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regulate DNA repair pathways and to be upregulated in several late-stage human cancers, including those of the breast, colon, glioblastoma, and soft tissue sarcoma (13–16). Overexpression of the IFN–STAT1 pathway is also associated with poor prognosis in different types of cancer (17–20), especially in breast cancers in which increased IFN–STAT1 pathway activity is considered a marker predicting resistance to chemotherapy and radiotherapy (17, 21). The actual function of STAT1 and associated regulatory mechanisms in cancer are not fully understood as well as the significance of the IFN–STAT1 pathway in SPEC.

In this study, we demonstrate constitutively active STAT1 expression in SPEC as compared with other histologic subtypes of endometrial cancers. We also present novel findings showing that in SPECs, STAT1 functions as a driver that modulates expression of downstream genes such as *MYC*, which in turn promotes the cellular capacity for proliferation, migration, invasion, and xenograft tumorigenecity. We therefore propose that STAT1 functions as a prosurvival factor in SPECs, in a manner important to tumor progression, and may be a novel target for molecular therapy in this disease.

Materials and Methods

Patients and tissues

Clinicopathologic information ($n = 294$) and specimens ($n = 91$) from patients treated for endometrial cancer between 2004 and 2012 at Kyoto University Hospital were obtained with written consent from each patient and used under protocols approved by the Kyoto University Institutional Review Board.

Tissue microarrays obtained from the BC Cancer Agency and Vancouver General Hospital (Vancouver, Canada) included specimens from 460 endometrial cancers in five tissue microarrays (355 endometrioid and 105 SPECs). These were examined independently and were used as an external validation. All patients provided informed written consent and the research was approved under the University of British Columbia and BC Cancer Agency.

Cell lines and culture

Human endometrial cancer cell lines, HEC1A (ATCC), HEC50B (JCRB), Ishikawa (National Hospital Organization, Japan), SPAC-1L (The Cancer Institute of the Japanese Foundation for Cancer Research, Japan) were used for further functional assays as described precisely in the Online Repository and were regularly tested for *mycoplasma* contamination, and were authenticated by STR analysis.

STAT1 knockdown

STAT1-specific short interfering RNAs (siRNA: FlexiTube siRNA Qiagen; catalogue no. SI02662884), *MYC*-specific siRNA (FlexiTube siRNA Qiagen; catalogue no. GS4609), and negative control siRNA (AllStars Negative Control siRNA; Qiagen) were transfected into cell lines using HiPerFect Transfection Reagent (Qiagen) as previously described (22). For establishing *STAT1* stably suppressed cells, STAT1-shRNA (HuSH 29mer shRNA pGFP-V-RS; Origene) and negative control shRNA (scrambled shRNA cassette; Origene) were transfected using Turbofectin 8.0 Transfection Reagent, and stably transfected cells were selected with puromycin treatment (0.5–1.0 μg/mL; Nacalai Tesque). A dominant-negative *STAT1* DNA plasmid (pBOS-STAT1-DN; Osaka University Graduate School of Medicine, Japan; ref. 23) was also transfected with Lipofectamine 2000 for obtaining *STAT1* dominant-negative cells, as described previously (24).

Microarray analysis

Gene expression microarray was generated from 63 endometrial cancer samples (GSE56026) using Affymetrix U133 Plus 2.0 gene chips (Affymetrix). The Significance Analysis of Microarrays (SAM) software (http://statweb.stanford.edu/~tibs/SAM/) was used to detect genes distinguishing type II cancers from type I cancers as described previously (25, 26). Supervised hierarchical clustering of these genes was performed and graphically viewed as a dendogram and heatmap using Cluster 3.0 and Java TreeView. The published microarray dataset TCGA UCEC_ 2013 (3) was also analyzed using this method, and the expression pattern for the group of genes commonly up- or downregulated in type II cancers was designated as a type II signature. The cBioPortal for Cancer Genomics database (http://www.cbioportal.org/public-portal/) was used to analyze genetic oncoprints of SPEC. Connectivity Map analysis (Cmap; http://www.broadinstitute.org/cmap/) was used to mine potential therapeutic agents for SPECs based on genes for which expression was altered by STAT1 suppression. Using a Bayesian binary regression model as previously reported (2), the MYC signature was generated and applied to our datasets *in vitro* and *in vivo* for assessing MYC pathway activity in endometrial cancers.

In vivo experiments

Subcutaneous xenografts were established in the flanks of female NOD-SCID mice (Nihon Clea) by inoculating 5×10^6 SPAC-1L cells with and without STAT1 alteration by dominant-negative and shRNA transduction methods. Tumor growth in inoculated mice was sequentially monitored twice a week for 8 weeks by measuring the volume of tumors.

Statistical analysis

Group comparisons were done using Mann–Whitney *U* tests. Prognostic analysis was performed using the log-rank test, Fisher exact test, and multivariate analysis. All statistical analyses were done using GraphPad Prism 5.5, SPSS ver. 22, and R software. Probability values below 0.05 were considered significant.

A complete description of the materials and methods, and any associated references are available in the Online Repository.

Results

Prognosis and gene profiling of endometrial cancers

A total of 294 patients were treated for endometrial cancers in Kyoto University from 2004 to 2012 (endometrioid grade 1,

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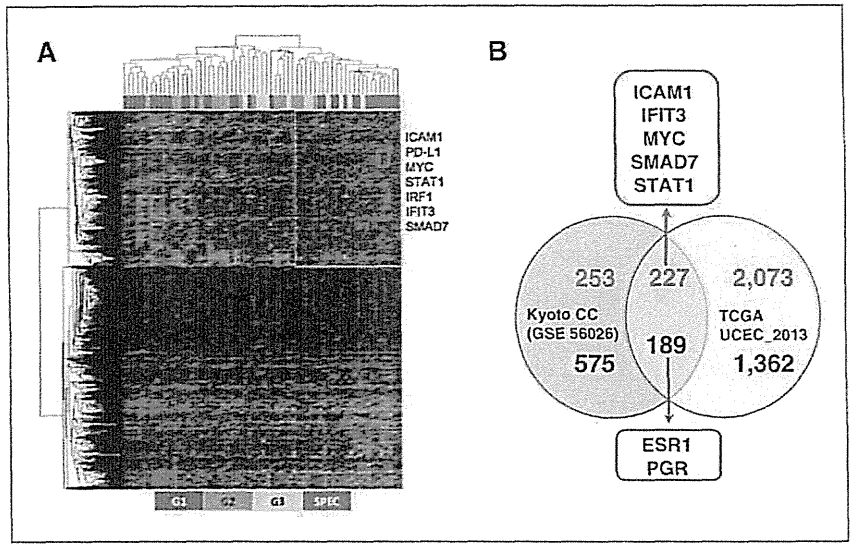

Figure 1. Microarray gene expression across histologic subtypes of endometrial cancers. A, supervised analysis of 1,244 genes for which expression in G3 and SPEC was significantly different from G1–G2 (*t* test, $P < 0.005$). Blue bar, G1 ($n = 22$); green bar, G2 ($n = 18$); yellow bar, G3 ($n = 11$); and red bar, SPEC ($n = 12$). SPECs are enriched in a subcluster within which *STAT1* and associated genes *ICAM1*, *PD-L1*, *MYC*, *IRF1*, *IFIT3*, and *SMAD7* are all highly expressed. B, a Venn diagram showing the overlap of 227 upregulated genes (red) and 189 downregulated genes (green) in the SPEC-enriched clusters of both the Kyoto University human endometrial cancer samples (CC) and TCGA UCEC_2013 microarray datasets. *STAT1* and associated genes are included among the 227 upregulated genes.

G1 $n = 148$; endometrioid grade 2, G2 $n = 55$; endometrioid grade 3, G3 $n = 53$; and SPEC $n = 38$). The disease-specific survival rates of patients bearing G3 and SPEC cancers were lower than patients with G1–G2 cancers ($P < 0.0001$; Supplementary Fig. S1A) and extra-uterine spread was more frequently observed in G3 and SPEC than in G1–G2 ($P < 0.0001$ and $P < 0.0001$, respectively; Supplementary Table S2). Thus, type II cancers, G3 and SPEC, exhibit unfavorable outcome with highly progressive features.

To investigate whether these distinct differences resulted from specific gene profiles, gene expression microarray analysis was conducted for 63 endometrial cancers (G1 $n = 22$; G2 $n = 18$; G3 $n = 11$; and SPEC $n = 12$). Unsupervised clustering analysis of gene expression revealed that SPECs compose a distinct cluster apart from the G1–G2 enriched clusters (Supplementary Fig. S1B). Furthermore, using supervised clustering with a total of 1,244 genes, the expression of G3 and SPEC was statistically different from G1–G2 (*t* test, $P < 0.005$). The 63 endometrial cancer samples were divided into two clusters, but SPECs were enriched in only one of the subclusters ($P < 0.0001$, Fig. 1A). The same result was found in the TCGA UCEC_ 2013 dataset. There were 416 overlapping genes between these datasets, whose gene expression profiles were subsequently designated as the "type II signature" (227 upregulated and 189 downregulated genes; Fig. 1B, Supplementary Fig. S1C, and Supplementary Table S3). Among these 227 upregulated genes, STAT1 and many STAT1-associated genes such as *MYC*, *ICAM1*, and *SMAD7* were highly expressed in the SPEC-rich cluster (Fig. 1A and B and Supplementary Table S3A). Conversely, *ESR1* and *PGR* were included among the 189 downregulated genes (Fig. 1B and Supplementary Table S3B). SAM analysis confirmed that STAT1 and its associated genes were highly expressed in the SPEC-rich cluster (Supplementary Fig. S1D and Supplementary Table S4).

STAT1 expression and clinical significance in endometrial cancers

The expression of STAT1 in 91 patients with endometrial cancer (mean age, 59.1 years) was assessed by immunohistochemistry (G1 $n = 35$; G2 $n = 16$; G3 $n = 18$; and SPEC $n = 22$; Table 1). The expression of STAT1 was observed within the nucleus of cancer cells with weak staining in G1–G2, weak to intermediate staining in G3, and strong staining in SPEC (Fig. 2A). The STAT1 expression score of G3 tumors was higher than that of G1 tumors ($P < 0.0001$), but SPEC staining was much higher than that of G3 tumors ($P < 0.01$) and G1–G2 tumors ($P < 0.0001$, Fig. 2B). These findings were also confirmed in the Vancouver tissue microarrays as an external validation, in which STAT1 was highly expressed in SPEC ($P < 0.0001$, Fig. 2C). The expression of *STAT1* mRNA was also higher in SPEC ($P < 0.05$), confirmed by similar results in our microarray data, the TCGA UCEC_2013 microarray dataset, and two additional datasets, GSE17025 and GSE24537 (Supplementary Fig. S2A–S2E). Intriguingly, around 77% (17 of 22) of SPEC cases contain infiltrated $CD8^+$ immune cells at the tumor front with strongly positive staining of ICAM1 and PD-L1 (Fig. 2A).

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Table 1. Clinicopathologic analysis of STAT1 expression in 91 endometrial cancers along with each known prognostic factor

Characteristics	Expression of STAT1[a] Low	High	P
Age			
≤60	30	20	0.1259
>60	18	23	
FIGO stage			
I	35	20	
II	7	1	0.0006
III	4	14	
IV	2	8	
Histology			
G1	32	3	
G2	9	7	<0.0001
G3	6	12	
Serous	1	21	
Myometrial invasion[b]			
No invasion	11	4	0.0229
≤1/2 (inner half)	19	9	
>1/2 (outer half)	16	24	
Lymphovascular space invasion[b]			
−	34	17	0.0193
+	12	18	
Lymph node metastasis[b]			
−	44	26	<0.0001
+	1	14	
Ascites[b]			
−	38	25	0.1628
+	9	12	
Recurrence risk			
Low	22	2	<0.0001
Intermediate	17	13	
High	9	28	

NOTE: Statistical significance was analyzed by the χ^2 test.
[a]STAT1 score: >1, high; ≤1, low.
[b]Some data were missing: 8 cases, 10 cases, 6 cases, and 7 cases, respectively.

The disease-specific survival of patients bearing STAT1-high cancers was significantly shorter than those bearing STAT1-low cancers ($P < 0.0001$, Fig. 2D), and this tendency was also confirmed in the Vancouver setting ($P < 0.05$; Supplementary Fig. S2G). Furthermore, as shown in Table 1, high expression of STAT1 was associated with known prognostic factors of tumor progression, including deep myometrial invasion ($P < 0.05$), lymphovascular space invasion ($P < 0.05$), lymph node metastasis ($P < 0.0001$), and consequently, high risk of recurrence ($P < 0.0001$). Multivariate analysis revealed that high STAT1 expression was a prognostic factor independent from histologic subtypes and FIGO stages ($P < 0.05$; Supplementary Table S5).

STAT1 pathway activity in endometrial cancer cells

To investigate the STAT1 pathway in endometrial cancers, mRNA levels of *STAT1* and STAT1-associated genes in endometrial cancer cells with/without modulation of *STAT1* was assessed by quantitative real-time PCR (qRT-PCR). Ishikawa, HEC1A, HEC50B, and SPAC-1L cell lines were used as representative of G1, G2, G3, and SPEC, respectively. *STAT1* encodes two alternatively spliced isoforms, STAT1α and STAT1β, which is induced by homodimeric IFNγ via its receptors, IFNγR1 and IFNγR2 (18). In endometrial cancer cell lines, *STAT1β* and *IFNγR2* mRNAs are generally expressed at higher levels than *STAT1α* and *IFNγR1*, and are highly expressed in SPAC-1L cells (Fig. 3A and E). *STAT1* mRNA expression was significantly upregulated by IFNγ but attenuated by siRNA-mediated suppression of *STAT1*. This modulation of *STAT1* expression was most prominent in SPAC-1L cells at both mRNA and protein levels but not in other representative cell lines (Fig. 3A and D, Supplementary Fig. S5D and E). Furthermore, *STAT1* expression was augmented by IFNγ in a dose-dependent manner (Fig. 3D and Supplementary Fig. S3A).

The mRNA expression levels of STAT1-associated genes, including *CD274* (also known as *PD-L1*), *ICAM1*, *IRF1*, *SMAD7*, and *CCL7* (also known as *MCP3*; refs. 27–29), were examined by qRT-PCR following treatment with IFNγ and/or *STAT1*-siRNA. Figure 3B and C show that IFNγ treatment upregulates *PD-L1* and *ICAM1* mRNA expression in SPAC-1L cells (>60-fold and nearly 15-fold, respectively) and HEC50B cells (2.4-fold and 1.5-fold, respectively). Increased expression was observed in a dose-dependent manner and was reversed by suppression of *STAT1* (Fig. 3D; Supplementary Fig. S3B and S3C). In SPAC-1L cells, similar IFNγ augmentation of mRNA expression was observed for *IRF1*, *SMAD7*, and *MCP3* (~12-fold, 2-fold, and 3-fold, respectively), and an observed attenuation of expression following treatment with STAT1-siRNA (Supplementary Fig. S3D–S3F). These results indicate that a SPEC cell line, SPAC-1L, is highly responsive to IFNγ induction through IFNγR to activate STAT1-associated genes.

STAT1 functions as a tumor prosurvival and proprogression gene in SPEC cells

STAT1 has previously been considered to function as a tumor suppressor to induce cell-cycle arrest and apoptosis in various types of cancers (30, 31). To determine how *STAT1* contributes to cell growth and survival in SPEC, various *in vitro* functional assays were performed using SPAC-1L cells following manipulation of *STAT1* activity. In addition to *STAT1*-siRNA transfected cells (*STAT1*-siRNA cells), SPAC-1L cells with stable suppression of *STAT1* were generated for further examination by introducing *STAT1*-specific shRNA (*STAT1*-shRNA) or a dominant-negative plasmid (*STAT1*-DN3 and *STAT1*-DN5 cells; Supplementary Fig. S4A–S4C).

To assess the potential role of STAT1 on prosurvival properties, assays for proliferation and colony formation in soft agar were performed. As a result, cellular proliferation was suppressed in a time-dependent manner in both *STAT1*-siRNA cells ($P < 0.0001$, Fig. 4A) and *STAT1*-DN5 cells ($P < 0.0001$; Supplementary Fig. S4D). Similarly, anchorage-independent

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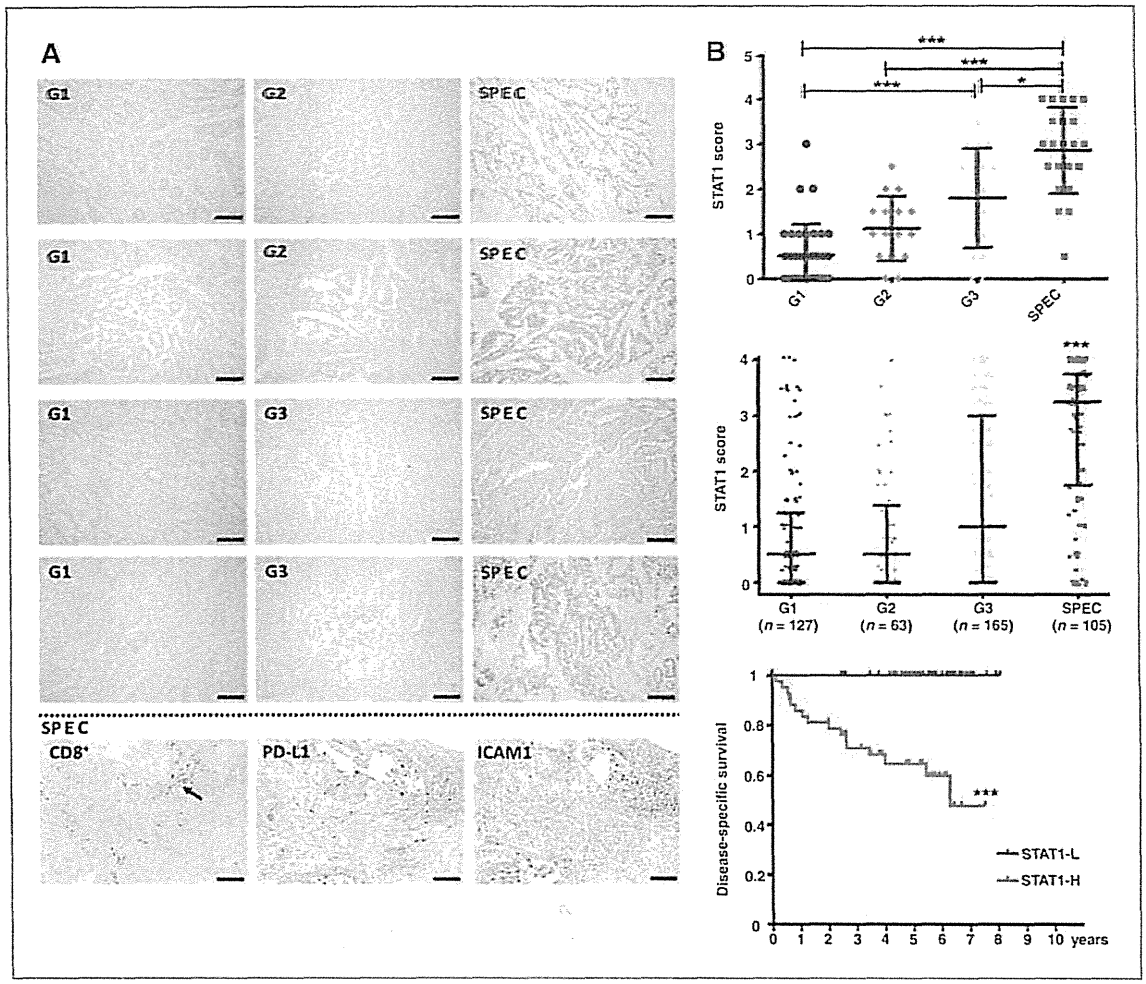

Figure 2. Immunohistologic analysis of STAT1 expression in human endometrial cancers. A, representative micrographs of STAT1 expression in each histologic subtype (×400). Top, left, G1; middle top, G2; middle bottom, G3; right, SPEC. The expression of STAT1 was observed in the cancer cells; near negative-weak in G1–G2, weak–intermediate in G3, and positive in SPEC. Below dashed line, immune microenvironment of SPEC. Left, $CD8^+$ expression; mid, PD-L1 expression; right, ICAM1 expression. Scale bars, 20 μm. Arrow, abundant $CD8^+$ cells surround the tumor invasive front with high expression of PD-L1 and ICAM1. B, the expression of STAT1 was scored on the basis of staining of cancer cells as follows: 0, negative; 1, weak positive (weak intensity and ≤25% area stained); 2, intermediate (weak intensity and 25%–50% area stained); 3, positive (prominent intensity and 50%–75% area stained); and 4, strong positive (prominent intensity and ≥75% area stained). STAT1 expression was significantly higher in high-grade endometrial cancers (G3 and SPEC; *, $P < 0.05$; ***, $P < 0.0001$); κ-agreement between observers are 0.823, 0.848, and 0.822, respectively. C, the expression of STAT1 from the Vancouver endometrial cancer tissue microarrays was scored on the basis of staining of cancer cells. STAT1 expression was higher in SPECs ($P < 0.0001$); κ-agreement between observers are 0.892, 0.982, and 0.910, respectively. D, Kaplan–Meier analysis of disease-specific survival for 91 patients with endometrial cancers in the Kyoto cohort with respect to intensity of STAT1 expression. STAT1-L, expression score ≤1; STAT1-H >1. The prognostic outcome is worst in STAT1-H ($P < 0.0001$).

growth capacity was impaired in *STAT1*-shRNA cells ($P < 0.0005$, Fig. 4B) and *STAT1*-DN5 cells ($P < 0.0001$: Supplementary Fig. S4E).

To assess the impact of STAT1 on progression, adhesion, and migration, Boyden-chamber assays were performed with IFNγ induction. Adhesive capacity was augmented by IFNγ ($P < 0.0001$), which was mitigated by *STAT1* suppression ($P < 0.0005$, Fig. 4C). Cellular adhesive capacity was also suppressed in STAT1-DN5 cells and STAT1-siRNA cells ($P < 0.0001$), whereas STAT1-DN3 cells did not show the same level of suppression (Supplementary Fig. S4F). The numbers of SPAC-1L cells attached to the single-layered HUVEC cells decreased with knockdown of *STAT1*, but attachment was recovered with concurrent IFNγ treatment. Second, SPAC-1L cellular motility over a 24-hour tracking period was accelerated by IFNγ treatment, although this acceleration was not observed in STAT1-shRNA cells (Fig. 4D). Boyden-chamber assays demonstrated that cellular invasive activity

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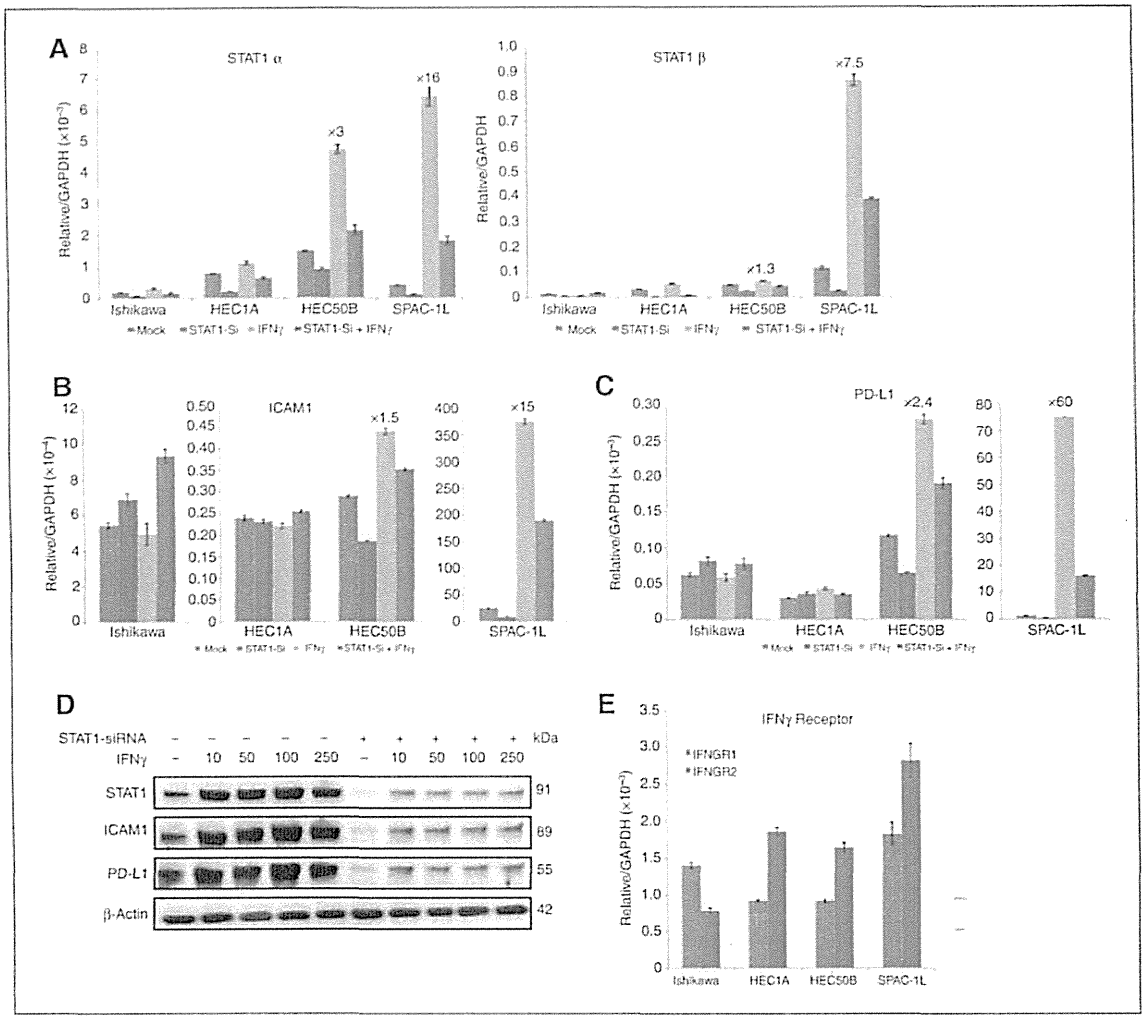

Figure 3. STAT1 pathway activity in endometrial cancer cells. A–C, expression of *STAT1*, *ICAM1*, and *PD-L1* mRNAs in endometrial cancer cell lines was assessed by qRT-PCR. Blue bar, mock-treated; red bar, *STAT1*-suppressed with siRNA; green bar, IFNγ treated; and purple bar, treated with both STAT1-siRNA and IFNγ. SPAC-1L, a cell line derived from SPEC, showed high responsiveness to IFNγ treatment with induction of *STAT1* (×16), *ICAM1* (×15), and *PD-L1* (×60) mRNAs. STAT1-siRNA treatment suppressed *STAT1*, *ICAM1*, and *PD-L1* mRNA expression in HEC50B (G3 cell line) and SPAC-1L. D, expression of STAT1 and proteins from STAT1-associated genes, ICAM1 and PD-L1, in SPAC1-L cells by Western blotting with/without STAT1-siRNA and/or IFNγ treatment. E, IFNγ receptor status within endometrial cancer cell lines. The expression of IFNγ receptor1 (*IFNGR1*) and IFNγ receptor2 (*IFNGR2*) was examined in four endometrial cancer cell lines by qRT-PCR.

was augmented by IFNγ ($P < 0.0005$), but this augmentation was remarkably suppressed by *STAT1* knockdown ($P < 0.0001$). Indeed, cellular invasion was suppressed in all cells with *STAT1* knockdown (Fig. 4E and Supplementary Fig. S4G). These *in vitro* results suggest that high expression of STAT1 in SPEC might contribute to aggressive cell behavior via enhanced proliferation and capacity for disease progression.

STAT1 pathway significance in tumorigenesis

To further determine the roles of the STAT1 pathway *in vivo*, NOD-SCID mice were used to compare the tumorigenic capacity of STAT1-shRNA cells with that of the parental SPAC-1L cells. Among 15 mice inoculated with STAT1-shRNA cells, xenograft tumors were observed in only four mice, all less than 50 mm^3 in size, whereas all 10 mice inoculated with SPAC-1L cells formed large tumors ($P < 0.0001$, Fig. 5A). This tumorigenic inhibition was also observed for STAT1-DN5 cells ($P < 0.0001$; Supplementary Fig. S5A). Thus, the suppression of *STAT1* expression inhibited tumor growth in NOD-SCID mice, likely via attenuation of oncogene function. The Connectivity Map (Cmap) analysis showed gene expression changes resulting from STAT1

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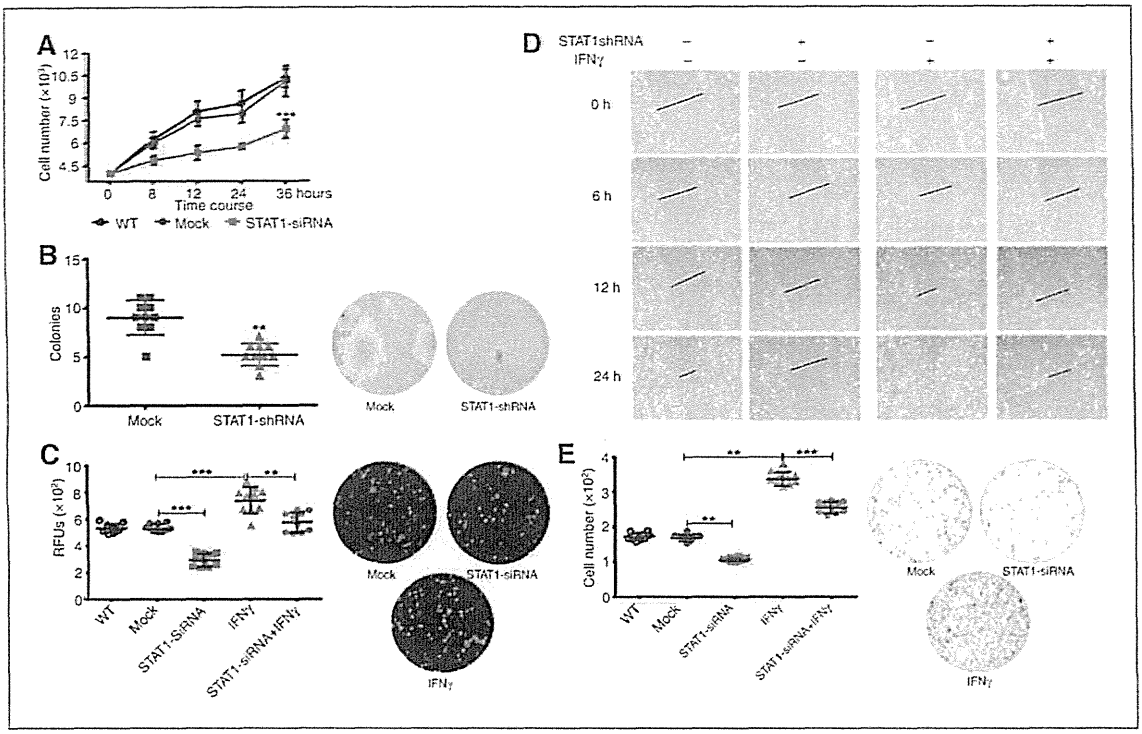

Figure 4. STAT1 functions to promote tumor cell survival and progression in SPEC cells. WT, nontreated cells; mock, negative control siRNA/shRNA–treated cells; STAT1-siRNA, STAT1-siRNA–treated cells; IFNγ, IFNγ-treated cells. A, STAT1 regulates cell proliferation. Proliferation in SPAC-1L cells was assessed by WST-1 assays in quintuplicate with or without STAT1-siRNA. Cell proliferation decreased significantly in STAT1-siRNA cells ($P < 0.0001$). B, *STAT1* inhibition decreases anchorage-independent growth. SPAC-1L cell colony formation in soft agar was assessed with or without *STAT1* knockdown. Left, colony numbers are significantly decreased by downregulating *STAT1* gene expression with STAT1-shRNA ($n = 10$, $P = 0.0003$). Right, representative pictures of cells cultured in soft agar. C, STAT1 modulates cell adhesion. After coculturing with HUVEC cells for 4 hours, cell adhesion was measured by spectrophotometer as relative fluorescence units (RFU). Left, downregulating STAT1 expression results in reduced SPAC-1L adhesion ($n = 10$; ***, $P < 0.0001$) and mitigates the inducing effect of IFNγ (**, $P = 0.0005$). Right, representative pictures of attached cells in each condition. D, STAT1 promotes cell migration. The effect of STAT1 on migration was assessed using wound-healing assays. The migration rate was evaluated in quadruplicate by measuring the gap between the cells most closely spaced on each leading edge at 0, 6, 12, and 24 hours postwounding. *STAT1* knockdown impairs migration of SPAC-1L cells and also lessens the inducing effect of IFNγ. E, STAT1 modulates invasion. Invasive potential of SPAC-1L cells was assessed using Boyden-chamber assays. Left, by downregulating *STAT1* using STAT1-shRNA, the number of invading cells was decreased ($n = 10$; **, $P < 0.0005$) and reduced the promoting effect of IFNγ (***, $P < 0.0001$). Right, representative micrographs of hematoxylin-stained cells that have invaded through the membrane.

suppression were not associated with changes induced by doxorubicin or paclitaxel but were associated with those induced by some bioactive molecules including sirolimus as the top-ranked candidate compound (Supplementary Table S6 and Supplementary Fig. S5B).

The *MYC* promoter region has STAT1 binding sites, and STAT1 has been reported to maintain basal expression of *MYC* during tumorigenesis (32). Both *MYC* and *STAT1* were highly expressed in SPECs (Fig. 1B), although the functional implications for these findings have not yet been clarified. We therefore investigated whether STAT1 regulates the oncogenic function of MYC. Similar to other genes, *MYC* mRNA expression was induced 12-fold following treatment with IFNγ ($P < 0.0001$; Fig. 5B and Supplementary Fig. S5G), but this induction was remarkably attenuated in STAT1-shRNA cells not only *in vitro* ($P < 0.0001$), but also in xenograft tumors (23-fold, $P < 0.0001$, Fig. 5C). Western blotting showed that induction of STAT1 was accompanied by upregulation of MYC, whereas suppression of STAT1 resulted in decreased MYC expression *in vitro* and *in vivo* (Fig. 5D). This was also true for the STAT1-DN5 cells, as MYC expression was not induced by IFNγ treatment (Supplementary Fig. S5C). In contrast, MYC expression was not remarkably changed in non-SPEC cell lines by STAT1 manipulation (Supplementary Fig. S5D and S5F–S5G). A predictive MYC signature score can be used to represent MYC pathway activity for a given specimen based on the expression levels of known MYC target genes (33). The MYC signature scores of STAT1-siRNA cells were significantly lower than that of SPAC-1L cells ($P < 0.05$), whereas that in SPECs was higher than that of endometrioid adenocarcinomas ($P < 0.05$ Supplementary Fig. S5D and S5E). To clarify the functional role

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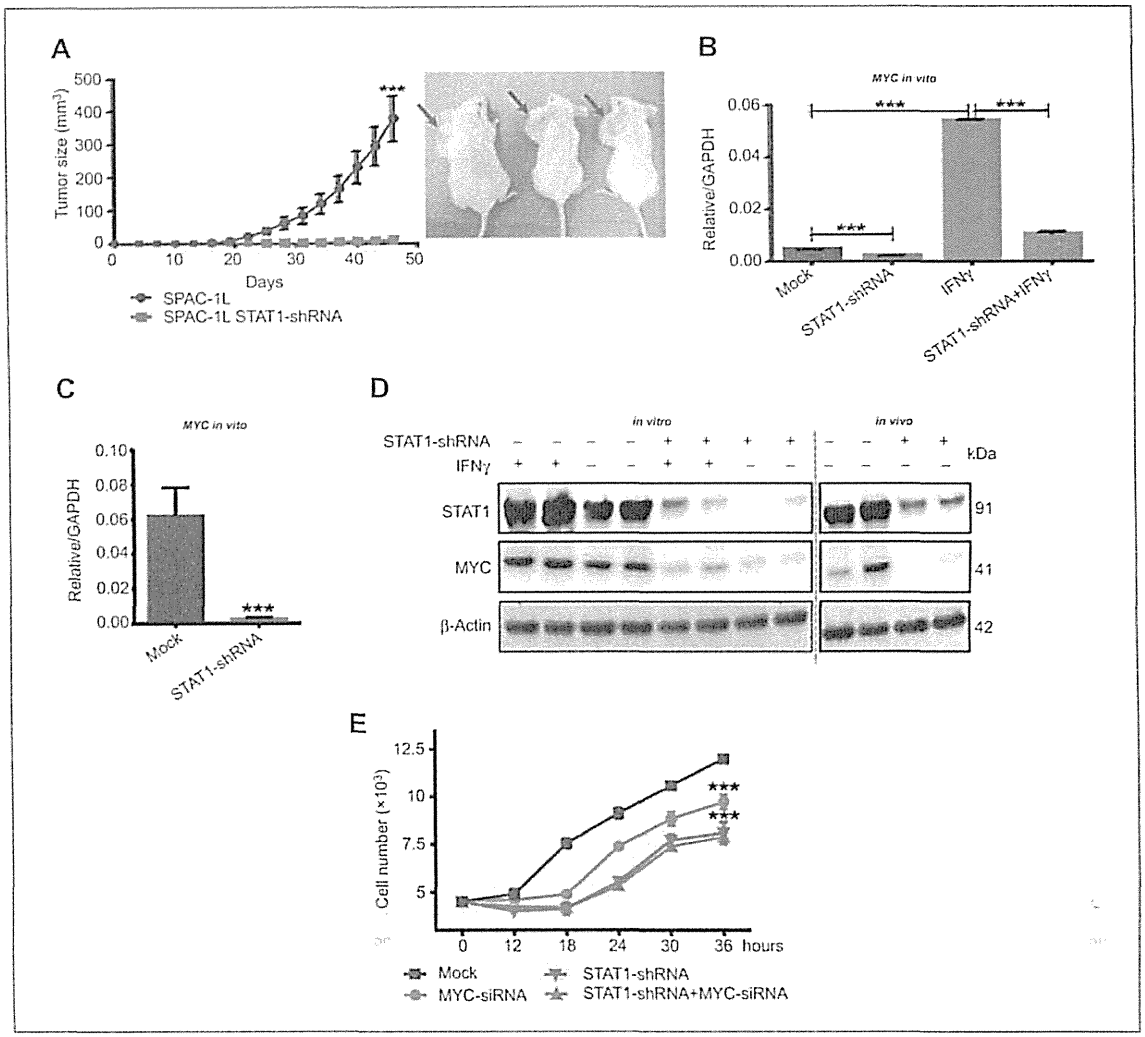

Figure 5. Significant role of the STAT1 pathway in tumorigenesis. A, *STAT1* downregulation inhibits tumorigenesis *in vivo*. NOD-SCID mice were subcutaneously inoculated with 5×10^6 SPAC-1L cells with and without STAT1 knockdown. Tumor growth was inhibited in mice inoculated with STAT1-shRNA cells ($n = 25$, $P < 0.0001$). Right, arrows indicate growing tumors of SPAC-1L cells. B, STAT1 regulates the *MYC* oncogene. *STAT1* knockdown suppressed *MYC* mRNA expression ($n = 5$; $P < 0.0001$) and diminished the inducing effect of IFNγ ($P < 0.0001$). C, *MYC* mRNA expression in xenograft tumors. *MYC* expression was repressed in STAT1-suppressed xenograft tumors ($P < 0.0001$). D, STAT1 regulates MYC *in vitro* and *in vivo*. MYC expression was assessed by Western blotting. MYC expression was suppressed by prior *STAT1* knockdown, *in vitro* and *in vivo*. E, proliferative capacity is regulated by STAT1 via MYC. Proliferation is inhibited by MYC-siRNA–mediated knockdown ($n = 10$; $P < 0.0001$), but double knockdown of *STAT1* and *MYC* showed no additive effect as compared with STAT1 knockdown alone in suppression of cellular proliferation ($n = 10$, *ns*).

of the IFNγ–STAT1–MYC axis, cell proliferation assays were performed under conditions of cosuppression of STAT1 and MYC *in vitro*. As shown in Fig. 5E, MYC suppression led to a significant reduction of SPAC-1L cellular proliferation ($P < 0.0001$). This inhibitory effect was more prominent in STAT1-shRNA cells ($P < 0.0001$). Cosuppression of *STAT1* and *MYC* showed no significant difference in inhibitory effect as compared with *STAT1* suppression alone. These results suggest that STAT1 contributes to regulation of oncogenic MYC expression to promote cancer cell proliferation and tumor growth.

Discussion

SPECs account for only 4% to 10% of endometrial cancer, are highly aggressive, and are difficult to treat effectively, whereas low-grade endometrioid adenocarcinoma comprises 80% of endometrial cancer and is frequently diagnosed

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at an early stage, at which point it is surgically curable (3, 34). As SPEC is chemorefractory and patients bearing SPEC usually exhibit unfavorable outcomes with multiple metastases and/or recurrence, SPEC has attracted a great deal of attention to determine characteristic malignant features that are distinct from endometrioid adenocarcinoma. We have previously described fludarabine as a potent therapeutic candidate for chemorefractory endometrial cancer (2). Its predicted efficacy was higher for G3 and SPEC than for G1–G2, but the precise mechanism explaining fludarabine efficacy or its therapeutic target(s) in this setting is unknown. Recent integrated genomic analysis demonstrated that SPEC exhibited specific genomic features that were quite different from the endometrioid subtype, but rather were shared with ovarian serous and basal-like breast carcinomas (3). However, the representative genes or pathways responsible for SPEC's aggressive malignant phenotype were not well clarified from these studies. Thus, further study of SPEC with regard to these findings is needed to determine the principal underlying genetic signature to not only improve understanding of SPEC-specific tumor biology but, more importantly, for developing novel targeted therapies based on this biology.

In our cohort setting, patients with SPEC exhibited poor outcome (Supplementary Fig. S1A) and a distinct gene expression profile (Fig. 1A) as compared with other subtypes of endometrial cancer. These results were compatible with those of the TCGA UCEC_2013 dataset (3); therefore, we sought to identify SPEC-specific signature genes that were commonly activated in the SPECs in our dataset and in the TCGA dataset. As the Venn diagram demonstrates in Fig. 1B, 227 genes were highly expressed in SPECs, including *STAT1* and STAT1-associated genes such as *MYC*, *SMAD7*, and *IFIT3*, which have been reported to be involved in carcinogenesis or cancer progression (32, 35, 36), and these genes were also detected as SPEC-specific upregulated genes in another SAM analysis (Supplementary Fig. S1D and Supplementary Table S4). In contrast, *ESR1* and *PGR* were among the downregulated genes, which is compatible with the hormone-independent feature of SPEC. Several genome-wide analyses have described distinctive gene expression patterns in SPEC from analysis of a single dataset without any external validation (37, 38); nevertheless, the SPEC driver genes or pathways were not conclusively identified. At the same time, interobserver reproducibility in subtype classification of type II endometrial cancers was poor (3, 39) and traditional subtype classification itself sometimes failed to distinguish SPEC from high-grade endometrioid endometrial carcinoma, as several G3 cases and two SPEC cases were not classified with their counterparts by clustering analysis in this study (Fig. 1A and Supplementary Fig. S1B and S1C). To address this issue, we identified STAT1 as a key molecule of the SPEC-signature that was commonly upregulated in both the datasets studied, and this was accompanied by the upregulation of putative downstream STAT1 gene targets. We confirmed high STAT1 expression in SPEC by external validation using GSE17025 and GSE24537 (Supplementary Fig. S2D and S2E; refs. 37, 38).

Immunohistochemical (IHC) staining confirmed high STAT1 expression in SPEC and demonstrated the association of STAT1 expression with worse clinical outcome accompanied by prognosis indicators, including deep myometrial invasion, lymphovascular space invasion, and lymph node metastasis (Fig. 2, Table 1). The most robust findings, that STAT1 was expressed significantly higher in SPECs than in the endometrioid subtype and that STAT1-high endometrial cancers had worse prognostic outcome (Fig. 2B and D), were externally validated by IHC finding in a large Vancouver cohort (Fig. 2C and Supplementary Fig. S2G). Thus, high expression of STAT1 is an indicator of recurrence, and is independently related to shorter disease-free survival, which is consistent with previous reports showing STAT1 overexpression or genes induced by IFNγ, is associated with poor clinical outcomes of breast cancers (19, 40). STAT1 expression in a SPEC cell line, SPAC-1L, was constitutively high, and augmented by IFNγ in a dose-dependent manner accompanied with similar augmentation of expression of several STAT1-associated genes (Fig. 3). STAT1-associated genes, such as *ICAM1*, *MYC*, *PD-L1*, are known to play roles in the progression and metastasis of other types of cancer through mechanisms specific to the type of malignancy (14, 29, 36, 41, 42). This augmentation by IFNγ was specifically prominent in SPAC-1L, and attenuated by suppressing *STAT1* expression. These results imply that overexpression of *STAT1* might be associated with the clinical aggressiveness of SPECs and that the IFNγ signal was transduced through STAT1, although the source of IFNγ *in vivo* is unknown.

Among the STAT1-associated genes, *ICAM-1*, *PD-L1*, and *SMAD7* are involved in cancer immunity (43), and proteomic analysis revealed multiple proteins associated with inflammation are overexpressed in the uterus with endometrial cancer (44). Immunohistochemical staining demonstrated that SPEC has a characteristic tumor microenvironment at its invasive front that is fully infiltrated by $CD8^+$ immune cells (Fig. 2A), which are a known source of IFNγ (41). As SPAC-1L cells have relatively high transcription of IFNγ receptors 1 and 2, the tumor microenvironment could be a potential source of persistent IFNγ *in vivo* that results in the constitutive activation of the STAT1 pathway for attenuating cancer immunity to promote progression in SPEC. Further experiments are required to determine the reason why this scenario is not the case for the endometrioid subtypes.

Previous studies showed that STAT1 activates antiproliferative and proapoptotic genes as a tumor suppressor (45); in contrast, STAT1 in SPEC appears to function as a tumor prosurvival gene. Alteration of STAT1 function using a dominant-negative plasmid and/or siRNA decreased the malignant characteristics of SPAC-1L cells, while restoration of these features occurred with IFNγ treatment to activate the STAT1 pathway (Fig. 4 and Supplementary Fig. S4D–S4G). With regard to IFNγ–STAT1 pathway genes, ICAM1 is involved in sphere formation and metastatic potential (46), and increased expression at invasive fronts is positively correlated with invasion and metastasis (47). SMAD7 is

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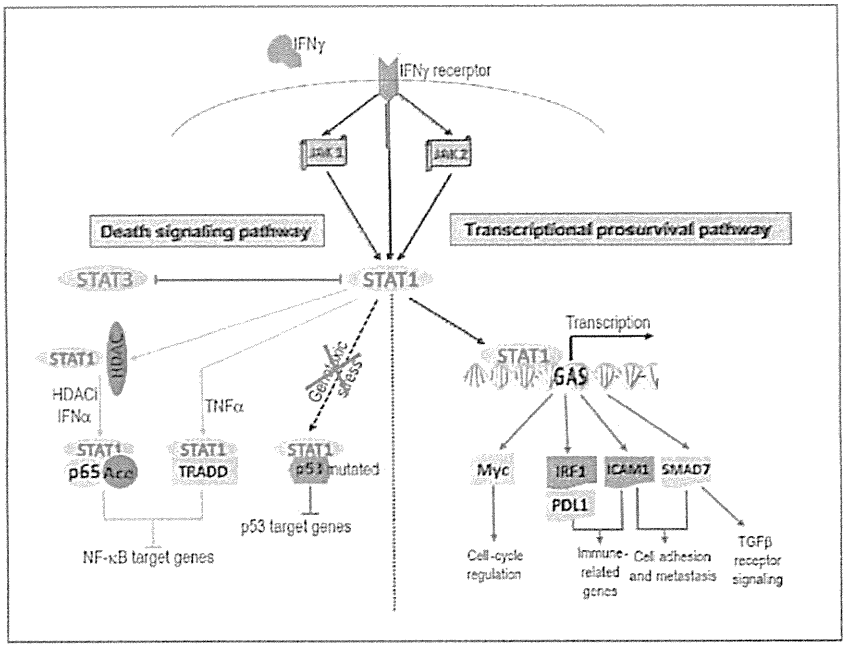

Figure 6. Schematic showing STAT1 roles as a driver gene of SPEC in modulating "transcriptional prosurvival pathways" to enhance malignant capacity. In response to IFNγ stimulation, dimerization of the IFN receptor allows phosphorylation and activation of JAKs (JAK 1 and JAK 2), and this is followed by STAT1 activation through phosphorylation, leading to the formation of a STAT1 homodimer. The STAT1 homodimer activates gene expression by binding to the IFNγ responsive element, GAS, as previously reported (9). This binding leads to activation of transcriptional activity in promoting some STAT1-associated genes, including *MYC*, *IRF1*, *PD-L1*, *SMAD7*, and *ICAM1*. MYC activation positively affects cell-cycle regulation; ICAM-1, PD-L1, and IRF1 are involved in cancer immunity (41) in the tumor microenvironment; SMAD7 functions in TGFβ signaling, and ICAM1 and SMAD7 affect metastatic capability. These effects suggest that STAT1 functions as a driver gene in a tumor prosurvival pathway. In contrast, reciprocal regulation between STAT1 and STAT3 has also been described as a "Death Signaling Pathway" (9); TP53 mutation is common in SPEC and is expected to inhibit this death signaling pathway.

involved in tumorigenesis of mesenchymal stem cells concomitant with upregulation of MYC under prolonged inflammatory IFNγ exposure (48). Cellular capacity for migration, anchorage-independent growth, and attachment are important prerequisites for tumor invasion and metastasis (49). The highly progressive features of SPEC might be partially due to constitutively high STAT1 expression and consequent upregulation of downstream STAT1 target genes under a highly orchestrated series of tumor microenvironment components, and we propose that constitutively high STAT1 expression in SPEC has a tumor-promoting role rather than a tumor-suppressing role. This is further supported by our observation that xenograft tumor growth was remarkably inhibited by repression of *STAT1* expression. Although metastasis was not observed in the NOD-SCID mouse model, this might be because progression outside of the primary tumor requires exogenous IFNγ. From these experiments, however, we can conclude that STAT1 pathway activation in SPEC cells is essential for tumor growth.

We then investigated the mechanism by which STAT1 works as a tumor-promoting gene as well as potential target molecules. MYC is one of the Yamanaka genes essential for cellular proliferation, and both STAT1 and STAT3 competitively bind to the MYC promoter to regulate expression (9, 32). Because the gene expression microarray data revealed that STAT3 is not highly expressed in SPEC, it was reasonable to consider that *MYC* might be regulated by STAT1 in SPEC as a growth-promoting driver gene. To support this notion, 108 of the 227 upregulated genes in the SPEC signature harbor the MYC binding site motif by GATHER analysis (VSMYCMAX_B; http://gather.genome.duke.edu/), and the predictive MYC activity signature score was statistically higher in SPEC than in other subtypes of endometrial cancers, whereas that in SPAC-1L cells was diminished with *STAT1* knockdown (Fig. 5 and Supplementary Fig. S5). As the suppressive effect on proliferation by double knockdown of *STAT1* and *MYC* was not superior to knockdown of *STAT1* alone (Fig. 5E), STAT1 may function as the master modulator of MYC activity. The cBioPortal for Cancer Genomics database indicated that aberration of the STAT1–MYC axis was more frequently found in SPECS (51%) than in endometrioid subtypes (17.6%; data not shown), whereas there were no SPEC samples with downregulation of STAT1 or MYC. As MYC upregulation was not observed in endometrioid cell lines (Supplementary Fig. S5D, S5F, and S5G), these results imply that more than half of SPECs have aberrant upregulation of the STAT1–MYC axis as a particular driver oncoprint of their aggressiveness. This STAT1-

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MYC signature may be viewed as a SPEC-specific target. Cmap analysis based on SPEC's specific gene signature predicted doxorubicin and paclitaxel as not effective for STAT1-high tumors, and many SPECs are indeed clinically resistant to these drugs. In contrast, sirolimus was identified as the top-most candidate drug for STAT1-high tumors out of the 72 candidates identified (Supplementary Table S6 and Supplementary Fig. S5B). As temsirolimus, a derivative of sirolimus, was identified as a potential drug for chemorefractory SPECs in our prior study (2), sirolimus might be a novel candidate for SPEC although further studies are warranted.

In summary, a genome-wide analysis together with functional assays revealed that *STAT1* is constitutively activated in SPEC. This activation may be the result of the tumor microenvironment and results in promotion of tumor growth and extra-uterine spread via sequential activation of STAT1 downstream genes. Generally, STAT1 has been considered as a tumor suppressor involved in the "death signaling pathway," but in this study of SPEC, STAT1 was identified as a master gene modulating "transcriptional prosurvival pathways" to enhance multiple malignant characteristics (Fig. 6). SPEC is highly aggressive and chemorefractory such that patients with SPEC often have poor outcomes, thus the development of novel treatment strategies based on the biology of this disease is an urgent need. Our findings support that targeting STAT1, the SPEC driver gene, may provide the means to improve poor outcomes for patients with SPEC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Novelty and Impact: We declare that all data are novel, developed by our own experiments, and have not been published or submitted for publication. We have revealed new insights regarding STAT1 as a driver of tumor progression in refractory serous papillary endometrial cancers (SPEC). Our study, based on bioinformatic analysis and *in vitro/in vivo* experiments, also suggests STAT1 functions as a tumor prosurvival gene rather than a tumor suppressor gene. These findings will inform much needed therapeutic strategies for SPEC by targeting the SPEC driver gene, *STAT1*, to improve the poor outcome of this disease.

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ORIGINAL PAPER

Menstrual cyclic change of metastin/GPR54 in endometrium

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Abstract Metastin/kisspeptin is encoded by *KISS1* and functions as an endogenous ligand of GPR54. Interaction of metastin with GPR54 suppresses metastasis and also regulates release of gonadotropin-releasing hormone, which promotes secretion of estradiol (E2) and progesterone (P4). We have previously demonstrated epigenetic regulation of *GPR54* in endometrial cancer and the potent role of metastin peptides in inhibiting metastasis in endometrial cancer. However, little is known about how the metastin–GPR54 axis is regulated in the endometrium, the precursor tissue of endometrial cancer. Endometrial stromal cells (ESCs) and endometrial glandular cells (EGCs) within the endometrium show morphological changes when exposed to E2 and P4. In this study, we show that metastin expression is induced in ESCs through decidualization, but is repressed in glandular components of atypical endometrial hyperplasia (AEH) and endometrial cancer relative to EGCs. The promoter of *GPR54* is unmethylated in normal endometrium and in AEH. These results indicate metastin may function in decidualized endometrium to prepare for adequate placentation but this autocrine secretion of metastin is deregulated during oncogenesis to enable tumor cells to spread.

Keywords Metastin · GPR54 · Decidualization · Endometrium · Endometrial cancer

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Introduction

Uterine endometrial cancer is the leading cause of malignant gynecological disease, and invasion from the endometrium into the deep myometrium and lymphovascular space is a critical life-threatening risk factor that is frequently followed by metastasis. In clinical settings, the rate of nodal metastases approaches 26 % in cases with deep myometrial invasion, and the 5-year survival rate for these cases is 30 % less than those without nodal metastasis. It is crucial to establish novel therapeutic approaches to prevent tumor invasion and metastasis in order to improve the prognosis of patients with endometrial cancer. Various molecules associated with tumor invasion have been intensively investigated as potential therapeutic targets. However, none of these efforts to establish metastasis-suppressing therapies have been successfully translated to clinical practice, even though novel metastasis-regulating molecules or pathways have been identified.

KISS1 was originally described as a metastasis suppressor in melanoma, and has since been identified as a candidate metastasis suppressor in several cancers [1, 2]. The *KISS1* gene encodes a number of metastin/kisspeptin peptides via alternative splicing that function as

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endogenous ligands for the G protein-coupled receptor, GPR54. One of the splice variants, metastin-10, can be chemically synthesized and has been proved safe for administration in vivo. We have previously described that decreased expression of GPR54 is frequently observed in histologically high-grade endometrial cancers and is a poor prognostic factor relevant to both invasive and metastatic capacity for such subtypes [1]. We also showed that metastin was ubiquitously expressed in cancer stromal cells and that administration of metastin-10 could suppress extrauterine tumor metastasis via the metastin–GPR54 axis. However, little is known about how the metastin–GPR54 axis is regulated in the human endometrium, the source of endometrial cancer.

The human endometrium exhibits dynamic changes in the shape and function of both endometrial stromal cells (ESCs) and endometrial glandular cells (EGCs) under ovarian hormonal controls. Previous reports revealed that in ESCs and EGCs there were significant changes in expression observed through the menstrual cycle for several proteins associated with cellular migration, including E-cadherin, Slug, Snail, and S100. There is cross-talk in neuroendocrine cells between the expression of metastin and GPR54 and the release of gonadotropin-releasing hormone (GnRH) to control the timing of puberty and the secretion of estradiol (E2) and progesterone (P4). ESCs demonstrate decidualization and secrete prolactin in late-secretory phase in response to long E2 and P4 exposure, and this phenomenon evokes the notion to consider the endometrium as an endocrine organ. There are two reports describing the expression of metastin and GPR54 in the endometrium [3, 4], but their conclusions are different and these reports did not investigate expression of these proteins specifically in ESCs and EGCs. In this study, the expression of metastin and GPR54 in ESCs and EGCs was investigated to clarify the regulation of the metastin–GPR54 axis in the endometrium and to determine feasibility of metastin-10 use as adjuvant therapy to prevent metastasis and preserve fecundity in young endometrial cancer patients.

Materials and methods

Tissue collection

Human endometrial tissues were obtained following written consent for the use of surgical specimens from patients who underwent hysteroscopy or hysterectomy under protocols approved by the Kyoto University Institutional Review Board. As the source of normal endometrium for immunohistochemical staining, formalin-fixed paraffin-embedded specimens containing the endometrial part of the uterus were used which had been excised from patients with regular menstrual cycles and no hormonal administration but who had a cervical intraepithelial neoplasm or myoma uteri ($n = 12$, age: 32–46 years old). Each endometrial specimen was examined histologically and dated according to published criteria [5]. Non-fixed endometrial specimens for isolated cell culture were obtained by scraping part of the endometrium from patients without endometrial disease or hormonal administration (Table 1) at the time of hysterectomy or endometrial curettage as previously reported [6].

Cell culture

Isolation of endometrial stromal cells (ESCs) and glandular cells (EGCs) was performed as described previously [6, 7]. Endometrial tissues from healthy donors with normal menstrual cycles (Table 1) were minced into small pieces <1 mm^3 and incubated at 37 °C in RPMI1640 (Nikken, Kyoto, Japan) supplemented with 10 % fetal bovine serum (FBS: Funakoshi, Tokyo, Japan), 0.5 % collagenase I (Wako Pure Chemicals, Osaka, Japan) and 0.05 % deoxyribonuclease I (DNAase I; Sigma-Aldrich, St. Louis, MO). After the enzymatic digestion, ESCs were present as single cells or small aggregates, which were repeatedly purified by differential sedimentation at unit gravity. On the other hand, EGCs remained in larger clumps, and these clumps were re-digested into a single cell suspension and incubated on a collagen type IV-coated plate (IWAKI, Tokyo, Japan). After >90 % purification [6, 7], ESCs and EGCs were independently maintained in the conditioned medium, phenol red-free RPMI1640 (Invitrogen, Carlsbad, CA) supplemented with 5000 U/ml penicillin, 5000 μg/ml streptomycin (Nacalai tesque, Kyoto, Japan), and 2 % charcoal/dextran-treated FBS (Thermo Fischer Scientific Inc., Walthum, MA). Endometrial stromal cells and EGCs were incubated for 12 days in this conditioned medium with 10^{-8} M 17-β estradiol (E2; Nacalai tesque) or 10^{-6} M medroxyprogesterone acetate (MPA; Sigma-Aldrich) alone, or with both reagents for further experimentation as previously reported [8–11]. These experiments were carried out independently three times, each in triplicate. An immortalized human endometrial glandular cell line, hEM cells, and a grade 1 endometrioid adenocarcinoma cell line, Ishikawa cells, were maintained as previously reported [1] and used for analysis after incubation in the conditioned media.

Real-time quantitative PCR analysis

Total RNA was extracted from the cultured cells or incised tissues with TRIzol reagent (Invitrogen, Carlsbad, CA) using the manufacturer's recommended protocol. First-

Table 1 Characteristics of donors with cyclic menstrual cycles who provided their endometrial tissue for the primary culture of ESCs and EGCs

Patient	Age	Cycle date	Procedure	Surgical indication	ESCs	EGCs
1	47	9	TAH	CIN3	+	−
2	43	17	TAH	Myoma uteri	+	−
3	34	20	TAH + RSO + pOM	Right ovarian tumor	+	+
4	33	10	RH + BSO + PeN	Cervical cancer stage Ib1	+	−
5	34	15	Hysteroscopy + curettage	Endometrial thickness	+	+
6	45	23	TAH	Myoma uteri	−	+
7	45	22	Hysteroscopy + curettage	Endometrial thickness	−	+

TAH total abdominal hysterectomy, *RSO* right salpingo-oophorectomy, *pOM* partial omentectomy, *RH* radical hysterectomy, *BSO* bilateral salpingo-oophorectomy, *PeN* pelvic lymphadenectomy, *CIN3* cervical intraepithelial neoplasia grade 3, *ESCs* endometrial stromal cells, *EGCs* endometrial glandular cells

strand cDNA was synthesized using the transcriptor high-fidelity cDNA synthesis kit (Roche, Basel, Switzerland). To monitor gene expression, quantitative reverse transcriptase (RT)-PCR amplification of human *IGFBP-1*, *Metastin*, *GPR54* and *GAPDH* mRNAs was performed using the LightCycler 480 II system (Roche). Primers were designed using Universal Probe Library Assay Design Center (https://qpcr.probefinder.com/organism.jsp) and their sequences are as follows: IGFBP-1, 5′-CCA TGT CAC CAA CAT CAA AAA-3′ (forward), 5′-CCT TGG CTA AAC TCT CTA CGA CTC-3′ (reverse); Metastin, 5′-GGT GGT CTC GTC ACC TCA G-3′ (forward), 5′-CTA GAA GTG CCT TGA GGC TTG-3′ (reverse); GPR54, 5′-TTC ATG TGC AAG TTC GTC AAC-3′ (forward), 5′-CAC ACT CAT GGC GGT CAG-3′ (reverse); GAPDH, 5′-AGC CAC ATC GCT CAG ACA C-3′ (forward), 5′-GCC CAA TAC GAC CAA ATC C-3′ (reverse). Cycling parameters were 95 °C for 10 min and 45 cycles of 95 °C for 10 s, 60 °C for 30 s and 72 °C for 1 s, followed by a cooling cycle of 40 °C for 30 s. The expression values for human *IGFBP-1*, *Metastin*, and *GPR54* mRNAs were estimated by dividing their threshold cycle (Ct) values by the GAPDH Ct values. The methylation status of the *GPR54* promoter in endometrial tissues (5 normal and 2 atypical endometrial hyperplasia; AEH) was examined by MS-PCR as previously described [1].

Immunohistochemistry

Immunohistochemical staining was done using the streptavidin–biotin peroxidase complex method as previously reported [12]. 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 1:100 dilution of anti-human GPR54 polyclonal antibody AKR-001 (Alomone Labs, Islael). The primary antibody was omitted for negative controls.

Microarray analysis and statistical analysis

GSE12446 and GSE29981, two independent endometrial tissue gene expression microarray datasets, were obtained from the gene expression omnibus website (http://www.ncbi.nlm.nih.gov/geo).

For statistical analysis, Mann–Whitney *U* test or Fisher's exact test was performed using GraphPad Prism 5 software. Probability values below 0.05 were considered significant.

Results

Expression of metastin and GPR54 in endometrial tissues

The expression of metastin and GPR54 was assessed by immunohistochemistry of proliferative phase ($n = 5$), early secretory phase ($n = 3$), late-secretory phase ($n = 4$) and menopausal ($n = 2$) endometrial tissues. Metastin exhibited weak expression in epithelial glands and was nearly absent in ESCs during the proliferative phase (Fig. 1a). This expression pattern was similar in the early secretory phase, but metastin expression in ESCs was prominent in the decidualized portion at late-secretory phase endometrial tissues (Fig. 1a). After menopause, the endometrium does not exhibit expression of metastin or GPR54, both in epithelial glands and stroma (Fig. 1a).

We previously reported on GPR54 expression in low-grade endometrioid adenocarcinoma [1]. To compare the intensity of expression, we examined staining of endometrium and adjacent grade 1 endometrioid adenocarcinoma on the same slide. The patient was 43 years old, and the non-cancerous endometrium was thick, exhibiting a mid-secretory appearance. GPR54 expression was weaker in the epithelial glands than in the adenocarcinoma lesion,

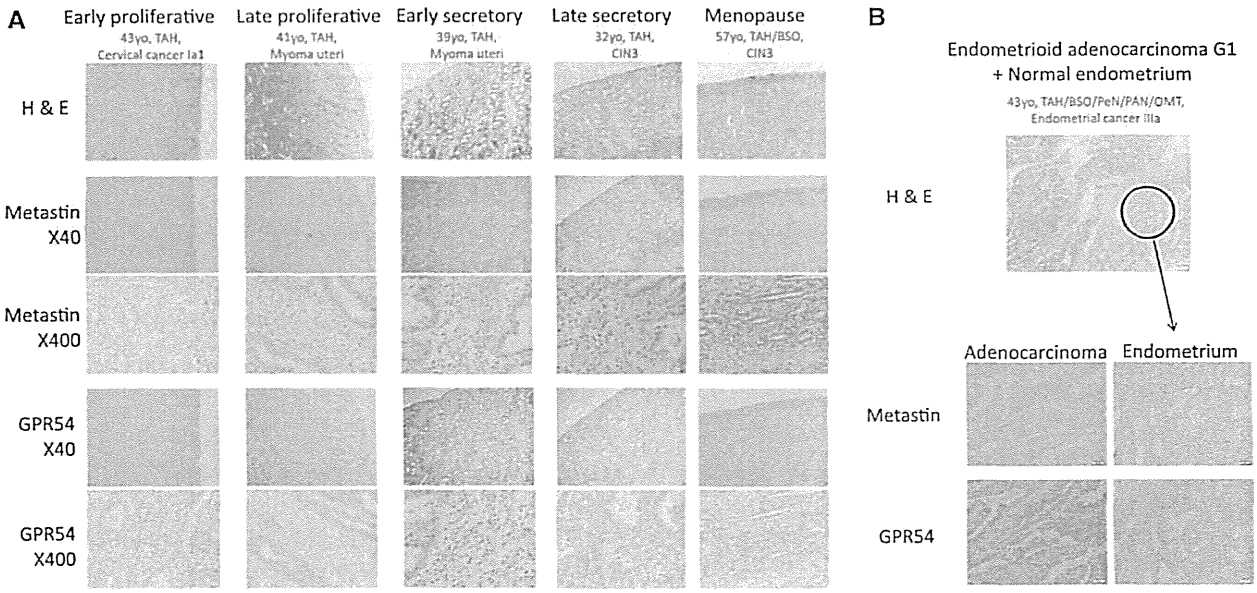

Fig. 1 Immunohistological analysis of metastin and GPR54 in human endometrial tissues. **a** Representative micrographs of metastin and GPR54 expressions in each menstrual phase. Metastin staining is weak in the epithelial glands but nearly absent in ESCs during the proliferative and early secretory phases, while metastin expression in ESCs is prominent in the decidualized component during late-secretory phase. No expression of metastin or GPR54 is observed both in epithelial glands and stroma during the menopausal phase. **b** Comparison of metastin and GPR54 expressions in co-localized endometrial tissue and endometrial cancer grade 1. GPR54 is weakly expressed in the epithelial glands relative to the adenocarcinoma lesion, whereas metastin expression is weak in both components. *H&E* hematoxylin and eosin staining, *yo* years old, *CIN3* cervical intraepithelial neoplasm grade 3, *TAH* total abdominal hysterectomy, *BSO* bilateral salpingo-oophorectomy, *PeN* pelvic lymphadenectomy, *PAN* paraaortic lymphadenectomy, *OMT* omentectomy

whereas metastin expression was weak in both regions (Fig. 1b).

Primary culture of endometrial cells and analysis of metastin and GPR54 expressions in ESCs

Primary cultures of endometrial tissues were established for seven donors listed in Table 1. None of these donors had a menstrual disorder or endometrial malignancy. Viable ESCs and EGCs were successfully isolated from five donors whose endometrium was in secretary phase, four in proliferative phase and four in early secretary phase.

Isolated ESCs were treated with hormone-conditioned medium for 12 days, and mRNA expression was examined by quantitative RT-PCR. *IGFBP1* is induced in ESCs through decidualization [13]. IGFBP1 expression was significantly augmented with E2 plus MPA treatment ($p = 0.0264$, Fig. 2a), while there was no significant induction with E2 or MPA alone ($p = 0.3929$). Mimicking decidualization, metastin expression in cultured ESCs was induced through treatment with E2 plus MPA ($p = 0.0179$, Fig. 2b). In contrast, GPR54 expression in ESCs was lower than that in an endometrioid adenocarcinoma grade 1 cell line, Ishikawa cells, and this was not augmented with treatment.

Metastin and GPR54 expressions in EGCs

We were unable to isolate EGCs from proliferative phase endometrium probably owing to limited glandular components. Metastin expression in endometrial glands appeared relatively higher during late-secretory phase than proliferative phase (Fig. 1a), and that in primarily cultured EGCs derived from secretory phase endometrium (sec-EGCs) was almost equivalent to that in primarily cultured ESCs (Supplementary Fig. 1A). In contrast with the result from ESCs, metastin expression in sec-EGCs was not augmented by treatment with E2 and MPA (Supplementary Fig. 1B). To consider the possibility that sec-EGCs were already differentiated, hEM cells were used as an alternative. However, metastin expression was not induced by E2 with or without MPA in hEM cells (Fig. 2b).

GPR54 expression in sec-EGCs appeared higher than that in ESCs, but was much lower than that in Ishikawa cells (Supplementary Fig. 1A). GPR54 expression in sec-EGCs or hEM cells was also not significantly augmented by E2 with or without MPA (Fig. 2b and Supplementary Fig. 1B). In a microarray dataset of normal endometrial tissues (GSE29981), GPR54 expression in micro-dissected endometrial glands gradually increased through the proliferative phase, and high GPR54 expression was more

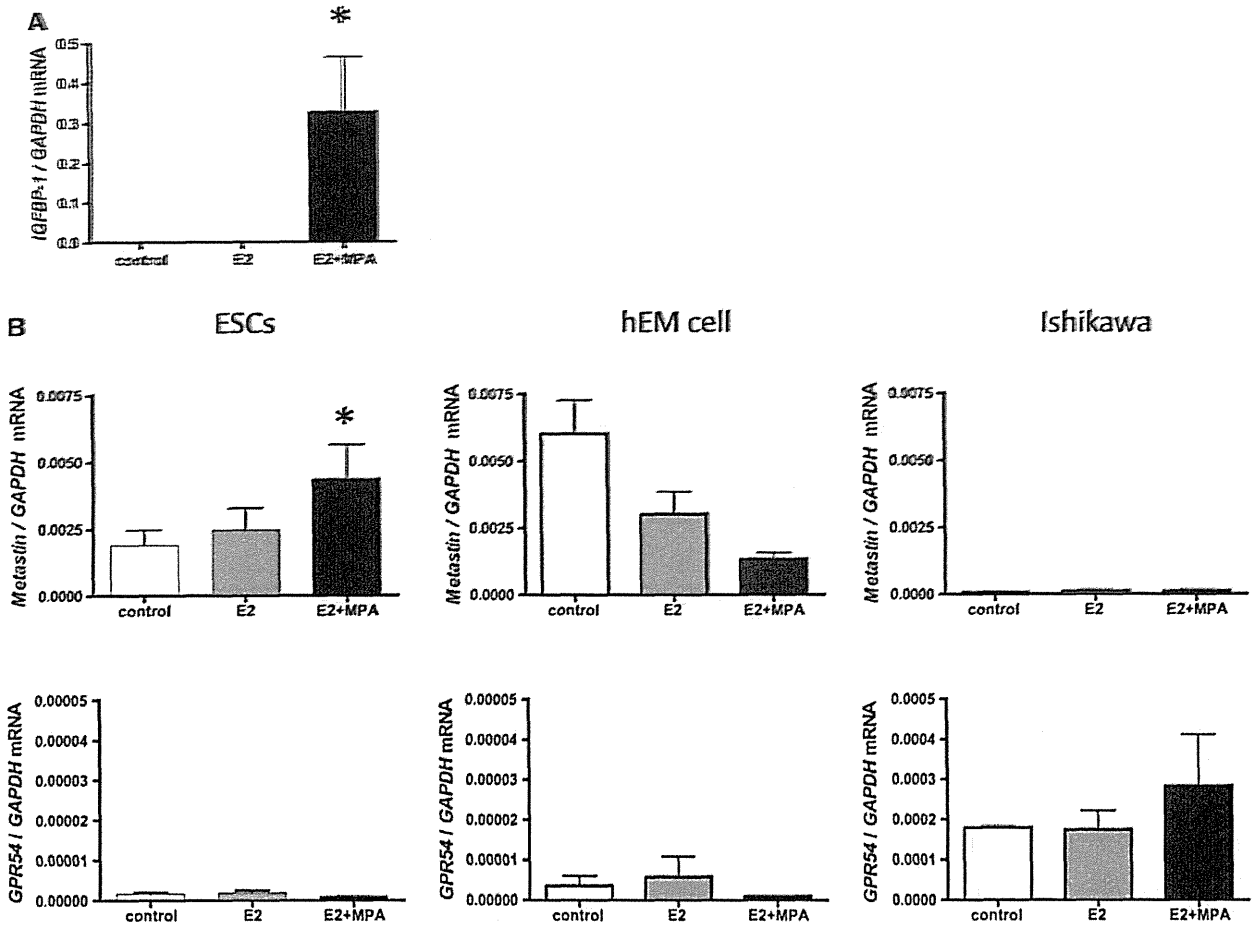

Fig. 2 mRNA expression in ESCs through 12 days of E2 and MPA treatment. **a** IGFBP1 expression in ESCs is significantly augmented following treatment with E2 plus MPA (*: $p = 0.0264$), while no induction is observed with E2 alone. **b** Metastin and GPR54 expressions in cultured ESCs (*left panels*), hEM cells (*middle panels*), and Ishikawa cells (*right panels*). Metastin is induced in ESCs following treatment with E2 plus MPA (*: $p = 0.0179$) but not in other cells. GPR54 expression is lower in ESCs and hEMA cells than in Ishikawa cells, and is not augmented following treatment with E2 alone or with E2 plus MPA

frequently observed from late-proliferative phase to early secretory phase (Supplementary Fig. 2A, $p = 0.01$, Fisher's exact test). In another microarray dataset of whole endometrium treated with E2 and MPA (GSE12446), high metastin expression was more frequently observed (Supplementary Fig. 2B, $p = 0.05$, Fisher's exact test). In GSE12446, *prolactin* expression was significantly higher following E2 and MPA treatment ($p = 0.0001$), indicating that the change in metastin expression occurred with decidualization.

GPR54 expression differences between normal endometrium and endometrial hyperplasia and promoter methylation status

GPR54 expression was markedly higher in grade 1 endometrioid adenocarcinoma (EmG1) and in Ishikawa cells relative to EGCs (Fig. 1a and Supplementary Fig. 1A). As EGCs are thought to transform into endometrial hyperplasia and EmG1 in a stepwise manner through the oncogenic process, *GPR54* expression in atypical endometrial hyperplasia (AEH) was examined by comparing alongside that in the normal endometrial glandular portion of the same specimens. Among seven AEH cases (age 50.1 ± 3.4 years), two cases exhibited higher relative *GPR54* expression in atypical hyperplastic glands than adjacent EGCs, whereas most cases exhibited similar expression in both. In two peri-menopausal patients, metastin was expressed in EGCs of dilated and elongated endometrial glands although it was scant in AEH (Fig. 3a).

The intensity of *GPR54* expression in endometrial cancer was attenuated by methylation in the promoter region although this region was unmethylated in hEM [1]. We therefore analyzed the methylation status of the *GPR54*

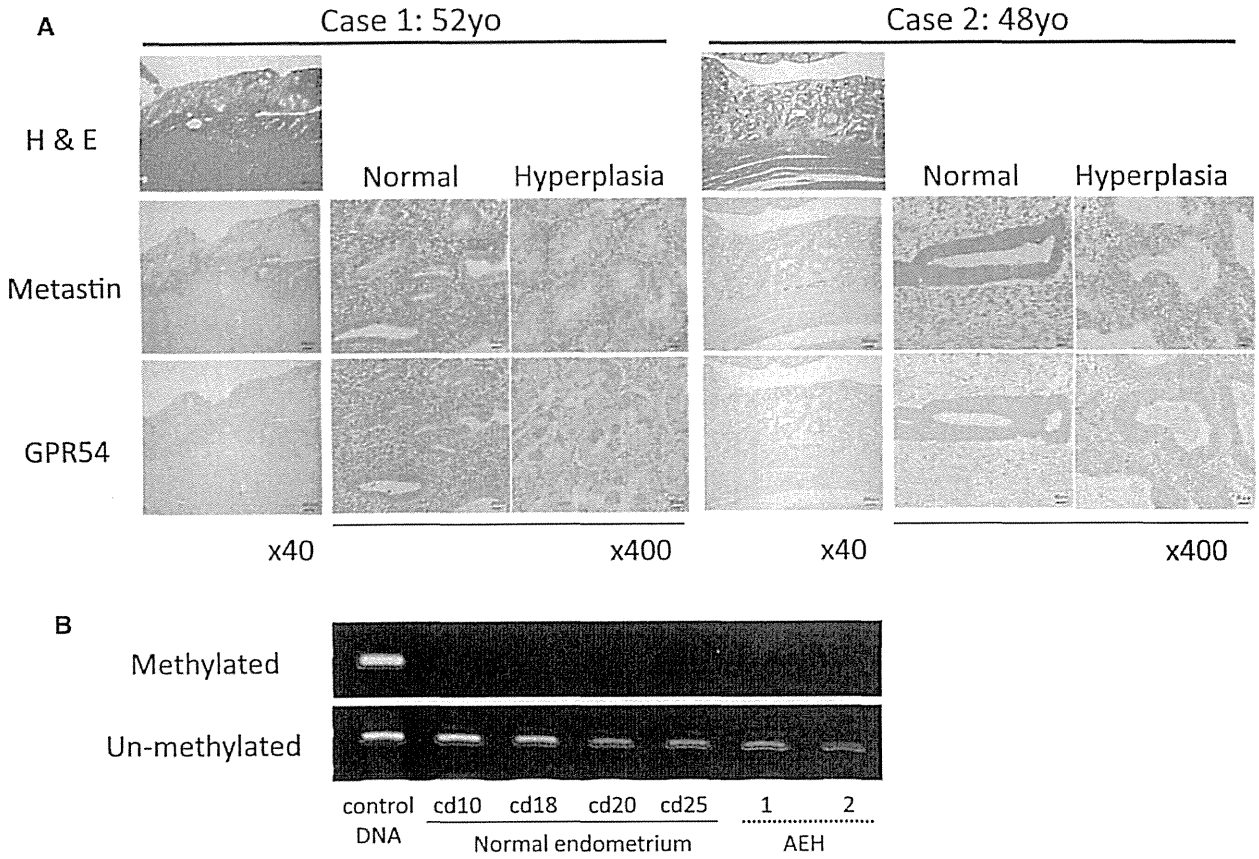

Fig. 3 Metastin and GPR54 expressions in pre-cancerous lesions. **a** Metastin and GPR54 expressions in endometrial hyperplastic lesions adjacent to normal endometrial glandular component from two representative AEH cases. GPR54 expression in the AEH lesion is higher relative to the adjacent EGCs, whereas metastin expression is scant in the AEH portion. **b** Methylation status of the GPR54 promoter region in AEH and in normal endometrium. Methylation is not observed in AEH cases or in normal endometrium specimens. *cd* cycle date

promoter region in AEH cases using MS-PCR. No *GPR54* promoter methylation was observed in AEH cases or in normal endometrium (Fig. 3b).

Discussion

Metastin is a biologically active peptide that was originally described as a metastasis suppressor and was identified from human placental extracts in 2001 [2], but its significance has been more recently described in reproductive research. A couple of studies have shown that sexual maturation does not occur in a pedigree in which there is loss of GPR54, an endogenous receptor of metastin, and *GPR54*-deficient mice do not display any of the characteristic physiological changes associated with sexual maturation of the testes and ovaries [14, 15]. To date, GPR54 is known to be expressed on neurons that secrete gonadotropin-releasing hormones (GnRH), and plays an important role in reproductive function through regulation by its ligand, metastin [16]. Metastin is a neuroendocrine peptide that functions upstream of the hypothalamus–pituitary–gonadal axis to regulate pulsatile secretions of GnRH from the hypothalamus. There are two types of metastin-secreting neurons in the arcuate nucleus (Arc) and the anteroventral periventricular nucleus (AVPV) in rodents [17, 18]. These neurons co-express estrogen receptor alpha (ERα) [19], and E2 differentially regulates the expression of *metastin* mRNA through ERα in distinct forebrain nuclei [20]. These metastin-secreting Arc and AVPV neurons are regulated by E2 in an inhibitory and stimulatory manner, respectively. Specifically, E2-mediated negative feedback on gonadotropin secretion in the Arc inhibits pulsatile GnRH/LH secretion but stimulates follicle maturation in the follicular period, whereas E2-mediated positive feedback in the AVPV induces the GnRH/LH surge resulting in ovulation [18, 21]. Thus, it is thought that metastin peptides are produced mainly in hypothalamus in vivo, but metastin is also present in peripheral organs such as the pancreas, which also implies extra-hypothalamic

production of metastin. In addition, serum metastin levels increase dramatically during pregnancy, implying that the metastin source is the placenta [22]. On the other hand, circulating metastin levels increase significantly in adult women compared with prepubertal and pubertal girls. Metastin levels are stable in males throughout puberty and adulthood [23], although metastin-10 stimulates gonadotropin release even in men [24]. These results support the notion that metastin may not only be produced in the hypothalamus but also produced in female-specific organs responsive to ovarian hormones.

The uterus and endometrium are regulated coordinately by the cyclical ovarian hormones, estrogen and progesterone. The endometrium is composed of endometrial epithelial cells (EGCs) and endometrial stromal cells (ESCs) that have various functions that through mutual interaction are geared toward receiving a fertilized egg after conception. That metastin and GPR54 are expressed by the endometrium is still not conclusive [3, 4], nor has the function of the metastin/GPR54 axis in the endometrium been clarified. We have shown that metastin is produced by EGCs and ESCs to a certain extent, and this is promoted when ESCs are decidualized by the coordinated effects of the sex steroid hormones, E2 and MPA. On the other hand, GPR54, an endogenous metastin receptor, is mainly expressed in EGCs, and GPR54 expression appears to not be affected by sex steroid hormones. Although further investigation is required to determine the mechanism, these findings suggest that endogenous metastin production is induced in ESCs through stimulation by sex steroid hormones to act on GPR54 in an autocrine/paracrine-like manner. Furthermore, this mechanism may be involved in embryo implantation and placentation, as the expression of metastin and GPR54 in the chorionic villi is prominent enough to be used as positive control in immunohistochemistry [1].

In the chorionic villus during early gestation, cytotrophoblasts adhere to the maternal decidualized endometrium and differentiate into extravillous trophoblasts (EVTs), forming a stratified structure called the cell column in which EVTs acquire the ability to invade the decidualized endometrium. In the floating villus, cytotrophoblasts differentiate into multinucleated syncytiotrophoblasts and take part in exchange of gas and nutrients [25]. Metastin is expressed in the stromal cells of the decidual endometrium and GPR54 was expressed in cytotrophoblasts [1, 22]. The reason that circulating metastin levels increase in pregnancy might result from the outer syncytiotrophoblasts being positioned adjacent to blood vessels, allowing easy passage of metastin into the maternal blood. A recent report described findings that decreased metastin expression in trophoblasts is associated with repeated pregnancy loss [26]. Another study showed that circulating metastin levels in early pregnancy are low in patients who later developed pre-eclampsia [27]. Repeated miscarriage and pre-eclampsia are considered to be a consequence of abnormal implantation of EVTs in early pregnancy. Metastin was previously reported to suppress villous motility [28], but these findings indicate that metastin secreted in decidua may adequately modulate the invasive activity of trophoblasts for ideal implantation and pregnancy maintenance.

Cancer invasion frequently shares invasive characteristics that are inherent to trophoblasts. We previously reported that GPR54 expression was inversely associated with tumor progression and histological grade of endometrial cancer [1]. With regard to GPR54-positive, low-grade cancers that comprise the majority of endometrial cancers, prognosis is fairly good and tumor invasion and metastasis are inhibited by metastin treatment. As low-grade cancers are known to arise through prolonged exposure to unopposed estrogen, it is remarkable that the source of metastin is the endometrium which is susceptible to sex hormones, and that endogenous metastin would play an inhibitory role in tumor progression. However, it has not been clarified whether or not endogenous metastin secretion is suppressed in endometrial cancers. In this study, metastin expression in endometrial cancer cells was lower than that in normal endometrium, while *GPR54* expression in cancer cells was higher than that in normal endometrium. Although it is still unclear why *GPR54* expression is paradoxically exaggerated in low-grade cancers, it is possible that prolonged estrogen exposure without progesterone might attenuate endogenous secretion of metastin in ESCs, which may make it easier for cancer cells to invade into the myometrium and metastasize beyond the uterus. Furthermore, *GPR54* expression in high-grade endometrial cancer is down-regulated through epigenetic or genetic mechanisms, and this down-regulation likely contributes to the more aggressive features of this subtype. Lymph node metastasis is a critical determinant of prognosis for women with endometrial cancers, and as this is fairly common even in low-grade subtypes that highly express GPR54, GPR54-targeted therapy is worthwhile developing for the control of lymph node metastasis.

Due to the recent increasing trends in obesity and later age at marriage, the number of young patients wishing to preserve fertility is increasing. As most of these patients bear low-grade cancers that appear localized within the endometrium by imaging, uterus-preserving therapy is sometimes employed with high-dose progestin administration and cyclic endometrial curettage. Thanks to this therapy, about 80 % of patients achieve a clinical response and more than 35 % subsequently bear children [29]. More than 40 % of patients, however, relapse during the follow-up period after achieving a clinical response. There is no

predictive marker for recurrence or preventive method that can block tumor progression during current uterus-preserving therapy [30]. Recently, several phase I–II endocrinology studies were conducted in which metastin or its analog was administered and reproducibly confirmed its safety and efficacy [31–33]. Utilizing characteristics of low-grade endometrial cancers, such as high GPR54 expression with poor metastin secretion from ESCs, metastin-10 treatment may be effective as an adjuvant therapy to prevent cancer cells from metastasizing during MPA therapy to preserve fertility in young patients.

Grade1 endometrioid adenocarcinoma is thought to arise from hyperplastic EGCs exposed to long-term unopposed estrogen. GPR54 expression was not epigenetically impaired in atypical endometrial hyperplasia (AEH), while metastin expression in AEH was lower than in the normal endometrial glandular component on the same specimen from several AEH patients. Atypical endometrial hyperplasia patients with menstrual disorders usually experience unopposed estrogen, and as such ESCs are expected to produce insufficient metastin. This attenuation of metastin may not only contribute to the pro-oncogenic status of AEH through initiation of the ability to acquire invasiveness but also to the persistent infertile status after mechanical eradication [34]. From this perspective, administration of metastin-10 might provide a means to support embryo implantation as well as to prohibit tumor progression.

In this study, we describe that metastin secretion is induced in ESCs through decidualization with E2 and MPA. Although further studies are needed, this process is expected to play an important role not only to maintain adequate placentation in pregnancy but also for the development of adjuvant therapy to prevent low-grade endometrioid adenocarcinoma from progression during fertility-sparing therapy with MPA.

Conflict of interest The authors declare no conflict of interest.

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