STAT1 Drives Tumor Progression in 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 prosurvival gene in SPEC, in a manner important to tumor progression, and that STAT1 may be a novel target for molecular therapy in this disease. Cancer Res; 74(22): 1–12. ©2014 AACR.

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).

Endometrials 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 with 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 histologic subtypes such as high-grade endometrioid adenocarcinoma, serous papillary and clear cell, which are estrogen-independent and more common in older, nonobese women. TP53, PPP2R1A, CHD4, FBXW7, SP0P mutations (3, 7), STK15 and HER2/neu amplification, p16 overexpression, downregulation 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 IFNγ 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 proimmune and antitumor transcription factor, based on its canonical role in IFNγ 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 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 tumorigeneity. 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 functional assays as described precisely in the Online Repository.

STAT1 knockdown

STAT1-specific short interfering RNAs (siRNA; FlexiTube siRNA Qiagen; catalogue no. SIO2662884), 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 dendrogram 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 × 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 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,
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 \((t\text{ test}, P < 0.005)\). The 63 endometrial cancer samples were divided into two clusters, but SPECs were enriched in only one of the subclusters \((P < 0.0001, \text{Fig. 1A})\). 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 \((G1 \ n = 35; G2 \ n = 16; G3 \ n = 18; \text{and SPEC } n = 22; \text{Table 1})\). 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 \((P < 0.0001, \text{Fig. 2B})\). These findings were also confirmed in the Vancouver tissue microarrays as an external validation, in which STAT1 was highly expressed in SPEC \((P < 0.0001, \text{Fig. 2C})\). The expression of STAT1 mRNA was also higher in SPEC \((P < 0.005)\), 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% \((17 \text{ of } 22)\) of SPEC cases contain infiltrated CD8+ immune cells at the tumor front with strongly positive staining of ICAM1 and PD-L1 (Fig. 2A).
Cancer Research

**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*</th>
<th>Low</th>
<th>High</th>
<th>( P )</th>
</tr>
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<tr>
<td>Age</td>
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<tr>
<td>(&lt; 60)</td>
<td>30</td>
<td>20</td>
<td></td>
<td>0.1259</td>
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<tr>
<td>( \geq 60)</td>
<td>18</td>
<td>23</td>
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<td>FIGO stage</td>
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<tr>
<td>I</td>
<td>35</td>
<td>20</td>
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<tr>
<td>IV</td>
<td>2</td>
<td>8</td>
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<tr>
<td>Histology</td>
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<tr>
<td>G1</td>
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<td>3</td>
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<td>&lt;0.0001</td>
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<tr>
<td>G2</td>
<td>9</td>
<td>7</td>
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<td>G3</td>
<td>6</td>
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<tr>
<td>Serous</td>
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<tr>
<td>Myometrial invasion ( b )</td>
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<tr>
<td>No invasion</td>
<td>11</td>
<td>4</td>
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<tr>
<td>&gt;1/2 (outer half)</td>
<td>16</td>
<td>24</td>
<td></td>
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<tr>
<td>Lymphovascular space invasion ( b )</td>
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<td></td>
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<tr>
<td>–</td>
<td>34</td>
<td>17</td>
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<td>+</td>
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<td></td>
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<tr>
<td>Lymph node metastasis ( b )</td>
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<td>&lt;0.0001</td>
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<tr>
<td>–</td>
<td>44</td>
<td>26</td>
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<tr>
<td>+</td>
<td>1</td>
<td>14</td>
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<td>Ascites ( b )</td>
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<tr>
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<td></td>
</tr>
<tr>
<td>High</td>
<td>9</td>
<td>28</td>
<td></td>
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</tbody>
</table>

NOTE: Statistical significance was analyzed by the \( \chi^2 \) test.

*STAT1 score: \( >1 \), high; \( \leq 1 \), low.

Subsequently, 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

**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, \( STAT1\alpha \) and \( STAT1\beta \), which is induced by homodimeric IFN\( \gamma \) via its receptors, IFN\( \gamma R1 \) and IFN\( \gamma R2 \) (18). In endometrial cancer cell lines, \( STAT1\beta \) and IFN\( \gamma R2 \) mRNAs are generally expressed at higher levels than \( STAT1\alpha \) and IFN\( \gamma R1 \), and are highly expressed in SPAC-1L cells (Fig. 3A and E). \( STAT1 \) mRNA expression was significantly upregulated by IFN\( \gamma \) 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 IFN\( \gamma \) in a dose-dependent manner (Fig. 3D and Supplementary Fig. S3A).

The mRNA expression levels of \( STAT1 \)-associated genes, including \( CD274 \) (also known as \( PD-L1 \)), \( ICAM1 \), \( IRF1 \), \( MAD7 \), and \( CCL7 \) (also known as \( MCP3 \); refs. 27–29), were examined by qRT-PCR following treatment with IFN\( \gamma \) and/or \( STAT1\)-siRNA. Figure 3B and C show that IFN\( \gamma \) treatment upregulates \( PD-L1 \) and \( ICAM1 \) mRNA expression in SPAC-1L cells (\( \geq 60 \)-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 IFN\( \gamma \) augmentation of mRNA expression was observed for \( IRF1 \), \( MAD7 \), and \( MCP3 \) (\( \sim 12 \)-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 IFN\( \gamma \) induction through IFN\( \gamma R \) 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...
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-DN3 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-IL cells attached to the single-layered HUVEC cells decreased with knockdown of STAT1, but attachment was recovered with concurrent IFNγ treatment. Second, SPAC-IL 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...
was augmented by IFN\(\gamma\) \((P < 0.0005)\), 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
in vitro shRNA cells not only butt hsi nduction wa sr emarka bly at tent uad i n S T A T 1 - with IFN mRNA expression was induced 12-fold following treatment.

We therefore investigated whether STAT1 regulates the implications for these findings have not yet been clarified. We therefore investigated whether STAT1 regulates the myonogenic 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). 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
of the IFN–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
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 the 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 genomewide 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. 1C and 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 ICAMI, 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. 1A), 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
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 (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, 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 oncprint 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 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 chemoresistant 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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