GPR54 Is a Target for Suppression of Metastasis in Endometrial Cancer

Hyun Sook Kang1, Tsukasa Baba1, Masaki Mandai1, Noriomi Matsumura1, Junzo Hamanishi1, Budiman Kharma1, Eiji Kondoh1, Yumiko Yoshioka1, Shinya Oishi2, Nobutaka Fujii2, Susan K. Murphy3, and Ikuo Konishi1

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, kisspeptin (KISS1), 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 the metastatic spread of endometrial cancers in combination with demethylating agents to induce GPR54 expression. Mol Cancer Ther; 10(4); 580–90. ©2011 AACR.

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 multicentered 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 antimetastatic 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 (kisspeptin) 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 to 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 2 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

Authors’ Affiliations: 1Department of Gynecology and Obstetrics, Kyoto University Graduate School of Medicine; 2Bioorganic Medicinal Chemistry, Graduate School of Pharmaceutical Science, Kyoto University, Kyoto, Japan; and 3Division of Gynecologic Oncology, Department of Obstetrics and Gynecology, Duke University Medical Center, Durham, North Carolina

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Corresponding Author: Masaki Mandai, Department of Gynecology and Obstetrics, Kyoto University Graduate School of Medicine, 54 Shogoin Kawahara-cho, Sakyo-ku, Kyoto, Kyoto 606-8507, Japan. Phone: +81-75-751-3269; Fax: +81-75-761-3967. E-mail: mandai@kuhp.kyoto-u.ac.jp

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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, 1 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 noncancer 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 done 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) or a 1:50 dilution of anti-human GPR54 polyclonal antibody LS-A1927 (MBL). 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 multihead 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 to 2 scale: 0, none-to-weak (almost negative); 1, intermediate [less positive than control, cytotoaphoblasts 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 × %weak) + (1 × %intermediate) + (2 × %strong)}/100.

Cell lines and culture

Human endometrial cancer cell lines AN3CA, HEC1A, HEC1B, HHUA, Ishikawa, and TEN (ATCC) were maintained in RPMI1640 (Nikken) supplemented with 10% FBS. KLE and RL95-2 (ATCC) were maintained in DMEM/Ham’s FI2 (Invitrogen) supplemented with 10% FBS (KLE) and with 10% FBS and 0.005 mg/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 reverse transcriptase-PCR analysis of GPR54

Total RNA was extracted from cell lines using the RNeasy Mini Kit (QIAGEN). To monitor gene expression, quantitative reverse transcriptase (RT)-PCR amplification of human GPR54 and GAPDH mRNAs was done using SYBR Premix Ex Taq (Takara) and analyzed on a Model 7000 Sequence Detector (Applied Biosystems). The following primers were used for analysis: GPR54, 5′-CGA CTT CAT CTG CAA GTT CGT C-3′ (forward), 5′-CAC ACT CAT CAT CGC 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 10 seconds and 40 cycles of 95°C for 5 seconds and 60°C for 31 seconds, followed by a dissociation cycle of 95°C for 15 seconds, 60°C for 20 seconds, and 95°C for 15 seconds. The expression of human GPR54 mRNAs was estimated by dividing the GPR54 threshold C_T values by the GAPDH C_T values.

Methylation analysis

To analyze 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 5 μmol/L 5-aza-DC (Sigma-Aldrich) for 72 hours. GPR54 expression was subsequently analyzed by quantitative PCR as described above.
GPR54 knockdown

GPR54 specific short interfering RNA (siRNA; Gene Solution siRNA catalogue 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 48 hours. The medium was aspirated and the cells were gently washed with RPMI1640 followed by transfection with the siRNAs. To confirm downregulation of GPR54 expression, quantitative RT-PCR was done 48 hours post-transfection as described above.

Proliferation assay

Ishikawa or HEC1A cells were seeded into 96-well (2 × 10^4 cells/well) tissue culture plates and incubated for 24 hours. The cell culture medium was replaced with fresh medium containing 10 μmol/L metastin-10 peptide or control peptide (16) and incubated for 24 hours. The resulting cell number was measured using a WST-1 assay kit (a modified MTT assay using a water-soluble tetrazolium salts, Premix WST-1, Takara) 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 6 cm 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 10 μmol/L metastin-10. The cells were then incubated for 24 hours to track cell motility. A total of 4 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 0 and 24 hours 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). Cell images were captured every 2 minutes for 6 hours and movies were assembled using Metamorph (Molecular Devices) as previously described (7).

Invasion assay

Invasive potential into a reconstituted basement membrane was assayed on 24-well companion plates (Becton Dickinson Labware) with an 8-μm pore polycarbonate filter coated with Matrigel (Becton Dickinson Labware) as described previously (17). The lower chamber contained 0.8 mL medium with 10% FBS as a chemoattractant. In the upper compartment, (1–2) × 10^6 cells in 0.2 mL medium were seeded and incubated with or without metastin-10 peptide at 37°C for 24 hours 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 10 μmol/L metastin-10 and/or siRNAs were generated in triplicate and RMA-normalized as described previously (18, 19). SAM was used to detect the genes specifically expressed in metastin-10 treated cells with a false discovery rate of less than 0.1 as described previously (18, 19). A metastasis gene signature was developed from 2 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; ref. 20) was also obtained from the website as above.

Orthotopic mouse model of endometrial cancer

Five- to 6-week-old CD1 Foxn1nu female mice were obtained from Oriental BioService, Inc. Animal care and experimental procedures were done in accordance with the guidelines of the Institute of Laboratory Animals Graduate School of Medicine, Kyoto University, Kyoto, Japan. Subcutaneous xenografts were established by inoculating 1 × 10^6 Ishikawa cells into the flanks. Four weeks after inoculation, subcutaneous tumors were removed and cut into ~2-mm³ 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 2 groups. Each group was treated for 15 days with intraperitoneal administration of 50 nmol metastin-10 or control, respectively, beginning 4 days after implantation. Six weeks later, paraaortic lymph node metastasis was examined as previously described (22) and tissues were preserved in RNAlater (Ambion) 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; AbD Serotec). 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 minutes, 45 cycles of 95°C for 10 seconds, 60°C for 30 seconds, and 72°C for 1 second, followed by a final 30-second extension at 40°C. Those lymph nodes with human β2-M C<sub>T</sub> < 33 were assessed as positive.

Statistical analysis

Comparison of the groups was done 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 done...
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 the International Federation of Gynecology and Obstetrics (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 (Fig. 1A), while it was weaker in grade 3 and serous adenocarcinoma (Fig. 1B, \( P = 0.0003 \) and 0.0128, respectively). On the other hand, metastin

Figure 1. Clinicopathological analysis of GPR54 and metastin expression in endometrial cancer. A, detection of GPR54 and metastin by immunostaining in endometrial cancers. Magnification, \( \times 400 \). Top, grade 1 endometrioid adenocarcinoma (G1); middle, grade 3 endometrioid adenocarcinoma (G3); bottom, serous papillary adenocarcinoma (Serous). Scale bars, 20 \( \mu \text{m} \). 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. *, \( P < 0.05 \).
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 (Fig. 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% CI, 1.211–2.026; \(P = 0.0113\)], FIGO stage (RR, 1.529; 95% CI, 1.023–2.285; \(P = 0.0190\)), histological type (RR, 1.742; 95% CI, 1.221–2.483; \(P = 0.0008\)), myometrial invasion (RR, 1.436; 95% CI, 1.025–2.012; \(P = 0.0270\)), and lymph node metastasis (RR, 1.610; 95% CI, 0.9593–2.703; \(P = 0.0291\)). 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 8 endometrial cancer cell lines was assessed by quantitative PCR (Fig. 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

<table>
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<tr>
<th>Characteristics</th>
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<th>(P) value</th>
<th>Expression of metastin</th>
<th>(P) value</th>
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![Figure 2. GPR54 expression in endometrial cancer cell lines. GPR54 expression was accessed in 8 endometrial cancer cell lines and hEM cells by quantitative PCR.](https://example.com/figure2.png)

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**Table 1** Clinicopathologic analysis of expression of GPR54 or metastin in 92 endometrial cancers along with each known prognostic factor including age, FIGO stage, histological grade, myometrial invasion, lymphovascular space invasion, and lymph node metastasis.
and GPR54+ HEC1A cells (P = 0.9768 and 0.9170, respectively, Supplementary Fig. S1). 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, Fig. 3A). Cell motility of Ishikawa cells with or without metastin-10 treatment was tracked using time-lapse microscopy over 6 hours. In the supplementary movie file, there are 2 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 show 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, Fig. 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, Fig. 3B).

To determine whether metastin has an invasion-inhibitory role through interaction with its receptor GPR54, we examined the functional consequence of GPR54

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**Figure 3.** 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 (black bars) at 0 and 24 hours postwounding, expressed as the percentage of the distance filled. Columns, mean of 15 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 10 μmol/L peptide (top, metastin-10; bottom, FTM080). The average of 5 fields was calculated for HEC1A cells (left), Ishikawa cells (center), and GPR54+ KLE cells (right). Columns, mean of 6 independent experiments; bars, SD; **, P < 0.001. C, the number of invasive Ishikawa cells was calculated following GPR54 suppression. Treatments are labeled below each column. Columns, mean of 6 independent experiments; bars, SD; *, P < 0.005.
knockdown using RNAi. We introduced 4 different 
GPR54 siRNAs into Ishikawa cells, each of which targeted 
independent sequences within GPR54 mRNA, and exam-
ined the efficiency of GPR54 knockdown using quantita-
tive RT-PCR. Among these siRNAs, the knockdown 
efficiency of siRNA4 was most effective (48.29% 
\(\pm\) 2.98%; Supplementary Fig. S2), and thus was selected 
for further analysis. There was no significant difference 
in matrigel invasion following GPR54 suppression 
(\(P = 0.3516\), Fig. 3C). Metastatin-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 methy-
lation (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 and hEM cells using quantitative 
PCR and MS-PCR. M, methylated; U, unmethylated. B, structure of 5-
aza-DC. C, GPR54 reactivation following 5-aza-DC pretreatment 
was subsequently analyzed by quantitative PCR. –, mock 
treated; D, 5-aza-DC treated. D, the ability to invade matrigel in 2 
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 6 
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 8 endometrial cancer cell lines and hEM cells using quantitative PCR and MS-PCR. M, methylated; U, unmethylated. B, structure of 5-aza-DC. C, GPR54 reactivation following 5-aza-DC pretreatment was subsequently analyzed by quantitative PCR. –, mock treated; D, 5-aza-DC treated. D, the ability to invade matrigel in 2 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 6 independent experiments; bars, SD; *, \(P < 0.005\).](image-url)
ability to invade matrigel in both GPR54− cells lines, HEC1A and AN3CA, was significantly inhibited after metastin-10 addition (Fig. 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:0050875) were Gene Ontology (GO) terms 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, Fig. 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 2 mm3 section of subcutaneous tumor, solid tumor growth was confirmed on the uterus as previously reported (Fig. 5B). On the cut surface, solid tumor grew from the uterine serosa toward the endometrium. The estrogen receptor was uniformly confirmed by the expression of human β2-microglobulin.

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:0050875) were Gene Ontology (GO) terms enriched among the upregulated 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 nonmetastatic 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 to II (P = 0.028, Supplementary Fig. S4). 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, Fig. 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 2 mm3 section of subcutaneous tumor, solid tumor growth was confirmed on the uterus as previously reported (Fig. 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 (Fig. 5C).
was observed in swollen paraaortic lymph nodes beneath the left renal vein, and they were positive for both human cytokeratin and the estrogen receptor (Fig. 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 ± 0.55 mm) than those without metastin-10 (4.1 ± 1.25 mm, \(P = 0.1686\)). 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 (\(P = 0.029\), Table 2). Together, the results obtained from the microarray analysis and the orthotopic endometrial cancer metastasis model shows the ability of metastin-10 to suppress metastasis of GPR54⁺ endometrial cancers \textit{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 7 representative metastasis suppressor genes divided the 146 endometrial cancers into 2 clusters, with high grade cancers enriched in cluster 1 (\(P = 0.0045\), Supplementary Fig. S5). 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, metastin 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 metastin 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 metastin-GPR54 axis is relevant to the migration and invasion of endometrial cancer cells \textit{in vitro}. Previous reports showed that metastin prevented migration or invasion in several cancers (23, 28–30) and human umbilical vein endothelial cell (31). In this study, we used metastin-10, a short peptide of 10 amino acids which is proteolytically processed from metastin. Metastin-10 is 10 times as active as metastin and regarded as a candidate for clinical use (16). Migration of the GPR54⁺ Ishikawa endometrial cancer cell line was significantly inhibited by metastin-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 metastin-10 treatment while cell division of treated cells appeared similar to that of nontreated 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

### Table 2. Results of nodal metastasis analysis

<table>
<thead>
<tr>
<th></th>
<th>Body weight, g</th>
<th>Diameter of uterine tumor, mm</th>
<th>Paraaortic node</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 10)</td>
<td>29.93 ± 0.76</td>
<td>7.25 ± 1.93</td>
<td>4.10 ± 1.25</td>
</tr>
<tr>
<td>Metastin-10 (n = 11)</td>
<td>29.15 ± 0.64</td>
<td>7.18 ± 0.70</td>
<td>2.45 ± 0.55</td>
</tr>
</tbody>
</table>

\(\text{Table 2. Results of nodal metastasis analysis}\)

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Metastin-10 Suppresses Metastasis of Endometrial Cancer

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 antimetastatic 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 were silenced by DNA methylation (14). In this study, promoter methylation was present in 7 endometrial cell lines and 5 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 5 completely methylated cell lines relative to the other 3 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 high 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 5 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 antimetastatic 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 nontreated groups indicates that metastin predominantly affects metastatic potential rather than tumor growth. This is the first animal model that shows 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.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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GPR54 Is a Target for Suppression of Metastasis in Endometrial Cancer

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


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