer along with each known prognostic factor including age, FIGO stage, histological grade, myometrial invasion, lymphovascular space invasion, and lymph node metastasis.

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Figure Legends

Figure1. Clinicopathological analysis of GPR54 and metastin expression in endometrial cancer. (A) Detection of GPR54 and metastin by immunostaining in endometrial cancers (x400). Top panel, grade 1 endometrioid adenocarcinoma (G1); middle panel, grade 3 endometrioid adenocarcinoma (G3); bottom, serous papillary adenocarcinoma (Serous). Scale bars, 20 μm. (B) Comparison of expression of GPR54 and metastin among histological subtypes. The expression of GPR54 and metastin was scored as follows: \( \{(0\times\% \text{none-to-weak}) + (1\times\% \text{intermediate}) + (2\times\% \text{strong})/100 \}. \) G2, grade 2 endometrioid adenocarcinoma; *, p<0.05; **, p<0.001. (C) Kaplan-Meier analysis of overall survival for expression of GPR54 and metastin with the log-rank test. *, p<0.05.

Figure2. GPR54 expression in endometrial cancer cell lines. GPR54 expression was assessed in eight endometrial cancer cell lines and hEM cells by quantitative PCR.

Figure3. Metastin suppresses cellular motility of GPR54+ endometrial cancer cells. (A) Migration rate of GPR54- HEC1A cells (left) and GPR54+ Ishikawa cells (right) was evaluated using a wound healing assay measuring the gap between the cells most closely spaced on each leading edge (red bars) at 0h and
24h post-wounding, expressed as the percentage of the distance filled. Columns, mean of fifteen independent experiments; bars, SD; *, p<0.05. (B) The number of cells able to invade through a matrigel-coated membrane was counted following mock treatment or treatment with 10μM peptide (top, metastin-10; bottom, FTM080). The average of five fields was calculated for HEC1A cells (left), Ishikawa cells (center), and GPR54+ KLE cells (right). Columns, mean of six independent experiments; bars, SD; *, p<0.05; **, p<0.001. (C) The number of invasive Ishikawa cells was calculated following GPR54 suppression. Treatments are labeled below each column. Columns, mean of six independent experiments; bars, SD; *, p<0.005.

Figure 4. **Methylation-dependent repression and reactivation of GPR54 expression.** (A) GPR54 expression and promoter methylation status was examined in eight endometrial cancer cell lines and hEM cells using quantitative PCR and MS-PCR. M, methylated; U, unmethylated. (B) GPR54 reactivation following 5-aza-DC pretreatment was subsequently analyzed by quantitative PCR. -, mock-treated; D, 5-aza-DC-treated. (C) The ability to invade matrigel in two GPR54- cell lines, HEC1A (white columns) and AN3CA (black columns), was examined following combined treatment with 5-aza-DC and metastatin-10. Left Y axis and right Y axis represent cell number of HEC1A cells and AN3CA cells, respectively. D, 5-aza-DC; D+m, 5-aza-DC and metastatin-10; Columns, mean of six
independent experiments; bars, SD; *, p<0.005.

Figure 5. *Metastin treatment suppresses GPR54+ endometrial cancer metastasis.* (A) Using the Bayesian binary method, metastasis signature probabilities were estimated for Ishikawa cells treated with metastin-10 and/or with GPR54-specific siRNAs. Treatments are labeled below each group. *, p<0.05. (B) Paraortic nodal metastasis resulting from an implanted uterine tumor. Orthotopic solid tumor growth of Ishikawa cells (circled in yellow) was observed six weeks after inoculation of a 2mm³ piece on the uterus, and the paraaortic lymph node (circled in red) beneath the left renal vein was swollen. (C) Hematoxylin and eosin staining (x100) alongside immunostaining (x400) with an anti-human cytokeratin antibody or an anti-estrogen receptor antibody of representative sections of an orthotopic tumor (left) and paraaortic node (right). ER, estrogen receptor. (D) Results of nodal metastasis analysis. The presence of tumor cells in lymph nodes was confirmed by the expression of human β2-microglobulin.
Figure 1

A

H&E | GPR54 | metastin

G1

G3

Serous

B

**

* 

score

GPR54

G1, G2, G3, Serous

metastin

C

Survival rate (%)

Days

GPR54 +

GPR54 -

metastin +

metastin -
Figure 2

The figure shows a bar graph comparing GPR54 transcription across different cell lines: HUA, RL5-2, HEC1A, HEC1B, Ishikawa, KLE, HeM cell, AN5CA, and TEN. The y-axis represents the level of GPR54 transcription, while the x-axis lists the cell lines.

GPR54 transcription levels are highest in the Ishikawa cell line, followed by KLE and HeM cell. The other cell lines show much lower transcription levels.
**Figure 3**

(A) HEC1A and Ishikawa cells were treated with control or metastin-10 for 0h and 24h, respectively. The images show the effects of metastin-10 on cell morphology.

(B) HEC1A, Ishikawa, and KLE cells were treated with control or metastin-10. The bar graphs depict the number of cells under each condition.

(C) Effects of metastin-10 and siRNA of GPR54 on cell numbers. The bars indicate the number of cells with or without the treatments.
Figure 5

A

![Graph showing the effect of siRNA on metastin-10 expression.](image)

B

![Image illustrating the effect on uterine tumor and lymph node.](image)

C

![Histological images of H&E, cytokeratin, and ER staining.](image)

D

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<th>Body Weight (g)</th>
<th>Diameter of Uterine Tumor (mm)</th>
<th>Diameter of Paraaortic Node (mm)</th>
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<td>Metastin-10 (n=11)</td>
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<td>7.18 +/- 0.70</td>
<td>2.45 +/- 0.55</td>
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* indicates a significant difference.
GPR54 is a target for suppression of metastasis in endometrial cancer

Hyun S Kang, Tsukasa Baba, Masaki Mandai, et al.

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