STAT1 drives tumor progression in serous papillary endometrial cancer

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Running title: STAT1 is an oncogene driver in serous papillary endometrial cancer

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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 pro-survival 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.

Keywords: STAT1, oncogene, tumor progression, endometrial cancer

Abbreviations: SPEC, Serous Papillary Endometrial Cancer

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Abstract

Recent studies of the interferon-induced transcription factor STAT1 have associated its dysregulation with poor prognosis in some cancers but its mechanistic contributions are not well defined. In this study, we report that the STAT1 pathway is constitutively upregulated in type II endometrial cancers. STAT1 pathway alteration was especially prominent in serous papillary endometrial cancers (SPEC) that are refractive to therapy. Our results defined a 'SPEC signature' as a molecular definition of its malignant features and poor prognosis. Specifically, we found that STAT1 regulated MYC as well as ICAM1, PD-L1, and SMAD7 as well as the capacity for proliferation, adhesion, migration, invasion, and in vivo tumorigenicity in cells with a high SPEC signature. Together our results define STAT1 as a driver oncogene in SPEC that modulates disease progression. We propose that STAT1 functions as a pro-survival gene in SPEC, in a manner important to tumor progression, and that STAT1 may be a novel target for molecular therapy in this disease.
Introduction

Endometrial cancer is one of the leading causes of gynecologic malignancy. It is the fourth most common malignancy among women in the United States, with an estimated 49,500 new cases and 8,200 deaths in 2013 (1), and in Japan, where the incidence has nearly doubled in the past decade (2). Most patients present with low grade and early stage disease with favorable prognosis, but cases of high-grade tumors are aggressive and frequently diagnosed with tumor spread beyond the uterus (3).

Endometrial cancers have generally been classified into two groups, type I and type II (4). Type I endometrial cancers are low grade endometrioid adenocarcinomas which feature high expression of estrogen receptor and a past history of unopposed estrogen associated with anovulation or obesity. Type I endometrial cancers usually have a favorable prognosis compared to type II endometrial cancers (5). Type I cancers often harbor alterations in oncogenes, such as PTEN, PIK3CA, ARID1A, K-ras, β-catenin and/or DNA mismatch repair genes (6). In contrast, type II endometrial cancers have worse outcomes and include histological subtypes such as high-grade endometrioid adenocarcinoma, serous papillary and clear cell, which are estrogen-independent and more common in older, non-obese women. TP53, PPP2R1A, CHD4, FBXW7, SPOP mutations (3,7), STK15 and HER2/neu amplification, p16 over-expression, down-regulation or loss of E-cadherin, and also loss of heterozygosity (LOH) have all been reported in type II endometrial cancers (5). These hallmarks of type II cancers do not entirely explain the aggressive nature of type II endometrial cancers, especially for serous papillary endometrial cancers (SPEC). SPEC is characterized by very aggressive progression with poor prognostic outcomes (5,8). Recent genome-wide analyses have revealed that SPEC exhibits gene expression profiles that are distinct from the endometrioid histologic subtype (3). Thus, it is important to
identify the SPEC driver genes or pathways responsible for the inherently aggressive phenotypes and to develop SPEC-specific therapies to target these driver genes or pathways.

Previous studies implicated the IFN-γ transcription factor signal transducer and activator of transcription 1 (STAT1) as a tumor suppressor, with transcription-dependent and transcription-independent mechanisms of regulation (9). STAT1 has been classically defined as a Th1 pro-immune and antitumor transcription factor, based on its canonical role in IFN-γ signaling and on studies using STAT1−/− tumor cells and mouse models (10-12). STAT1 has also been described to regulate DNA repair pathways and to be up-regulated 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 where 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 to other histological 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 pro-survival factor in SPECs, in a manner important to tumor progression, and may be a novel target for molecular therapy in this disease.
Material and Methods

Patients and tissues.

Clinicopathological information (n=294) and specimens (n=91) from patients treated for endometrial cancer between 2004-2012 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, Rockville, MD), HEC50B JCRB, Osaka, Japan), 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, Rockville, MD, USA) 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) (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, Santa Clara, CA). The
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 heat map 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 down-regulated 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, Kyoto, Japan) by inoculating 5×10⁶ 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’s 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 2004-2012 (endometrioid grade 1; 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 Figure 1A) 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 2). 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 Figure 1B). Furthermore, using supervised clustering with a total of 1244 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 sub-clusters \((p<0.0001\), Figure 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 up-regulated and 189 down-regulated genes, Figure 1B, Supplementary Figure 1C, and Supplementary Table 3). Among these 227 up-regulated genes, STAT1 and many STAT1-associated genes such as \textit{MYC}, \textit{ICAM1}, and \textit{SMAD7} were highly expressed in the SPEC-rich cluster (Figure 1A-B and Supplementary Table 3A). Conversely, \textit{ESRI} and \textit{PGR} were included among the 189 down-regulated genes (Figure 1B and
Supplementary Table 3B). SAM analysis confirmed STAT1 and its associated genes were highly expressed in the SPEC-rich cluster (Supplementary Figure 1D and Supplementary Table 4).

**STAT1 expression and clinical significance in endometrial cancers.**

The expression of STAT1 in 91 endometrial cancer patients (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 (Figure 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 \( (p<0.0001, \text{ Figure 2B}) \). These findings were also confirmed in the Vancouver tissue microarrays as an external validation, in which STAT1 was highly expressed in SPEC \( (p<0.0001, \text{ Figure 2C}) \). 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 data sets, GSE17025 and GSE24537 (Supplementary Figure 2A-E). Intriguingly, around 77% (17/22) of SPEC cases contain infiltrated CD8\(^+\) immune cells at the tumor front with strongly positive staining of ICAM1 and PD-L1 (Figure 2A).

The disease specific survival of patients bearing STAT1-high cancers was significantly shorter than those bearing STAT1-low cancers \( (p<0.0001, \text{ Figure 2D}) \), and this tendency was also confirmed in the Vancouver setting \( (p<0.05, \text{ Supplementary Figure 2G}) \). 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.
Multivariate analysis revealed high STAT1 expression was a prognostic factor independent from histological subtypes and FIGO stages ($p<0.05$, Supplementary Table 5).

**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 (Figure 3A and E). *STAT1* mRNA expression was significantly up-regulated 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 (Figure 3A and D, Supplementary Figure 5D-E). Furthermore, *STAT1* expression was augmented by IFNγ in a dose dependent manner (Figure 3D and Supplementary Figure 3A).

The mRNA expression levels of STAT1-associated genes, including *CD274* (also known as *PD-L1*), *ICAM1*, *IRF1*, *SMAD7*, and *CCL7* (also known as *MCP3*) (27-29), were examined by qRT-PCR following treatment with IFNγ and/or *STAT1*-siRNA. Figure 3B and 3C show that IFNγ treatment up-regulates *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 (Figure 3D, Supplementary Figure 3B-C). 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 Figure 3D-F). 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 pro-survival and pro-progression 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 Figures 4A-C).

To assess the potential role of STAT1 on pro-survival 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, Figure 4A) and STAT1-DN5 cells (p<0.0001, Supplementary Figure 4D). Similarly, anchorage-independent growth capacity was impaired in STAT1-shRNA cells (p<0.0005, Figure 4B) and STAT1-DN5 cells (p<0.0001, Supplementary Figure 4E).
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 ($p<0.0005$, Figure 4C). Cellular adhesive capacity was also suppressed in STAT1-DN5 cells and STAT1-siRNA cells ($p<0.0001$), while STAT1-DN3 cells did not show the same level of suppression (Supplementary Figure 4F). 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. Secondly, SPAC-1L cellular motility over a 24-hour tracking period was accelerated by IFNγ treatment, while this acceleration was not observed in STAT1-shRNA cells (Figure 4D). Boyden-chamber assays demonstrated that cellular invasive activity 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 (Figure 4E and Supplementary Figure 4G). 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 to 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, while all 10 mice inoculated with SPAC-1L cells formed large tumors ($p<0.0001$, Figure 5A). This tumorigenic inhibition was also observed for STAT1-DN5 cells ($p<0.0001$, Supplementary Figure 5A). 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 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 6 and Supplementary Figure 5B).

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 (Figure 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, Figure 5B and Supplementary Figure 5G), 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, Figure 5C). Western Blotting showed that induction of STAT1 was accompanied by up-regulation of MYC, while suppression of STAT1 resulted in decreased MYC expression *in vitro* and *in vivo* (Figure 5D). This was also true for the STAT1-DN5 cells, as MYC expression was not induced by IFNγ treatment (Supplementary Figure 5C). In contrast, MYC expression was not remarkably changed in non-SPEC cell lines by STAT1 manipulation (Supplementary Figure 5D and 5F-G). 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-IL cells (*p*<0.05), while that in SPECs was higher than that of endometrioid adenocarcinomas (*p*<0.05, Supplementary Figure 5D-E). To clarify the functional role of the IFNγ-STAT1-MYC axis, cell proliferation assays were performed under conditions of
co-suppression of STAT1 and MYC in vitro. As shown in Figure 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 ($p<0.0001$). Co-suppression of STAT1 and MYC showed no significant difference in inhibitory effect as compared to STAT1 suppression alone. These results suggest that STAT1 contributes to regulation of oncogenic MYC expression to promote cancer cell proliferation and tumor growth.

Discussion

Serous papillary endometrial cancers (SPEC) account for only 4-10% of endometrial cancer are highly aggressive and difficult to treat effectively, while low grade endometrioid adenocarcinoma comprises 80% of endometrial cancer, is frequently diagnosed at an early stage at which point it is surgically curable (3,34). As SPEC is chemo-refractory 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 chemo-refractory 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 are 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 Figure 1A) and a distinct gene expression profile (Figure 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 Figure 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 up-regulated genes in another SAM analysis (Supplementary Figure 1D and Supplementary Table 4). In contrast, ESR1 and PGR were among the down-regulated 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, inter-observer 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 (Figure 1A and Supplementary Figure 1B-C). To address this issue, we identified STAT1 as a key molecule of the SPEC-signature that was commonly up-regulated in both datasets studied, and this was accompanied by up-regulation of
putative downstream STAT1 gene targets. We confirmed high STAT1 expression in SPEC by 
external validation using GSE17025 and GSE24537 (Supplementary Figure 2D and E) (37,38).

Immunohistochemical staining (IHC) 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 (Figure 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 (Figure 2B, 2D), were externally validated 
by IHC finding in a large Vancouver cohort (Figure 2C and Supplementary Figure 2G). 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 IFN\(\gamma\), 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 
IFN\(\gamma\) in a dose-dependent manner accompanied with similar augmentation of expression of 
several STAT1 associated genes (Figure 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 
IFN\(\gamma\) was specifically prominent in SPAC-1L, and attenuated by suppressing STAT1 expression. 
These results imply that over-expression of STAT1 might be associated with the clinical 
aggressiveness of SPECs and that the IFN\(\gamma\) signal was transduced through STAT1, although the 
source of IFN\(\gamma\) 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 (Figure 2A),
which are a known source of IFN\(\gamma\) (41). As SPAC-1L cells have relatively high transcription of
IFN\(\gamma\) receptors 1 and 2, the tumor microenvironment could be a potential source of persistent
IFN\(\gamma\) \textit{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 anti-proliferative and pro-apoptotic genes
as a tumor suppressor (45); in contrast, STAT1 in SPEC appears to function as a tumor pro-
survival 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 IFN\(\gamma\) treatment to activate the STAT1 pathway (Figure 4 and Supplementary
Figure 4D-G). With regard to IFN\(\gamma\)-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 involved in tumorigenesis of
mesenchymal stem cells concomitant with up-regulation of MYC under prolonged inflammatory
IFN\(\gamma\) 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 up-regulation 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 tumor suppressing role.
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). Since 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 up-regulated genes in the SPEC signature harbor the MYC binding site motif by GATHER analysis (V$MYCMAX_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, while that in SPAC-1L cells was diminished with STAT1 knockdown (Figure 5 and Supplementary Figure 5). As the suppressive effect on proliferation by double knockdown of STAT1 and MYC was not superior to knockdown of STAT1 alone (Figure 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), while there were no SPEC samples with down-regulation of STAT1 or MYC. As MYC up-regulation was not observed in endometrioid cell lines (Supplementary Figure 5D, F, G), these results imply that more than half of SPECs have aberrant up-regulation of the STAT1-MYC axis as a particular driver oncoprint of their
aggressiveness. This STAT1-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 6 and Supplementary Figure 5B). 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 pro-survival pathways” to enhance multiple malignant characteristics (Figure 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.

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References


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

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Expression of STAT1&lt;sup&gt;Θ&lt;/sup&gt;</th>
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<td>≤60</td>
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<td>20</td>
</tr>
<tr>
<td>&gt;60</td>
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<tr>
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<td>20</td>
</tr>
<tr>
<td>II</td>
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<tr>
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<tr>
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<tr>
<td>G2</td>
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<tr>
<td>G3</td>
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<tr>
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<tr>
<td>Myometrial invasion§</td>
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<tr>
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<td>4</td>
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<tr>
<td>≤1/2 (inner half)</td>
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<td>9</td>
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<tr>
<td>&gt;1/2 (outer half)</td>
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<tr>
<td>Lymphovascular space invasion§</td>
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<tr>
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<td>34</td>
<td>17</td>
</tr>
<tr>
<td>+</td>
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<td>18</td>
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<tr>
<td>Lymph node metastasis§</td>
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<tr>
<td>–</td>
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<td>26</td>
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<tr>
<td>+</td>
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<tr>
<td>Ascites§</td>
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<tr>
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<td>25</td>
</tr>
<tr>
<td>+</td>
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<tr>
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<tr>
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※Statistical significance were analyzed by Chi-square. Θ STAT1 score >1: high; ≤1: low
§ Some data were missing; 8 cases, 10 cases, 6 cases, and 7 cases, respectively
Figure Legends

Figure 1 Microarray gene expression across histological 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 \textit{STAT1} and associated genes, \textit{ICAM1}, \textit{PD-L1}, \textit{MYC}, \textit{IRF1}, \textit{IFIT3}, and \textit{SMAD7} are all highly expressed. (B) A Venn diagram showing the overlap of 227 up-regulated genes (in red) and 189 down-regulated genes (in green) in the SPEC enriched clusters of both the Kyoto University human endometrial cancer samples (CC) and TCGA UCEC_2013 microarray datasets. \textit{STAT1} and associated genes are included among the 227 up-regulated genes.

Figure 2 Immunohistological analysis of STAT1 expression in human endometrial cancers. (A) Representative micrographs of STAT1 expression in each histological subtype (x400). Upper panels: 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 based on 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; \(*p<0.05, **p<0.0001\); \(\kappa\)-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 based on staining of cancer cells. STAT1 expression was higher in SPECs \((p<0.0001); \kappa\)-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).

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 quantitative real time 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 (x16), ICAM1 (x15), and PD-L1 (x60) 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 quantitative real time PCR.

Figure 4 STAT1 functions to promote tumor cell survival and progression in SPEC cells. WT, non-treated 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 down-regulating 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 co-culturing with HUVEC cells for four hours, cell adhesion was measured by spectrophotometer as relative fluorescence units (RFUs). left: Down-
regulating 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 post-wounding. 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 down-regulating STAT1 using STAT1-shRNA, the numbers of invading cells is decreased (n=10, **p<0.0005), and reduces the promoting effect of IFNγ (***p<0.0001). right: Representative micrographs of Hematoxylin stained cells that have invaded through the membrane.

**Figure 5** Significant role of the STAT1 pathway in tumorigenesis.

(A) STAT1 down-regulation inhibits tumorigenesis in vivo. NOD-SCID mice were subcutaneously inoculated with 5x10^6 SPAC-1L cells with and without STAT1 knockdown. Tumor growth was inhibited in mice inoculated with STAT1-shRNA cells (n=25, p<0.0001). Arrows in the right picture 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 to STAT1 knockdown alone in suppression of cellular proliferation (n=10, ns).

Figure 6 Schematic showing STAT1 roles as a driver gene of SPEC in modulating “transcriptional pro-survival 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 IFN-γ 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 TGF-β signaling, and ICAM1 and SMAD7 affect metastatic capability. These effects suggest that STAT1 functions as a driver gene in a tumor pro-survival 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.
Figure 1

A

B

ICAM1
IFIT3
MYC
SMAD7
STAT1

253
227
2073

189

575

1362

Kyoto CC
(GSE 56026)

TCGA
UCEC_2013

ESR1
PGR

STAT1; B. Kharma, et al., 2014
Figure 2

A

B

C

D

STAT1; B. Kharma, et al., 2014
Figure 3

A

B

C

D

E

STAT1; B. Kharma, et al., 2014
**STAT1; B. Kharma, et al., 2014**

**Figure 4**

**A**

Graph showing the time course of cell number (in 10^6) over 36 hours. The graph compares WT, Mock, and STAT1-siRNA conditions. The y-axis represents cell number (in 10^6), and the x-axis represents time (0, 8, 12, 24, 36 hours). The data includes error bars indicating variability.

**B**

Bar graph showing the number of colonies formed by Mock and STAT1-shRNA conditions. The graph includes error bars indicating variability.

**C**

Graph showing the expression of STAT1-siRNA and IFNγ conditions. The y-axis represents pSU R (arbitrary units), and the x-axis represents different treatment conditions (WT, Mock, STAT1-siRNA, IFNγ). The graph includes error bars indicating variability.

**D**

Images showing the effects of STAT1shRNA and IFNγ on cell morphology at different time points (0 hr, 6 hr, 12 hr, 24 hr). The images compare Mock and STAT1-shRNA conditions.

**E**

Graph showing the cell number (in 10^6) under different treatment conditions. The graph includes error bars indicating variability.
Figure 5

A) Tumor size (mm$^3$) over time for SPAC-1L and SPAC-1L STAT1-shRNA.

B) MYC expression levels in vitro.

C) MYC expression levels in vivo.

D) Western blot analysis of STAT1, MYC, and β-actin expression levels in vitro and in vivo. KDa:
- STAT1: 91
- MYC: 41
- β-actin: 42

E) Cell number growth over time for Mock, STAT1-shRNA, MYC-siRNA, and STAT1-shRNA+MYC-siRNA.
STAT1; B. Kharma, et al., 2014

Figure 6
STAT1 drives tumor progression in serous papillary endometrial cancer

Budiman Kharma, Tsukasa Baba, Noriomi Matsumura, et al.

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