# Transcriptomic analysis of hormone-sensitive patient-derived endometrial cancer spheroid culture defines Efp as a proliferation modulator

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## ARTICLE INFO

## ABSTRACT

Estrogen-responsive endometrial cancer (EC) is prevalent in uterine cancer. Its precise molecular mechanisms remain to be elucidated partly because of limited availability of estrogen-sensitive EC models recapitulating clinical pathophysiology. We previously established EC patient-derived cancer cell (EC-PDC) spheroid culture with high expression of estrogen receptor α (ERα). Using this EC-PDC, we study the transcriptional regulation and function of estrogen-responsive finger protein (Efp), a prototypic tripartite motif (TRIM) protein that modulates protein degradation and RNA processing. Intense estrogen-dependent *EFP* mRNA induction and high ERα occupancy to *EFP* estrogen responsive element (ERE) were observed in EC-PDC. Luciferase reporter gene assay showed that the ERE facilitates *EFP* transcriptional activity estrogen-dependently. siRNA-mediated Efp silencing in EC-PDC resulted in suppressed spheroid proliferation and altered gene expression profile, featuring downregulation of genes related to cell cycle (e.g., *CDK6*) and inflammation/immune responses (e.g., *IL10RA*, *IL26*, and *IL6ST*) while unaffected expression of cancer stemness-related markers. Taken together, EC-PDC spheroid culture is a powerful EC tool that enables to dissect Efp-mediated ERα signaling pathways as an estrogen-sensitive EC model. This study provides an insight into alternative EC therapeutic strategies targeting ERα-Efp axis.

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## 1. Introduction

Uterine cancer is one of the most common gynecological cancer, with the number of related deaths continuing to rise in recent decades [1]. More than 80% of patients with uterine cancer have endometrial cancer (EC). On the basis of pathological features, 80—90% of ECs are categorized as hormone-sensitive endometriod subtype [2]. Prolonged and unopposed estrogen exposure has been indicated as a risk factor for endometriod EC. While endocrine therapy is not a standard treatment for endometriod EC, elucidation of precise molecular mechanisms underlying hormone-dependent EC tumorigenesis will facilitate the understanding of the disease pathophysiology and the development of alternative clinical management.

We previously reported that estrogen-responsive finger protein (Efp, also known as TRIM25) promotes the proliferation of estrogen receptor α (ERα)-positive Ishikawa EC cells [3,4]. Efp belongs to the family of tripartite motif (TRIM) proteins. The TRIM motif, which defines this superfamily, comprises a RING domain, one or two B-box domains and an associated coiled-coil domain (RBCC) [5,6]. In Ishikawa cells, Efp contributes to 14-3-3σ protein degradation as observed in estrogen-naïve breast cancer cells [7], and the activation of nuclear factor-κB (NF-κB)-mediated transcription. Recently, another molecular aspect of Efp has been shown as a critical RNA-binding protein in gene regulatory networks [8—10] associated with breast cancer metastasis [8]. These findings suggest that Efp is a key modulator of estrogen-dependent signaling pathways that contribute to EC tumorigenesis.

Conventional cancer cell lines maintained in two-dimensional cultures are useful cancer models widely used for molecular studies and drug discovery. In terms of hormone-sensitive EC cells, the availability has been limited except Ishikawa cells, even in which ERα expression is often downregulated during long-term culture [11]. Thus, hormone-sensitive EC models that recapitulate clinical EC pathophysiology has been long expected from the view point of endocrine oncology. We recently established EC patient-derived cancer cell (EC-PDC) spheroid culture with high expression of ERα [12]. The three-dimensional culture of EC-PDC exhibits cancer stemness character and estrogen-dependent proliferation, suggesting that this model is particularly useful to evaluate estrogen signaling pathways that contribute to EC tumorigenesis.

In the present study, we questioned whether TRIM proteins including Efp are critical targets of ERα and contribute to estrogen-dependent proliferation of EC-PDC model. Transcriptomic analysis of EC-PDC spheroid culture showed that *EFP* is the most significantly upregulated TRIM family gene in response to estrogen. Estrogen-dependent promoter activity of *EFP* via its functional estrogen response element (ERE) has been shown in EC cells. Knockdown of Efp by its specific siRNAs substantially suppressed EC-PDC spheroid proliferation and downregulated the expression of proliferation-related genes, such as cell cycle-related genes and inflammation/immune-related genes. The present study defines molecular mechanisms of ERα-induced Efp actions in hormone-sensitive EC and will provide an insight into alternative EC therapeutic strategies targeting ERα-Efp axis.

## 2. Materials and methods

### 2.1. Cell culture

Ishikawa cells and EC-PDC spheroid culture were previously described [3,12]. Surgical specimens from patients with EC were obtained from the Saitama Medical University International Medical Center under a protocol approved by the institutional review board (#12–096), and written informed consent was obtained from all patients.

### 2.2. siRNA transfection

siRNA duplexes targeting Efp (siEfp #A and #B) and a non-targeting control siRNA (siControl) were previously described [3,13]. Cells were transfected with siRNA at a final concentration of 10 nM using RNAiMAX (Invitrogen). The day of transfection was defined as day 0.

### 2.3. Spheroid growth assay

Five thousand of EC-PDC cells were seeded in ultra-low attachment 96-well plates (Corning) per well. After siRNA transfection, PDC spheroid growth was evaluated using CellTiter-Glo 3D Cell Viability Assay kit (Promega) with TriStar$^2$ S LB 942 Multimode Reader (Berthold Technologies). The detected luminescent values were normalized to the corresponding values at day 1.

### 2.4. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

RNA was extracted from EC-PDC spheroid culture and Ishikawa cells using ISOGEN reagent (Nippongene). Gene expression levels were examined by RT-qPCR with gene-specific primers (Supplementary Table S1). The comparison between the amounts of PCR products of the target genes relative to *GAPDH* or *36B4* was carried out by the comparative cycle threshold method [14].

### 2.5. Microarray analysis

EC-PDC spheroid culture was treated with 100 nM 17β-estradiol (E2) or vehicle (ethanol) for 48 h and transcriptomic analysis was performed using Affymetrix GeneChip (Human Clariom S Array) in the previous study [12] and the data were deposited to GEO

**Table 1**
Estrogen regulation of TRIM family members in EC-PDC.

| Gene[a] | Also known as | Fold change[b] |
|---|---|---|
| *TRIM25* | Estrogen-responsive finger protein (Efp) | 2.41 |
| *TRIM16L* | TRIM70 | 1.99 |
| *TRIM11* | | 1.89 |
| *TRIM55* | Muscle-specific RING Finger protein 2 (MURF-2) | 1.85 |
| *TRIM47* | Gene overexpressed in astrocytoma (GOA) | 1.65 |
| *TRIM34* | Interferon-responsive finger protein 1 (IFP1) | 1.54 |
| *TRIM38* | Zinc finger protein RoRet | 1.51 |
| *TRIM44* | | 1.49 |
| *TRIM17* | Testis RING finger protein (TERF) | 1.47 |
| *TRIM72* | Mitsugumin 53 (MG53) | 1.43 |
| *TRIM1* | Midline 2 (MID2) | 1.39 |
| *TRIM69* | Testis-specific ring finger (Trif) | 1.38 |
| *TRIM73* | TRIM50B | 1.36 |
| *TRIM45* | | 1.36 |
| *TRIM16* | Estrogen-responsive B box protein (EBBP) | 1.36 |
| *TRIM68* | 56-kDa protein structurally related to Ro/SSA antigen (SS-56) | 1.32 |
| *TRIM4* | | 1.31 |
| *TRIM26* | α-Fetoprotein (AFP) | 1.26 |
| *TRIM74* | TRIM50C | 1.25 |
| *TRIML1* | | 1.25 |
| *TRIM22* | Stimulated *trans*-acting factor of 50 kDa (STAF50) | 1.23 |
| *TRIM39* | Testis-abundant finger protein (TFP) | 1.21 |
| *TRIM46* | Tripartite, fibronectin type-III and C-terminal SPRY motif protein (TRIFIC) | 1.20 |
| *TRIM42* | Protein phosphatase 1, regulatory subunit 40 (PPP1R40) | 1.20 |
| *TRIM49D1* | TRIM49D,TRIM49D2; TRIM49DP,TRIM49D1P,TRIM49D2P | 1.20 |
| *TRIM10* | Hematopoietic RING finger 1 (HERF1) | 1.19 |
| *TRIM49* | TRIM49A,TRIM49L2 | 1.19 |
| *TRIM61* | | 1.17 |

**Table 1** (continued)

| Gene[a] | Also known as | Fold change[b] |
|---|---|---|
| TRIM21 | 52 kDa ribonucleoprotein autoantigen Ro/SSA | 1.17 |
| TRIM63 | Muscle-specific RING Finger protein 1 (MURF-1) | 1.16 |
| TRIM49D2 | TRIM49L,TRIM48L1,TRIM49D1,TRIM49L1,TRIM49D2P | 1.16 |
| TRIM62 | Ductal epithelium−associated ring chromosome 1 (DEAR1) | 1.15 |
| TRIM23 | ADP-ribosylation factor domain protein 1 (ARD1) | 1.15 |
| TRIM48 | | 1.15 |
| TRIM67 | TRIM9-like protein (TNL) | 1.14 |
| TRIM19 | Promyelocytic leukemia (PML) | 1.11 |
| TRIM6 | Interferon-responsive finger protein (IFP1) | 1.11 |
| TRIM43B | | 1.11 |
| TRIM66 | Transcriptional intermediary factor 1δ (TIF1D) | 1.10 |
| TRIM37 | Mulibrey nanism (MUL) | 1.09 |
| TRIM20 | Mediterranean fever (MEFV) | 1.08 |
| TRIM65 | | 1.08 |
| TRIM2 | | 1.06 |
| TRIM33 | Transcription intermediary factor 1γ (TIF1γ) | 1.05 |
| TRIM43 | TRIM43A | 1.05 |
| TRIM50 | TRIM50A | 1.03 |
| TRIM49B | | 1.03 |
| TRIM71 | Abnormal cell LINeage (LIN-41) | 1.01 |
| TRIM29 | Ataxia-Telangiectasia Group D Complementing (ATDC) | 1.01 |
| TRIM56 | | 1.01 |
| TRIM5 | | 1.01 |
| TRIM8 | Glioblastoma expressed RING finger protein (GERP) | 1.00 |
| TRIM13 | Ret finger protein 2 (RFP2) | 1.00 |
| TRIM64B | | 0.97 |
| TRIM7 | Glycogenin-interacting protein 1 (GNIP1) | 0.97 |
| TRIM31 | | 0.96 |
| TRIM14 | | 0.94 |
| TRIM49C | TRIM49L2 | 0.94 |
| TRIM3 | Brain expressed ring finger (BERP) | 0.93 |
| TRIM76 | Cardiomyopathy associated 5 (CMYA5) | 0.93 |
| TRIM58 | | 0.92 |
| TRIM36 | Haploid germ cell-specific RING finger protein (HAPRIN) | 0.90 |
| TRIM64 | | 0.88 |
| TRIM32 | Zinc-finger protein HT2A | 0.87 |
| TRIM40 | | 0.86 |
| TRIM59 | Tumor suppressor TSBF1, TRIM57 | 0.85 |
| TRIM64C | | 0.83 |
| TRIM52 | | 0.83 |
| TRIM28 | Transcriptional intermediary factor 1β (TIF1β) | 0.82 |
| TRIM41 | RING-finger protein that interacts with C kinase (RINCK) | 0.81 |
| TRIM35 | Hemopoietic lineage switch protein 5 (HLS5) | 0.78 |
| TRIM27 | Ret finger protein (RFP) | 0.78 |
| TRIM24 | Transcription intermediary factor 1α (TIF-1α) | 0.77 |
| TRIM60 | | 0.76 |
| TRIM15 | | 0.75 |
| TRIM54 | Muscle-specific RING finger protein 3 (MURF-3) | 0.72 |
| TRIML2 | SPRY domain-containing protein 6 (SPRYD6) | 0.69 |
| TRIM18 | Midline 1 (MID1) | 0.64 |
| TRIM77 | TRIM77P | 0.58 |
| TRIM9 | SNAP-25-interacting RING finger protein (SPRING) | 0.57 |
| TRIM51 | SPRY domain containing 5 (SPRYD5A) | 0.29 |

[a] Names of TRIM family members are indicated.
[b] Values are indicated as fold change relative to vehicle treated cells.

database (GSE127238). Using this data, TRIM family members were ranked by estrogen-induced fold change values relative to vehicle-treated cells in the present study.

EC-PDC was also treated with 10 nM siEfp #A or siControl for 60 h and gene expression levels were analyzed by Human Clariom S Array. Microarray data are available in the Gene Expression Omnibus (GEO) database with the accession number GSE164724. Gene expression values were plotted with the log2 fold change (siEfp #A vs siControl) against log2 expression (siControl). The enrichment analyses for signature gene sets influenced by siEfp #A were conducted by gene set enrichment analysis (GSEA) using GSEA 4.1.0 software with gene set c2 (cp.kegg.v.7.2.symbols.gmt) [15]. Genes with a Yes value in the CORE ENRICHMENT index were screened.

### 2.6. Luciferase assay

EFP promoter region corresponding with −173 to −1 upstream of the translation initiation site was amplified by PCR and then subcloned into a luciferase reporter plasmid psiCHECK2 (Promega) with exchange of SV40 enhancer/promoter [16,17]. Double-stranded oligonucleotides corresponding with ERE sequence at the 3′-UTR of EFP (wtERE) and its mutated ERE (mutERE) were created by annealing of oligonucleotides: 5′-ATTCAG<u>GGGTCATGGT</u>-<u>GACC</u>CTGATC-3′ and 5′-GATCG<u>GGTCACCATGACCC</u>TGAAT-3′ for wtERE; and 5′-ATTCAG**TTT**CATGGT<u>GATT</u>CTGATC-3′ and 5′-GATCA-G**AA**TCACCATGA**AA**CTGAAT-3′ for mutERE where the underlines show half-site ERE and the bold shows mutated sequences. These annealed wtERE and mutERE oligonucleotides were inserted into the psiCHECK2 containing the EFP promoter region and named EFP-

Luc-wtERE and *EFP*-Luc-mutERE, respectively. Ishikawa cells were seeded onto 24-well plates at a density of $2 \times 10^4$ cells/well and cultured overnight in phenol-red free Dulbecco's modified Eagle's medium (DMEM) containing 10% charcoal-dextran stripped fetal bovine serum (FBS). Cells were transfected with 100 ng of psi-CHECK2, *EFP*-Luc-wtERE, or *EFP*-Luc-mutERE plasmids and cultured with 100 nM E2 or vehicle (ethanol) for 48 h. The luciferase assay was performed using Dual-Luciferase Reporter Assay System (Promega).

### 2.7. Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assay was performed as described previously [18]. Ishikawa cells and EC-PDC spheroid culture were treated with 100 nM E2 for 0, 90, and 180 min. Cells were then fixed in 1% formaldehyde for 5 min at room temperature. Chromatin was sheared to an average size of 500 bp by sonication using Bioruptor ultrasonicator (Cosmo-Bio). Lysates were rotated at 4 °C overnight with anti-ERα antibody and precipitated DNA fragments dissociated from proteins were quantified by quantitative real-time PCR using the StepOnePlus™ Real-Time PCR System (Applied Biosystems) based on SYBR Green I fluorescence. Primer pairs specific for *EFP* ERE, Forkhead box protein A1 (*FOXA1*) ERE, and background region were described in Supplementary Table S1.

### 3. Results

#### 3.1. EFP is the most prominently estrogen-inducible TRIM family gene in EC-PDC spheroid culture

To explore estrogen-dependent alteration of gene expression in EC tumorigenesis, we performed microarray analysis for ERα-positive EC-PDC spheroid culture treated with estrogen or control vehicle in the previous study [12]. Using this data, we focused on the expression of TRIM family genes as we previously showed that Efp contributes to estrogen-dependent proliferation of EC cells [3]. Of 82 human TRIM protein genes, 7 genes including *EFP/TRIM25*, *TRIM16L*, *TRIM11*, *TRIM55*, *TRIM47*, *TRIM34*, and *TRIM38* showed >1.5-fold upregulation in response to E2 treatment and *EFP* was the most markedly upregulated gene among them (Table 1).

#### 3.2. Estrogen-dependent transcription of EFP in ERα-positive EC cells

We next examined time-dependent alteration of *EFP* mRNA expression by E2 treatment in EC-PDC spheroid culture as well as in ERα-positive Ishikawa cells (Fig. 1A and B). RT-qPCR showed that higher expression of *EFP* mRNA after E2 treatment was observed in EC-PDC than in Ishikawa cells. The substantial upregulation of *EFP* mRNA expression was observed 4 h after E2 treatment in both EC-PDC and Ishikawa cells, although high *EFP* mRNA level was sustained in EC-PDC whereas rather transient in Ishikawa cells (Fig. 1A and B).

We previously showed that the transcriptional activity of *EFP* could be increased by E2 treatment through estrogen-dependent activation of a functional ERE located in the 3′-UTR of the gene in breast cancer cells [17]. Therefore, we examined the estrogen responsiveness of *EFP* ERE with its promoter in Ishikawa cells using luciferase reporter plasmids including basal *EFP* promoter region (−173 to −1 bp upstream of its translation initiation site) and wild-type or mutated EREs (Fig. 1C). E2-dependent increase of luciferase activity was observed in wild-type ERE-containing vector whereas not in mutated ERE-containing vector, suggesting that *EFP* transcriptional activity is estrogen-dependent via its 3′-UTR ERE (Fig. 1D).
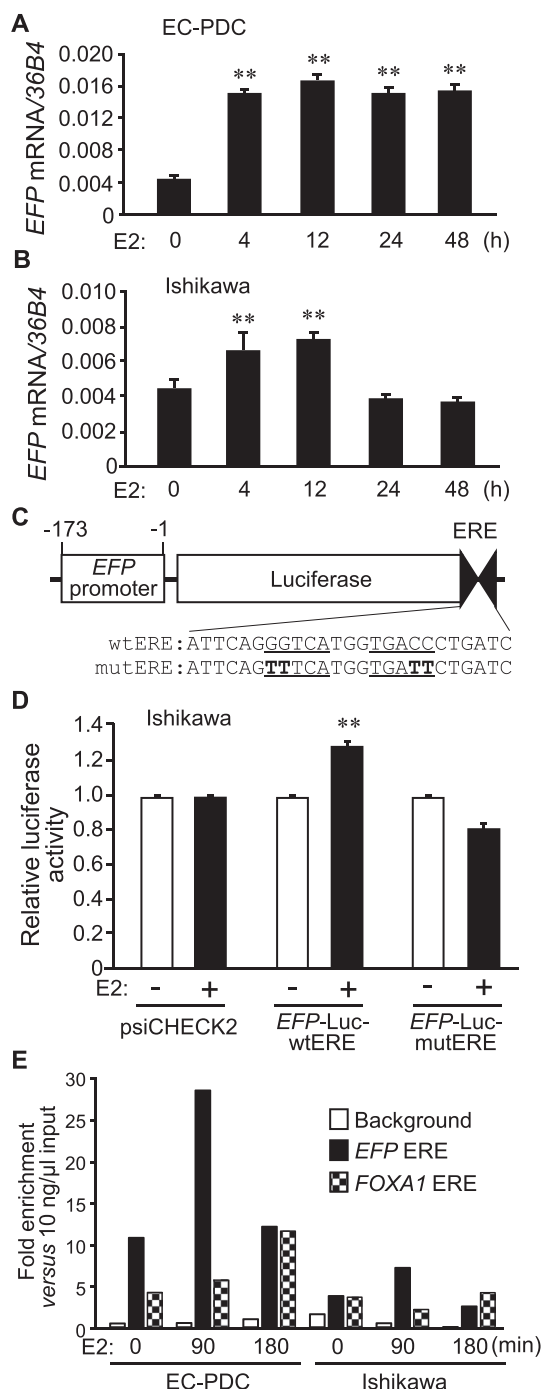


**Fig. 1.** Efp is regulated by estrogen in EC cells. (**A** and **B**) Estrogen-induced expression of *EFP* mRNA. RT-qPCR analysis was performed to detect expression levels of *EFP mRNA* in EC-PDC (A) and Ishikawa cells (B) treated with 100 nM 17β-estradiol (E2). (**C**) Schematic representation of luciferase reporter construct possessing *EFP* promoter region and estrogen responsive element (ERE) in 3′-untranslated region (UTR). Wild type (wtERE) and mutated ERE (mutERE) sequences were indicated. (**D**) Estrogen-induced promoter activity and functional ERE of *EFP*. Ishikawa cells were transfected with luciferase reporter plasmid, *EFP*-Luc-wtERE or *EFP*-Luc-mutERE, or parental vector psiCHECK2, and then cultured with 100 nM E2 or vehicle (ethanol). After 48 h, luciferase assay was performed. The results are represented as mean fold changes ± SD relative to the corresponding level of each reporter in E2 (−) control ($n = 3$). \*\*$P < 0.01$, using student's $t$-test. (**E**) ERα recruitment on ERE of *EFP*. EC-PDC and Ishikawa cells treated with 100 nM E2 for 0, 90, 180 min were subjected to chromatin immunoprecipitation (ChIP) assay using anti-ERα antibody. Immunoprecipitated DNA was analyzed by real-time qPCR. The results are shown as the averaged fold enrichment *versus* 10 ng/μl input ($n = 2$).

The recruitment of ERα to the *EFP* ERE was evaluated by ChIP-qPCR in Ishikawa cells and EC-PDC treated with 100 nM E2, using a known *FOXA1* ERE as a reference. ERα recruitment to the *EFP* ERE was increased at 90 min after E2 treatment in both Ishikawa cells and EC-PDC, while the ERα binding was much enriched in EC-PDC compared with Ishikawa cells (Fig. 1E).

### 3.3. Efp regulates growth and gene expression in EC cells

To clarify the role of Efp in EC-PDC spheroid proliferation, we transfected Efp-specific siRNAs (siEfp #A and #B) into EC-PDC culture as these siRNAs efficiently downregulate *EFP* levels in EC cell lines [3]. In EC-PDC, both siEfp #A and #B substantially repressed *EFP* mRNA expression (Fig. 2A) and significantly suppressed spheroid proliferation compared with EC-PDC transfected with control siRNA (siControl)(Fig. 2B).

To identify the downstream signals of Efp in EC-PDC spheroid culture, expression microarray analysis was performed in EC-PDC transfected with siEfp #A or siControl. The log2 fold changes in gene expression values (siEfp #A *versus* siControl) were plotted against the log2 gene expression values in the culture treated with siControl (Fig. 2C). Candidate genes were selected on the basis of outstanding fold-change values and high expression values: 1.585 and 9 for downregulated genes (Supplementary Table S2) and 1.585 and 5.3 for upregulated genes (Supplementary Table S3), respectively. We focused on siEfp #A-downregulated genes, among which Efp interactor or downstream gene is involved such as CDK6 [8,9] and IL6ST [3]. Based on the result of microarray analysis, we performed Gene Set Enrichment Analysis (GSEA) to dissect pathways

influenced by siEfp #A using KEGG gene sets. Interestingly, *CDK6* ranked second in the list of genes contributing to a positive enrichment score in the cell cycle pathway (Fig. 2D). *IL6ST* was listed as a gene contributing positively to the enrichment score in the cytokine-cytokine receptor interaction pathway, together with members of the *IL10* family (*IL26* and *IL19*) and its receptor *IL10RA* (Fig. 2E).

### 3.4. Efp modulates cell cycle- and immune-related genes

We further evaluated the effects of siEfp transfection on gene expression in EC-PDC by RT-qPCR. We examined *CDK6* and *CDK4* because both are critical kinases that interact with D-type cyclins and play crucial roles in cell cycle initiation and proliferation of hormone receptor-positive breast cancer. Notably, siEfp significantly decreased *CDK6* mRNA level (Fig. 3A) whereas not *CDK4* level (Fig. 3B), indicating distinct regulation processes between the two kinases. Moreover, the mRNA levels of the immune-related genes *IL10RA*, *IL26,* and *IL6ST* were substantially repressed in EC-PDC transfected with siEfp compared with that transfected with siControl (Fig. 3C–E). On the other hand, siEfps has no significant effect on expression levels of cancer stemness-related markers, *SOX2* and *OCT3/4* (Fig. 3F and G).

## 4. Discussion

In the present study, we demonstrated that *EFP* is an estrogen-inducible gene that contributes to the proliferation of EC-PDC. The transcriptional activity of the *EFP* was induced by estrogen in
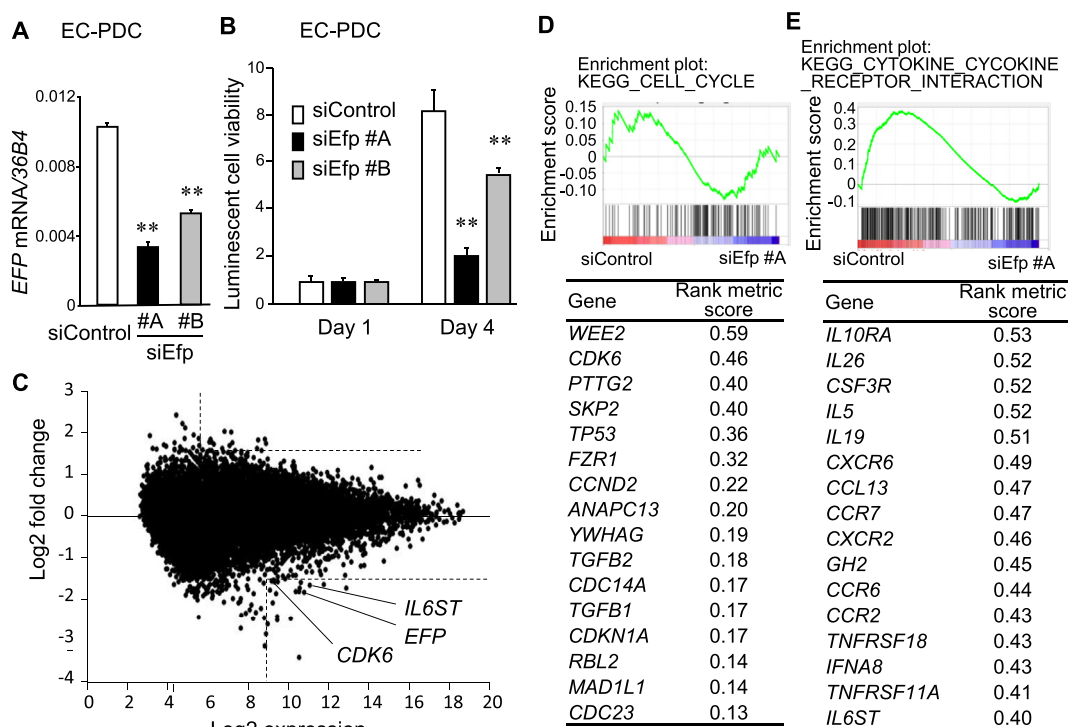


**Fig. 2.** Efp regulates growth and gene expression in EC-PDC. (**A**) Efp siRNAs (siEfp #A and #B) decreased *EFP* mRNA expression. Cells were transfected with siEfp #A and #B or control siRNA (siControl) for 60 h at a concentration of 10 nM and then the expression levels of *EFP* mRNA were evaluated by RT-qPCR and normalized to the corresponding *36B4* levels. (**B**) Efp knockdown resulted in inhibition of EC-PDC spheroid proliferation. EC-PDC cells were transfected with indicated siRNAs (10 nM each). CellTiter-Glo luminescent cell viability assay was performed at the indicated time points after transfection. Data were shown as fold change relative to the corresponding value for day 1. The results are presented as means ± SD (*n* = 3). **P < 0.01, using student's *t*-test. (**C**) Altered gene expression in response to Efp siRNA treatment. Expression microarray analysis was performed using RNAs from EC-PDC treated with 10 nM siEfp #A or siControl for 48 h. Gene expression values were plotted with the log2 fold change (siEfp #A *versus* siControl) against log2 expression (siControl). (**D** and **E**) Gene set enrichment analysis (GSEA) based on the result of microarray analysis. Enrichment analyses for signature gene sets influenced by siEfp #A were conducted by GSEA 4.1.0 software with gene set c2 (cp.kegg.v.7.2.symbols.gmt). GSEA based on Kyoto Encyclopedia of Genes and Genomes (KEGG) gene sets revealed the enrichment of cell cycle pathway (D) and the cytokine-cytokine receptor interaction pathway (E), and top 16 genes contributing to those pathways are listed.
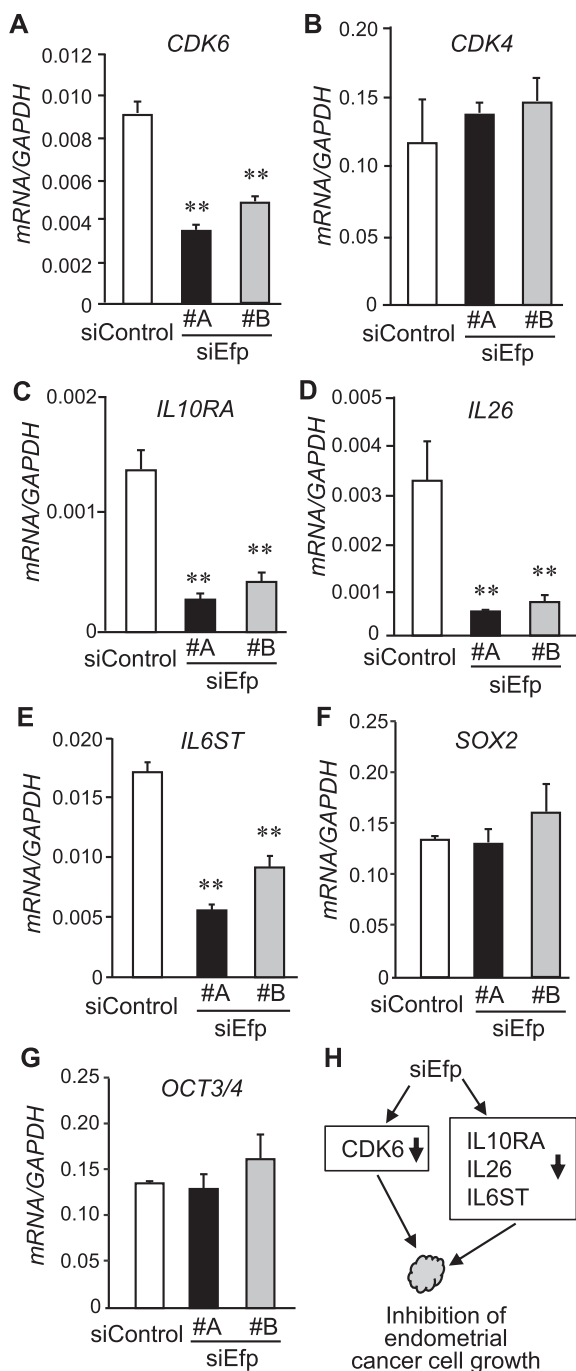
**Fig. 3.** Efp modulates cell cycle- and immune-related genes. EC-PDCs were transfected with siEfp #A, #B, or siControl for 60 h. RT-qPCR was performed to analyze the expression levels of *CDK6* (**A**)*, CDK4* (**B**)*, IL10RA* (**C**)*, IL26* (**D**)*, IL6ST* (**E**)*, SOX2* (**F**), and *OCT3/4* (**G**). Results are shown as means ± SD ($n = 3$). \*\**P* < 0.01, using Student's *t*-test. (**H**) Model for the function of Efp siRNA in EC-PDC. siEfp could suppress EC-PDC growth by decreasing the expression of cell cycle-related gene *CDK6*, and inflammation/immune-related genes *IL10*RA, *IL26*, and *IL6ST*.

protein kinases, mediating cell cycle progression from the G1 to the S phase [19]. These kinases phosphorylate the tumor suppressor retinoblastoma protein (RB1), which leads to the release of E2 factors (E2Fs) from the RB1—E2F complex and cell cycle entry into the S phase [20]. Inhibitors of CDK4/6 kinases have been recently applied to cancer management [21], as shown to improve the prognosis of patients with hormone receptor-positive and human epidermal growth factor receptor 2 (HER2)-negative advanced breast cancer [19,22]. Attention has been also paid to the clinical relevance of CDK4/6 in EC management. As a potential diagnostic target, enhanced CDK4/6-specific activity can be useful as it is significantly correlated with shorter progression-free survival times and an independent prognostic factor of poor outcomes in patients with low-risk EC not received adjuvant chemotherapy [23]. In terms of EC therapeutic option, CDK4/6 inhibitor abemaciclib can be applied to ECs with high D-type cyclin levels due to genomic alteration as the drug exhibited enhanced sensitivity [24]. CDK4/6 inhibitor palbociclib showed antitumor effects on PTEN-deficient ECs in a preclinical study [25] and clinical trials of CDK4/6 inhibitors are ongoing for EC [26]. Interestingly, our transcriptomic analysis indicated that Efp silencing preferentially influences *CDK6* rather than *CDK4* expression. Differential function of CDK6 not observed in CDK4 has been reported to efficiently phosphorylate pyruvate kinase M2 (PKM2) and phosphofructokinase (PFKP), thereby reducing the activity of these enzymes and leading to the metabolic reprogramming of glycolytic intermediates into the pentose phosphate pathway and serine pathway [27]. While CDK4 and CDK6 coordinately behave in cell cycle machinery, CDK6 may exhibit its distinct functions that contribute to tumorigenesis and its expression may be modulated by Efp in EC tumors. Our results highlight the importance of the Efp-CDK6 pathway in EC biology and may suggest the clinical relevance of CDK4/6 inhibitors in EC management.

In this study, we identified inflammation/immune-related genes *IL10*RA and *IL26* as new Efp-regulated genes in EC based on transcriptomic analysis using patient-derived model EC-PDC with Efp silencing. We previously identified *IL6ST* as another Efp target in EC cells [3]. IL10RA, a subunit of IL10 receptor, is associated with immune diseases, such as inflammatory bowel disease and atopic dermatitis [28,29]. IL26 belongs to the IL10 family and is overexpressed in numerous chronic inflammatory diseases [30]. Although the number of reports describing the role of these genes in cancer is limited, a positive correlation between IL10RA and expression of Ki67 proliferation marker has been shown in colorectal cancer [31]. IL26 has been found to stimulate the growth of gastric cancer cells which express IL-10RA through signal transducer and activator of transcription 3 (STAT3) activation [32]. IL26 expression is associated with a poor prognosis in patients with hepatocellular carcinoma [33]. Interestingly, IL26 was shown to be a significant inflammatory cytokine in promoting the neutrophil-mediated proliferation and metastasis of triple-negative breast cancer cells [34]. Overall, we speculate that *EFP/TRIM25* is a key estrogen-responsive gene in EC cells and promotes tumorigenesis by modulating multiple signaling pathways involved in cell cycle progression and immune responses. Efp would be a promising molecular target for potential EC therapeutic strategies.

cooperation with ERα binding to *EFP* 3′-UTR ERE. Efp silencing by its siRNAs decreased EC-PDC spheroid proliferation and down-regulated cell cycle-related gene *CDK6* and inflammation/immune-related genes including *IL10*RA, *IL26, and IL6ST*. These results suggest that Efp promotes estrogen-sensitive EC by modulating its downstream targets related to EC tumorigenesis (Fig. 3H).

Cell cycle control is a critical factor involved in cell proliferation. In this context, CDK6 as well as CDK4 are D-type cyclin-dependent

## Declaration of competing interest

## Acknowledgements

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2021.02.066.

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