

Regulation and reversal of paclitaxel resistance via the STAT1-mediated apoptotic pathway in ovarian cancer

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Abstract. Ovarian cancer (OC) is the most lethal disease in women. Resistance to paclitaxel (PTX) is the main cause of treatment failure in patients with OC. The STAT1 protein is a transcription factor implicated in a variety of cellular processes. The present study explored the function and regulatory mechanism of STAT1 in the reversal of PTX resistance *in vivo* and *in vitro*. The OC cell lines SK-OV-3 and OVCAR-3 and their counterpart PTX-resistant OC cell lines SK3R-PTX and OV3R-PTX were applied. The Tet-On STAT1-overexpression plasmids were constructed using the technique of the Tet-On gene expression system and were packaged by lentivirus. RNA and protein were detected by reverse transcription-quantitative PCR (RT-qPCR) and western blot analysis, respectively. OC cell mRNA-sequencing and subsequent RT-qPCR verification revealed that STAT1 expression was downregulated in PTX-resistant cells compared with their sensitive counterparts ($P < 0.01$), except for STAT1 β expression in SK3R cells ($P > 0.05$). Cell viability was assessed using a CCK-8 assay and PTX sensitivity was detected based on their IC₅₀ values. Overexpression of STAT1 sensitized PTX responses and decreased the tumor volume in xenograft mice. Bioinformatics analysis indicated that STAT1 had favorable effects on the overall survival of patients with OC. Apoptotic cells were detected using flow cytometry.

STAT1 α overexpression increased the percentage of apoptotic cells to 53.20 ± 0.92 and $36.74 \pm 0.77\%$ in OV3R-PTX and A2780-PTX cells, respectively, after 1 μ M PTX treatment for 24 h. Mechanistically, overexpression of STAT1, especially STAT1 α , confirmed by western blot and immunofluorescence staining, induced apoptosis by increasing apoptotic molecules such as Fas cell surface death receptor (FAS) and caspase-8 (CASP8), which was abolished in the presence of a caspase blocker (Z-VAD-FMK). Furthermore, the dual-luciferase assay confirmed that STAT1 directly bound to the promoter regions of the FAS and CASP8 genes. Thus, the present data demonstrated that STAT1 was a key mediator of the PTX chemotherapy response. Low STAT1 expression was a marker of PTX resistance, whereas overexpression of STAT1 sensitized OC cells to PTX and promoted apoptosis via the FAS/CASP8 signaling pathway. These findings may provide a potential therapeutic strategy to reverse PTX resistance in OC patients by targeting STAT1.

Introduction

Ovarian cancer (OC) is the most lethal disease of the female reproductive system (1,2). The initial approach for OC treatment is tumor reduction followed by chemotherapy, including platinum and paclitaxel (PTX) (3). Although PTX has shown good effects in the clinical practice of OC treatment, some patients eventually develop drug resistance, which is the main cause of treatment failure in patients with OC (4,5). The *ABCB1*-encoded drug transporter P-glycoprotein is considered to induce chemoresistance (6); however, a previous study has revealed that there are other mechanisms responsible for PTX resistance (7), which might provide a renewed sense of optimism for the clinical management of patients with OC with PTX resistance.

Apoptosis, a form of programmed cell death, influences tumor cell fate decisions (8). Dysregulation in the apoptotic pathway of OC cells may cause the occurrence of chemoresistance (9). Apoptosis can be categorized into two main types: Intrinsic and extrinsic apoptosis (10,11). Intrinsic apoptosis is triggered when the outer membrane of the mitochondria becomes leaky and allows cytochrome *c* to escape from the mitochondria, forming an apoptosome and activating

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Abbreviations: CASP8, caspase-8; Dox, doxycycline; FAS, Fas cell surface death receptor; GEO, Gene Expression Omnibus; GFP, green fluorescent protein; GSEA, Gene Set Enrichment Analysis; NC, negative control; OC, ovarian cancer; PTX, paclitaxel; RT-qPCR, reverse transcription-quantitative PCR; TCGA, The Cancer Genome Atlas

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caspase-9 (12). Extrinsic apoptosis is activated via membrane receptors, also referred to as death receptors, such as Fas cell surface death receptor (FAS; also referred to as CD95 and APO-1), tumor necrosis factor receptor-1 and Toll-like receptors, forming signaling complexes and activating caspase-8 (CASP8) (12,13). The induction of apoptosis is a hallmark of PTX-treated cancer cells (14) and exploiting this pathway represents a potential strategy to reverse PTX resistance (15). Nevertheless, the specific mechanisms of chemoresistance reversal via apoptosis require further elucidation.

STAT1 is a transcription factor (TF) involved in diverse biological processes, including the regulation of malignant behavior in various cancer types (16,17). The *STAT1* gene is located at 2q32.2 in the human genome with a total length of 45,215 bp and has two major transcripts, STAT1 α and STAT1 β , which are generated through alternative splicing (18,19). Our previous study has shown that STAT1 α is the main functional variant that serves an important role in the development of OC (20). Furthermore, using our Target Finder of Transcription Factor (TFoTF) tool (21), some apoptotic genes were predicted to be target genes of STAT1. Based on this prediction, the present study aimed to elucidate the mechanisms of STAT1-mediated apoptosis to reverse PTX resistance in OC cells.

Materials and methods

Cell culture. The human OC cell lines utilized in the present study are cataloged with Research Resource Identifiers (<https://scicrunch.org/resources>; Table SI). Each cell line underwent authentication through short tandem repeat profiling and was consistently screened to ensure it was free from pathogen and mycoplasma contamination. SK-OV-3, OVCAR-3 and 293T cells were purchased from Shanghai Fuheng Biotechnology Co., Ltd. A2780 cells and their PTX-resistant counterpart A2780-PTX cells were purchased from Nanjing KeyGen Biotech Co., Ltd. The PTX-resistant OC cell lines SK3R-PTX and OV3R-PTX were developed from their parental cell lines SK-OV-3 and OVCAR-3 as previously described (22). For cell culture, 293T, SK-OV-3, SK3R-PTX, A2780 and A2780-PTX cells were maintained in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Invitrogen; Thermo Fisher Scientific, Inc.). OVCAR-3 and OV3R-PTX cells were maintained in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 20% FBS. All cells were cultured at 37°C in a humidified atmosphere with 5% CO₂.

Construction of Tet-On STAT1-overexpression plasmids and lentiviral infection. Based on our previously generated STAT1-overexpression plasmids (20), two novel STAT1 α and STAT1 β plasmids containing green fluorescent protein (GFP) were constructed using the Tet-On gene expression system (23) and packaged into lentiviruses. The lentiviral plasmid pLenti-TET-on-GFP-puro (gift from Professor Yanyan Zhan, Cancer Research Center, School of Medicine, Xiamen University, Xiamen, China) was co-transfected with the second-generation packaging plasmid psPAX2 and the VSV-G envelope plasmid pMD2.G (both from Addgene, Inc.) into the 293T interim cells. For a standard 10-cm dish, the

transfection mixture contained 4.0 μ g of the lentiviral plasmid, with a mass ratio of 4:3:1 for the pLenti-TET-on-GFP-puro, psPAX2 and pMD2.G plasmids, respectively. Transfection was carried out at 37°C with 5% CO₂ for 6–8 h, after which the medium was replaced. Lentiviral particle-containing supernatant was collected at 48 and 72 h post-transfection, pooled, clarified by filtration through a 0.45- μ m filter. The empty vector (pLenti-tet-on-GFP) was used as a negative control (NC). The sequences of primers used for Tet-On plasmid cloning are listed in Table SII. The inserted sequences of the two variants are detailed in Table SIII. The maps of plasmids (pLenti-tet-on-GFP, pLenti-tet-on-STAT1 α -GFP and pLenti-tet-on-STAT1 β -GFP) and the validation of STAT1 protein expression after induction in the presence of 8 μ g/ml doxycycline (at 37°C for 4 days; Dox; Beyotime Biotechnology) by western blotting are shown in Fig. S1. The STAT1-overexpressing cells were generated after lentiviral infection at an MOI of 5 per well in a 6-well plate for 48 h and further culture with puromycin (5 μ g/ml) for 10 days to ensure stable integration. GFP expression was visualized and confirmed using fluorescence microscopy. The excitation and emission wavelengths for GFP were 488 and 509 nm, respectively.

Cell viability and PTX sensitivity assays. PTX-resistant cells (SK3R-PTX, OV3R-PTX and A2780-PTX cells) and their parental sensitive cells (SK-OV-3, OVCAR-3 and A2780 cells) were seeded in 96-well plates at a density of 5 $\times$ 10³ cells/well. Lentivirus-infected SK3R-PTX, OV3R-PTX and A2780-PTX cells with plasmids (pLenti-tet-on-GFP, pLenti-tet-on-STAT1 α -GFP and pLenti-tet-on-STAT1 β -GFP) were replated into 96-well plates at a density of 5 $\times$ 10³ cells/well and induced in the presence of 8 μ g/ml Dox at 37°C for 4 days. After treatment with varying concentrations of PTX (0, 0.0001, 0.001, 0.01, 0.1, 1 and 10 μ M) at 37°C for 48 h, cell viability was assessed using a Cell Counting Kit-8 (CCK-8; Beyotime Biotechnology). After 2 h of incubation, absorbance was measured at 450 nm using a Multiscan Spectrum (BioTek; Agilent Technologies, Inc.). Finally, a nonlinear fit was performed using the Hill model, and the absolute IC₅₀ value was calculated. To assess PTX sensitivity, each cell type was treated with a dose of PTX corresponding to its IC₅₀ values at 37°C. Cell viability was determined using a dose of 0.01 μ M for SK3R-PTX cells, 0.1 μ M for OV3R-PTX cells and 0.01 μ M for A2780-PTX cells at 0, 24 and 48 h at 37°C.

Xenograft mouse model. The animal experiments were approved by the Laboratory Animal Welfare and Ethics Committee of the Shanghai Public Health Clinical Center, Fudan University (approval no. 2020-A027-01; Shanghai, China). A total of 32 female BALB/c nude mice (3 weeks old with an initial body weight of ~17 g; SPF Biotechnology Co., Ltd.) were randomly assigned to four groups (n=8/group): Empty vector + saline, overexpression vector (oe-)STAT1 α + saline, empty vector + PTX and oe-STAT1 α + PTX. Each mouse received a subcutaneous injection of lentivirus-infected SK3R-PTX cells (5 $\times$ 10⁶ cells in 100 μ l FBS-free DMEM) into the right flank, and mice were housed under specific pathogen-free conditions in a controlled environment (temperature, 22 $\pm$ 2°C; humidity, 50 $\pm$ 10%; 12/12-h light/dark cycle) with

ad libitum access to food and autoclaved water. Measurements of body weight and tumor size (calculated as long diameter $\times$ short diameter²/2) were performed every 2 days. When the tumor size reached ~ 50 mm³ (14 days after tumor cells injection), the mouse was administered an intratumoral injection of PTX (10 mg/kg; marked as day 0), which was repeated once after 4 days. Starting from the first PTX injection, animals drank pre-prepared water containing 2 mg/ml Dox and 1.25% sucrose (24). On day 8, each mouse was euthanized by administering 200 mg/kg pentobarbital via intraperitoneal injection. Subsequently, images of mice were captured using a small-animal live imaging system (Guangzhou Biolight Biotechnology Co., Ltd.). After sacrificing, the tumors were excised and images captured.

Treatment with an apoptosis inhibitor. STAT1-overexpressing PTX-resistant cells were maintained in complete medium with 0.1 μ M PTX in 96-well plates (5,000 cells/well) and treated with the apoptosis inhibitor Z-VAD-FMK (20 μ M; Beyotime Biotechnology) or solvent control DMSO (Beyotime Biotechnology) at 37°C for 48 h. The viability of cells was determined using a CCK-8 assay as aforementioned. The experiments were repeated three times.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). Total RNA of cells was isolated using the RNA-Quick Purification Kit (Shanghai Yeasen Biotechnology Co., Ltd.). The RNA sample (500 ng) was reverse transcribed into cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics) at 42°C for 1 h. RT-qPCR was carried out with BeyoFast SYBR Green RT-qPCR Mix (Beyotime Biotechnology) according to the manufacturer's protocols. The primer sequences for RT-qPCR are listed in Table SIV. The PCR cycling settings included an initial denaturation step (95°C for 1 min), followed by 40 cycles of denaturation (95°C for 10 sec) and annealing/extension (60°C for 30 sec). The threshold cycle (Cq) value was measured using the 7300 real-time PCR system (version 1.4; Applied Biosystems; Thermo Fisher Scientific, Inc.). β -actin served as the internal control for normalizing gene expression. Relative target gene expression was calculated using the $2^{-\Delta\Delta Cq}$ method (25). The experiments were repeated at least three times.

Protein extraction and western blot analysis. Total protein of cells was isolated using SDS Lysis Buffer (Nanjing KeyGen Biotech Co., Ltd.) supplemented with phenylmethanesulfonyl fluoride and phosphatase inhibitors (Nanjing KeyGen Biotech Co., Ltd.). The concentration of extracted protein was determined using a BCA protein assay kit (Beyotime Biotechnology) and a microplate spectrophotometer (Epoch; BioTek; Agilent Technologies, Inc.). Total protein (20 μ g/lane) was run on a 10% SDS-PAGE gel and subsequently transferred to a PVDF membrane (MilliporeSigma). The membrane was blocked with 5% non-fat milk for 1 h at room temperature and then incubated with a primary antibody overnight at 4°C, followed by incubation with a secondary antibody for 1 h at room temperature. The following primary antibodies were used: Anti-STAT1 (cat. no. 42H3; dilution, 1:1,000) from Cell Signaling Technology, Inc.; and anti-FAS (cat. no. 13098-1-AP; dilution, 1:1,000), anti-CASP8 (cat. no. 66093-1-Ig; dilution,

1:1,000; this antibody can recognize pre- and cleaved-CASP8) and anti- β -actin (cat. no. 66009-1-Ig; dilution, 1:5,000) from Proteintech Group, Inc. The following secondary antibodies were used: HRP-conjugated goat anti-mouse IgG (cat. no. SA00001-1; dilution, 1:5,000) and HRP-conjugated goat anti-rabbit IgG (cat. no. SA00001-2; dilution, 1:5,000) from Proteintech Group, Inc. Protein bands were visualized using the BeyoECL Moon chemiluminescence kit (cat. no. P0018FS; Beyotime Biotechnology), and images were captured and analyzed using the Tanon-4500 imaging system (Tanon Science and Technology Co., Ltd.).

Apoptosis assay. STAT1-overexpressing PTX-resistant cells were cultured in complete medium. After treatment with PTX (0, 0.1, 1 and 10 μ M) at 37°C for 24 h, cells were harvested, washed with PBS, resuspended in 500 μ l 1X binding buffer, and stained with 3 μ l FITC Annexin V (BD Biosciences) and 5 μ l PI (BD Biosciences) for 15 min. Apoptotic cells were detected using flow cytometry (GALLIOS™; Beckman Coulter, Inc.) according to the manufacturer's instructions. Data were evaluated and analyzed using ModFit software (v 3.1; Verity Software House, Inc.). The experiments were repeated three times.

Immunofluorescence staining. Cells were plated in a 35-mm confocal culture dish featuring a 20-mm glass bottom at 1×10^5 cells/dish and cultured in medium with 8 μ g/ml Dox at 37°C for 4 days. Once the cells reached 50-70% confluence, they were fixed with 4% paraformaldehyde at room temperature for 15 min and subsequently washed with PBS for 5 min. Following permeabilization with 0.1% Triton X-100 in PBS for 15 min, the cells were treated with QuickBlock Blocking Buffer for Immunol Staining (Beyotime Biotechnology) for 1 h at room temperature. Cells were incubated with either rabbit anti-FAS antibody (cat. no. 13098-1-AP; dilution, 1:400; Proteintech Group, Inc.) or mouse anti-CASP8 antibody (cat. no. 66093-1-Ig; dilution, 1:400; Proteintech Group, Inc.) at 4°C for 12 h, followed by incubation with rabbit anti-STAT1 antibody (cat. no. 42H3; dilution, 1:4,000; Cell Signaling Technology, Inc.) at 4°C for another 12 h. Subsequently, the cells were treated with Alexa Fluor 594-conjugated goat anti-rabbit IgG (cat. no. 8760S; dilution, 1:1,000) or Alexa Fluor 647-conjugated goat anti-mouse IgG (cat. no. 4410S; dilution, 1:1,000) secondary antibody (Cell Signaling Technology, Inc.) for 2 h at room temperature in the dark. After staining the nucleus with DAPI (Beyotime Biotechnology) for 5 min at room temperature, fluorescence images were acquired using a fluorescence microscope (Olympus Corporation) and analyzed using Adobe Photoshop (version 24.3.0; Adobe Systems, Inc.).

Dual-luciferase reporter gene assay. The plasmid of the firefly luciferase reporter gene was constructed by the insertion of PCR products of FAS or CASP8 cDNAs amplified using a promoter region as a template from cultured cells. The thermocycling conditions were: initial denaturation at 95°C for 1 min, 15 cycles of denaturation at 95°C for 15 sec, annealing at 65°C (the temperature was reduced by 1°C after each cycle) for 15 sec and extension at 72°C for 30 sec, 25 cycles of denaturation at 95°C for 15 sec, annealing at 58°C for 15 sec and 7 extension at 2°C for 30 sec and final extension at 72°C for

5 min. The template DNA were extracted using a TIANamp Genomic DNA Kit (Tiangen Biotech Co., Ltd.) according to the standard manufacturer's instructions. PrimeSTAR Max DNA Polymerase (cat. no. R047A; Takara Bio, Inc.) was used for PCR, and experiments were performed according to the manufacturer's instruction. The sequences of primers are listed in Table SV. 293T cells were co-transfected with pGL4.10 firefly luciferase reporter and pRL-TK *Renilla* luciferase reporter plasmids (Promega Corporation) and oe-STAT1 α/β plasmid or empty vector using Lipo8000™ Transfection Reagent (cat. no. C0533-0.5ml; Beyotime Biotechnology). Luciferase activity was determined using a Dual-Luciferase Reporter Gene Assay Kit (Shanghai Yeasen Biotechnology Co., Ltd.) and a Fluoroskan Ascent FL (Thermo Fisher Scientific, Inc.) after 48 h of transfection. The experiments were conducted according to the manufacturer's protocol and were repeated three times. Results were calculated and normalized using the following formula: Relative activity (fold change)=mean value of experimental group (FLUC/RLUC)/mean value of control group (FLUC/RLUC), where FLUC indicates firefly luciferase readings and RLUC indicates *Renilla* luciferase readings.

Bioinformatics analysis. To distinguish gene expression between A2780-PTX and A2780 cells, mRNA-sequencing was performed (26). Total RNA was extracted by RNA-Quick Purification Kit (ESScience) according to the manufacturer's instructions. mRNA-sequencing analysis was performed by Gene Denovo Co. Ltd. (Guangzhou, Guangdong, China). The differentially expressed TFs based on mRNA-sequencing were analyzed using Gene Ontology (GO) using the 'clusterProfiler' (ver. 4.18.1) (27) and 'GOplot' (ver. 1.0.2) (28) R packages (ver. 4.2.2; <https://www.r-project.org/>). The target genes of STAT1 were predicted by TFoTF (ver. 1.0) with default settings, and position weight matrix (PWM) scores were calculated to evaluate the regulatory relations (21). The PWM of each TF was obtained using the R package JASPAR2020 (version 0.99.10; <http://jaspar.genereg.net/>). The pan-cancer transcriptome expression data were obtained from The Cancer Genome Atlas (TCGA; <https://www.cancer.gov/ccg/research/genome-sequencing/tcga>). Samples from each type of cancer were classified into a STAT1-high group (expression levels >80th percentile in all types of cancer) and a STAT1-low group (expression levels <20th percentile in all types of cancer). The differences between the STAT1-high and -low groups in terms of functional annotations were analyzed using Gene Set Enrichment Analysis (GSEA; version 4.2.2; <https://www.gsea-msigdb.org/gsea/index.jsp>) and hallmark gene sets (version 7.5.1) (29). Kaplan-Meier survival analysis was performed using the 'lifelines' Python package (ver. 0.30.0) (30) based on the OC transcriptome expression data and survival data downloaded from TCGA (TCGA-OV). The parameters were set as default following the official user guide. The cut-off values were defined as: Patients with gene expression levels >80th percentile were considered to be high-expressed, and gene expression levels <20th percentile were considered to be low-expressed. The significance was determined by log-rank test. The chromatin immunoprecipitation-sequencing (ChIP-seq) peak data (GSM320736) were obtained from Gene Expression Omnibus (GEO; <https://www.ncbi.nlm.nih.gov/geo/>) (31,32) and

analyzed by peak annotation analysis using the 'ChIPseeker' (ver. 1.32.1) (33) and 'GenomicFeatures' (ver. 1.62.0) R packages (34). Transcriptome expression data (GSE51373) of chemotherapy-sensitive and -resistant patients downloaded from the GEO database were analyzed for differential gene expression analysis (35). The Pearson correlation between the cell line transcriptome expression and chemotherapeutic agent sensitivity was analyzed with the 'SciPy' Python package (ver. 1.16.3) (36). The drug sensitivity data were sourced from CellMiner (<https://discover.nci.nih.gov/cellminer/>). The Python version used was 3.12.11 (<https://www.python.org/>).

Statistical analysis. Data were analyzed using the 'SciPy' and 'statsmodels' packages (ver. 0.14.4; <https://www.statsmodels.org/stable/index.html>) in the Python environment, if not otherwise stated, and were plotted using the 'Matplotlib' Python package (ver. 3.9.0; <https://matplotlib.org/>) and the 'ggplot2' R package (ver. 4.0.0; <https://ggplot2.tidyverse.org/>) using GraphPad Prism 8 software (Dotmatics). The data are presented as the mean $\pm$ SD or standard error of the mean. The linear trend test was performed using GraphPad Prism 8. Differences between two groups were analyzed using an unpaired Student's t-test. Differences among more than two groups were analyzed using one-way or two-way ANOVA followed by Tukey's Honestly Significant Difference test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

STAT1 is a marker of PTX resistance and enhances PTX sensitivity in OC cells. PTX resistance was confirmed in the resistant cell lines (SK3R-PTX, OV3R-PTX and A2780-PTX) by demonstrating reduced sensitivity across a range of PTX concentrations compared with their parental counterparts (SK-OV-3, OVCAR-3 and A2780) (Fig. 1A). After treatment with different dose of PTX for 48 h, the absolute IC₅₀ values of PTX were 0.044 μ M in SK3R-PTX cells vs. 0.012 μ M in SK-OV-3 cells, 4.016 μ M in OV3R-PTX cells vs. 1.800 μ M in OVCAR-3 cells, and 0.480 μ M in A2780-PTX cells vs. 0.001 μ M in A2780 cells. Screening of differentially expressed TF genes between A2780 and A2780-PTX cells using mRNA-sequencing data from the JASPAR database showed that 34.2% of TF genes were changed at the expression level (fold change, <0.4 or >2.5; $P < 0.05$). Among the 588 differentially expressed TF genes, 48.8% were downregulated and 51.2% were upregulated (Fig. 1B). Further analyses of TF genes with significantly altered gene expression showed that *STAT1* expression was lower in PTX-resistant A2780-PTX cells (Fig. 1C). The significantly altered expression of TF genes was classified by JASPAR annotation and GO term analyses and these differentially expressed TFs between A2780 and A2780-PTX cells may reflect the process of PTX resistance (Fig. S2A-C). STAT1 mRNA expression was lower in PTX-resistant cells than their counterpart PTX-sensitive cells ($P < 0.01$), except for STAT1 β expression in SK3R cells ($P > 0.05$), based on RT-qPCR (Fig. 1D), and these changes were confirmed at the protein level based on total STAT1 expression as analyzed using western blotting (Fig. 1E). Overexpression of STAT1 using oe-STAT1 (mainly

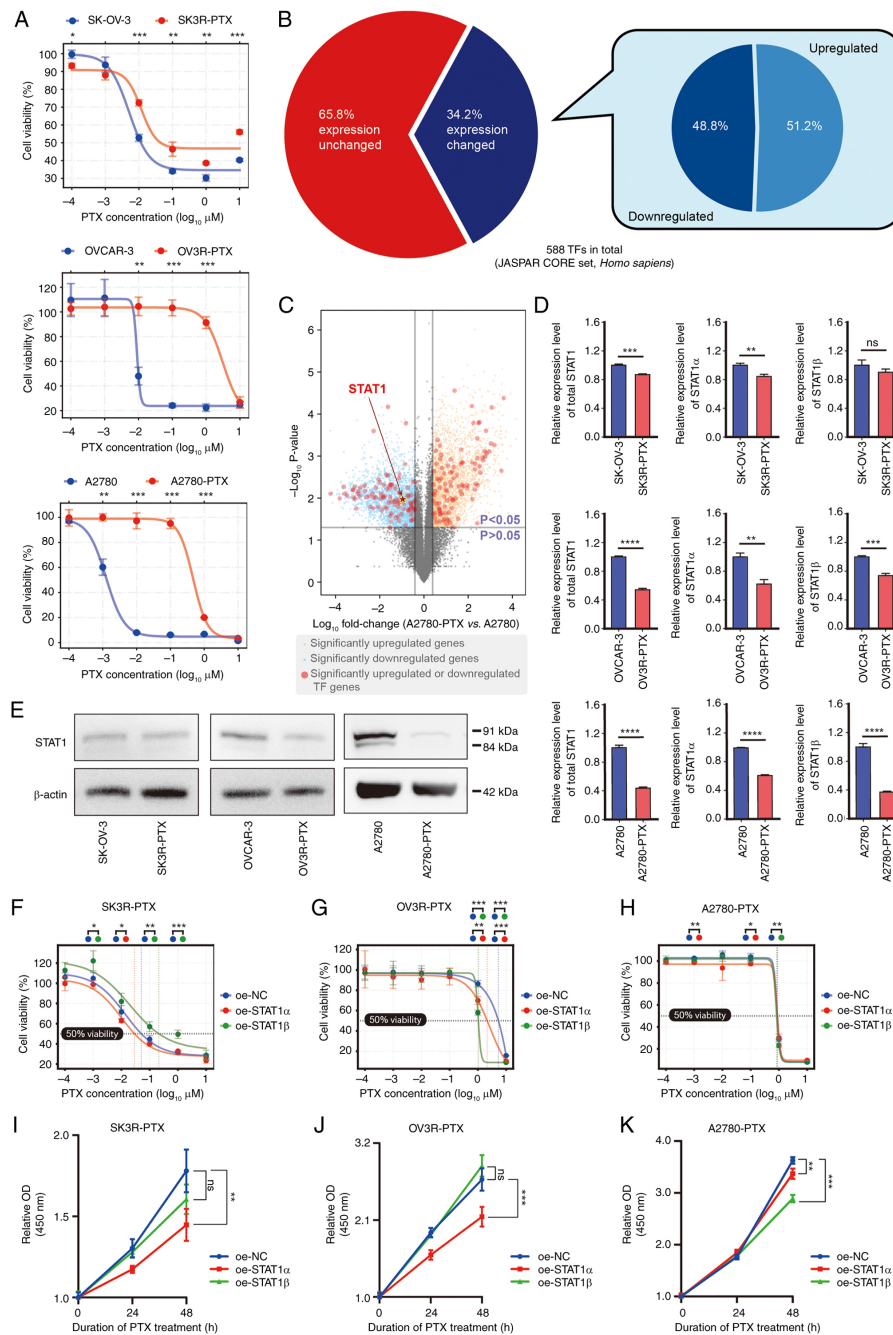


Figure 1. Detection of STAT1 expression, and effect of STAT1 on PTX sensitivity and viability in PTX-resistant OC cells (SK3R-PTX, OV3R-PTX and A2780-PTX cells) and their sensitive counterpart cells (SK-OV-3, OVCAR-3 and A2780 cells). (A) Validation of PTX resistance in OC cells. The dots and curves in the graphs indicate the relative cell viability at the corresponding PTX concentrations and non-linear regression fitting curves. Differences between sensitive cells and resistant cells were analyzed using two-way ANOVA followed by a Tukey's HSD test. Data are presented as the mean $\pm$ SD (n=3). (B) Differentially expressed TF genes in PTX-sensitive and PTX-resistant OC cells. The pie chart on the left represents the percentage of TF genes that were differentially expressed at a significant level (expression FC >2.5 and P<0.05). The pie chart on the right represents the percentage of upregulated and down-regulated TF genes. (C) Volcano plot showing the alteration of TF gene expression between A2780 and A2780-PTX cells based on the mRNA-sequencing results (n=3). The gray horizontal line represents the P-value of 0.05, and the two gray vertical lines represent the locations of FC >2 in gene expression (A2780-PTX vs. A2780 cells). TF genes with significantly altered expression (defined as FC <0.4 or >2.5 and P<0.05) were marked with red dots. The red star indicates STAT1. (D) Detection of total STAT1, STAT1 α and STAT1 β mRNA expression in three paired cells [PTX-sensitive cells (SK-OV-3, OVCAR-3 and A2780) vs. counterpart resistant cells (SK3R-PTX, OV3R-PTX and A2780-PTX)] by reverse transcription-quantitative PCR. Expression values for each group were normalized using β -actin as an internal reference. Differences between PTX-sensitive cells and PTX-resistant cells were analyzed using unpaired Student's t-test. Data are presented as the mean $\pm$ SD (n=3). (E) Detection of STAT1 protein expression in three paired cells by western blotting. Detection of PTX sensitivity in (F) SK3R-PTX, (G) OV3R-PTX and (H) A2780-PTX cells after overexpression of STAT1 α or STAT1 β in the presence of 8 μ g/ml Dox and different doses of PTX. The dots and curves in the graphs indicate the relative cell viability based on the Cell Counting Kit-8 assay. Blue, red and green vertical dotted lines in each panel indicated the absolute IC₅₀. Differences among groups were analyzed by two-way ANOVA followed by Tukey's HSD test. Data are presented as the mean $\pm$ SD [n=4 for (F and G); n=3 for (H)]. Determination of growth inhibitory effect of PTX in (I) SK3R-PTX, (J) OV3R-PTX and (K) A2780-PTX cells after induction of STAT1 overexpression by Dox. A cell viability assay was performed. Differences among multiple groups were analyzed by one-way ANOVA followed by Tukey's HSD test. Data are presented as the mean $\pm$ SD (n=3). *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001 resistant cells vs. sensitive cells in each pair in (A) and (D) or oe-STAT1 α / β vs. oe-NC in (F-K). Dox, doxycycline; FC, fold change; HSD, Honestly Significant Difference; NC, negative control; ns, not significant; OC, ovarian cancer; OD, optical density; oe, overexpression vector; PTX, paclitaxel; TF, transcription factor.

STAT1 α) lentiviral infection increased the PTX sensitivity (Fig. 1F-H) and decreased cell viability (Fig. 1I-K) in three PTX-resistant cell lines. Specifically, the absolute IC₅₀ values of PTX were 0.050, 0.029 and 0.211 μ M in SK3R-PTX cells, 5.329, 2.132 and 1.040 μ M in OV3R-PTX cells, and 0.883, 0.882 and 0.831 μ M in A2780-PTX cells after infection with empty vector, oe-STAT1 α and oe-STAT1 β , respectively. Overexpression of STAT1 after Tet-On system-based plasmid lentiviral infection was verified in three PTX-resistant cell lines treated with or without 8 μ g/ml Dox for 4 days as immunofluorescent signals were captured by immunofluorescence microscopy (Fig. S3A-C).

Overexpression of STAT1 inhibits tumor formation in xenograft mice and is a favorable factor in the overall survival of patients. To clarify the biological effect of STAT1 on PTX resistance of tumors, an *in vivo* xenograft mouse model was used. Considering that STAT1 α was the major transcript in OC cells (Fig. S4A-B), STAT1 α -overexpressing SK3R-PTX cells were generated. As aforementioned, SK3R-PTX cells were stably infected with oe-STAT1 α or control vector, and were subcutaneously injected into nude mice (Fig. 2A). The body weight of the mice in each group was compared at each timepoint and was not significantly changed during the experimental intervention (Fig. 2B and C). Overexpression of STAT1 significantly decreased the tumor volume regardless of PTX treatment at post-intervention day 8 (Fig. 2D and E). Furthermore, overexpression of STAT1 sensitized PTX responses *in vivo* (Fig. 2F) as the tumor weight in the oe-STAT1 α group was markedly lower than the vector group after PTX treatment. Since STAT1-GFP expression in the cells used for the subcutaneous implantation was inducible in the presence of Dox, small-animal live imaging was used to observe tumor formation. The fluorescence signal was significantly lower in the STAT1 α -overexpressing group than in the vector control group without or with PTX treatment (Fig. 2G and H). These data further indicated that oe-STAT1 α inhibited tumorigenicity and increased PTX sensitivity in resistant cells. To clarify the impact of STAT1 in patients with OC, clinical datasets were downloaded from GEO and TCGA and analyzed. The expression levels of STAT1 were significantly lower in chemoresistant patients compared with chemosensitive patients (Fig. 2I). The survival analysis using TCGA datasets revealed a significant difference in the overall survival of patients with OC between the STAT1-high and STAT1-low expression groups ($P=0.018$; Fig. 2J). The median survival time in the STAT1-high and STAT1-low groups was 1,446 and 1,163 days, respectively. The hazard ratio in the STAT1-high group compared with the STAT-low group was 0.641 ($P=0.019$). These data indicated that STAT1 was a favorable factor in the overall survival of patients with OC.

Overexpression of STAT1 reverses PTX resistance through the apoptosis pathway. Since overexpression of STAT1 decreased OC cell viability in some cell lines (Fig. 1F-K), a wide-ranging analysis of the biological functions of STAT1 in pan-cancer was performed. GSEA revealed that high STAT1 expression was closely associated with 'IL6 JAK STAT3 signaling', 'PI3K AKT mTOR signaling', and 'apoptosis' in a number of cancer types (Fig. 3A). Further analysis of

'HALLMARK_APOPTOSIS' between STAT1 high and low expression (expression level <20th percentile) samples of OC using GSEA revealed that apoptosis was significantly enriched (Fig. 3B). Overexpression of STAT1 α decreased the viability of SK3R-PTX, OV3R-PTX and A2780-PTX cells in the presence of PTX, whereas these effects of STAT1 α were completely abolished in OV3R-PTX and A2780-PTX cells and partially abolished in SK3R-PTX cells in the presence of a caspase blocker (Z-VAD-FMK; 20 μ M) (Fig. 3C). DMSO was used as a control for comparison. Because STAT1 is a TF protein, TFoTF was used to predict target genes of STAT1 using the apoptosis gene set 'HALLMARK_APOPTOSIS', which was downloaded from the GSEA database (Hallmark gene sets; Ver 7.5.1) (29). FAS and CASP8, the two key genes of the apoptosis pathway, were ranked among the highest-scoring genes and might be target genes of STAT1 (Fig. 3D). Furthermore, Pearson correlation analysis revealed that FAS and CASP8 exhibited a weakly negative correlation with PTX resistance (Fig. 3E), which was confirmed by TFoTF with high scores (Fig. 3F). These data indicated that STAT1 affected PTX resistance mainly through the apoptosis pathway.

PTX enhances the expression of STAT1 and apoptotic factors, and overexpression of STAT1 induces PTX-resistant cell apoptosis. The effect of PTX on the expression levels of STAT1 and apoptotic factors CASP8 and FAS in PTX-sensitive cells was first confirmed. Time-course (6, 12, 18 and 24 h) and dose-dependent (0.001, 0.01 and 0.1 μ M) experiments showed that STAT1, CASP8 and FAS mRNA expression was upregulated in PTX-sensitive cells after PTX treatment, albeit at different levels in OVCAR-3, A2780 cells and SK-OV-3 cells (Figs. 4A and B, and S5A). The expression values of each gene at different time points were further analyzed and confirmed using the linear trend test. The mRNA expression levels of STAT1, CASP8 and FAS were gradually increased in the time-course experiment after PTX treatment (Figs. 4C and D, and S5B), and the trends were more significant at specific PTX concentrations, such as 0.001 μ M in OVCAR-3 and A2780 cells and 0.1 μ M in SK-OV-3 cells. Notably, a decrease in STAT1 β expression was observed in SK-OV-3 cells after PTX treatment at 12, 18 and 24 h compared with 6 h (Fig. S5A). Next, it was confirmed that overexpression of STAT1 enhanced PTX sensitivity to induce PTX-resistant cell apoptosis. Flow cytometry was performed to detect PTX-induced apoptosis in oe-STAT1-infected OV3R-PTX and A2780-PTX cells compared with oe-NC cells (Fig. 4E and F). Quantitative analysis of flow cytometry data showed that overexpression of STAT1 α induced apoptosis under different PTX treatments. oe-STAT1 α , compared with oe-NC, increased the percentage of apoptotic cells to 53.20 $\pm$ 0.92% and 36.74 $\pm$ 0.77% in OV3R-PTX and A2780-PTX cells, respectively, after 1 μ M PTX treatment for 24 h ($P<0.01$) (Fig. 4G and H).

Overexpression of STAT1 upregulates CASP8 and FAS expression. Since PTX can induce apoptosis (14), and since PTX can upregulate STAT1 (Fig. 4), the present study subsequently investigated the effect of STAT1 on CASP8 and FAS expression in PTX-resistant cells. The Dox-induced overexpression of STAT1 α and STAT1 β was confirmed by RT-qPCR in SK3R-PTX, OV3R-PTX and A2789-PTX cells after infection

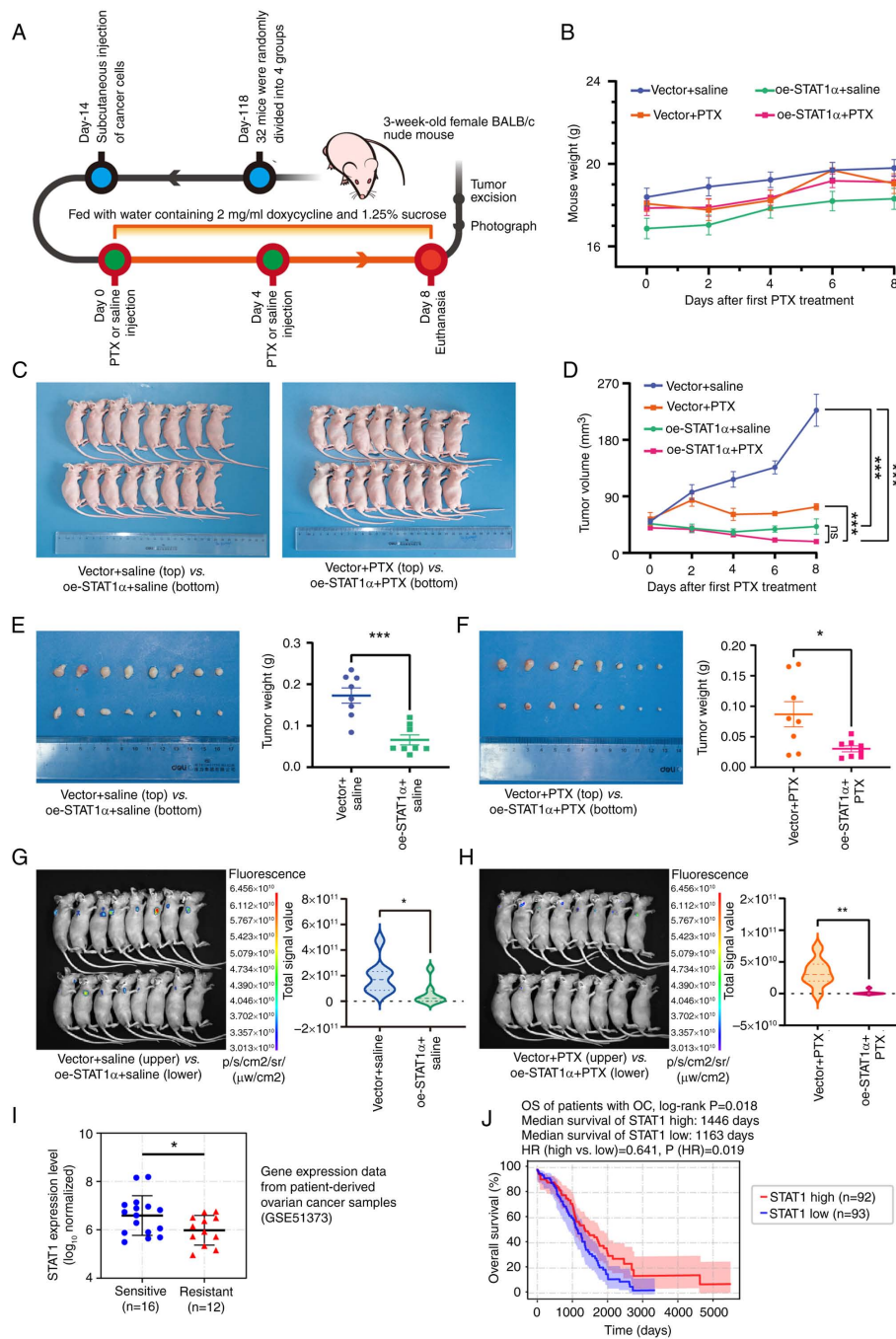


Figure 2. Effect of STAT1 on tumor formation in xenograft mice and OS in patients with OC. (A) Brief schematic diagram of the flow of the animal experiments. (B) Measurement of the body weight of the mice in each group during the experimental intervention. Differences among the four groups were analyzed by one-way ANOVA followed by Tukey's HSD test at each timepoint. Data are presented as the mean $\pm$ SEM (n=8 per group). (C) Images of empty vector or oe-STAT1 α -overexpressing mice without or with PTX injection. The top row shows the empty vector control group and the second row shows the STAT1 α -overexpressing group. (D) Comparison of the volume of subcutaneous tumors after saline or PTX treatment. Differences among the four groups were analyzed by one-way ANOVA followed by Tukey's HSD test. Data are presented as the mean $\pm$ SEM (n=8 per group). (E) Images of the tumors after the euthanasia of mice with saline injection on the left. Comparison of the weight of debulked tumors is shown on the right. (F) Images of the tumors after the euthanasia of mice with PTX injection on the left. Comparison of the weight of debulked tumors is shown on the right. The top row shows the empty vector control group and the second row shows the STAT1 α -overexpressing group. Differences between the two groups were analyzed using Student's t-test. Data are presented as the mean $\pm$ SEM (n=8 per group). (G) Images from small-animal live imaging after saline injection are shown on the left. The vector control group is shown in the top row and the STAT1 α -overexpressing group is shown in the second row. Superimposed color patches on the right indicate the intensity of the green fluorescent protein signal. (H) Images from small-animal live imaging after PTX injection are shown on the left. The vector control group is shown in the top row and the STAT1 α -overexpressing group is shown in the second row. Superimposed color patches on the right indicate the intensity of the green fluorescent protein signal. Differences between the two groups were analyzed using Student's t-test. Data are presented as the mean $\pm$ SEM (n=8 per group). (I) Differential mRNA expression of STAT1 between samples from chemotherapy-sensitive OC cases (n=16) and chemotherapy-resistant OC cases (n=12). Data were extracted from Gene Expression Omnibus (GSE51373). Differences between the two groups were analyzed using Student's t-test. Data are presented as the mean $\pm$ SD. (J) Difference in OS of patients with OC (data were downloaded from TCGA; TCGA-OV dataset; <https://www.cancer.gov/ccg/research/genome-sequencing/tcga>) between STAT1-high expression (n=92) and STAT1-low expression (n=93) groups. The survival times in these two groups were compared using the log-rank test. The HR was calculated using Cox's proportional hazard model and the significance of the HR was compared using the log-likelihood ratio test. *P<0.05; **P<0.01; ***P<0.001. HR, hazard ratio; HSD, Honestly Significant Difference; OC, ovarian cancer; ns, not significant; oe, overexpression vector; OS, overall survival; PTX, paclitaxel; SEM, standard error of the mean; TCGA, The Cancer Genome Atlas.

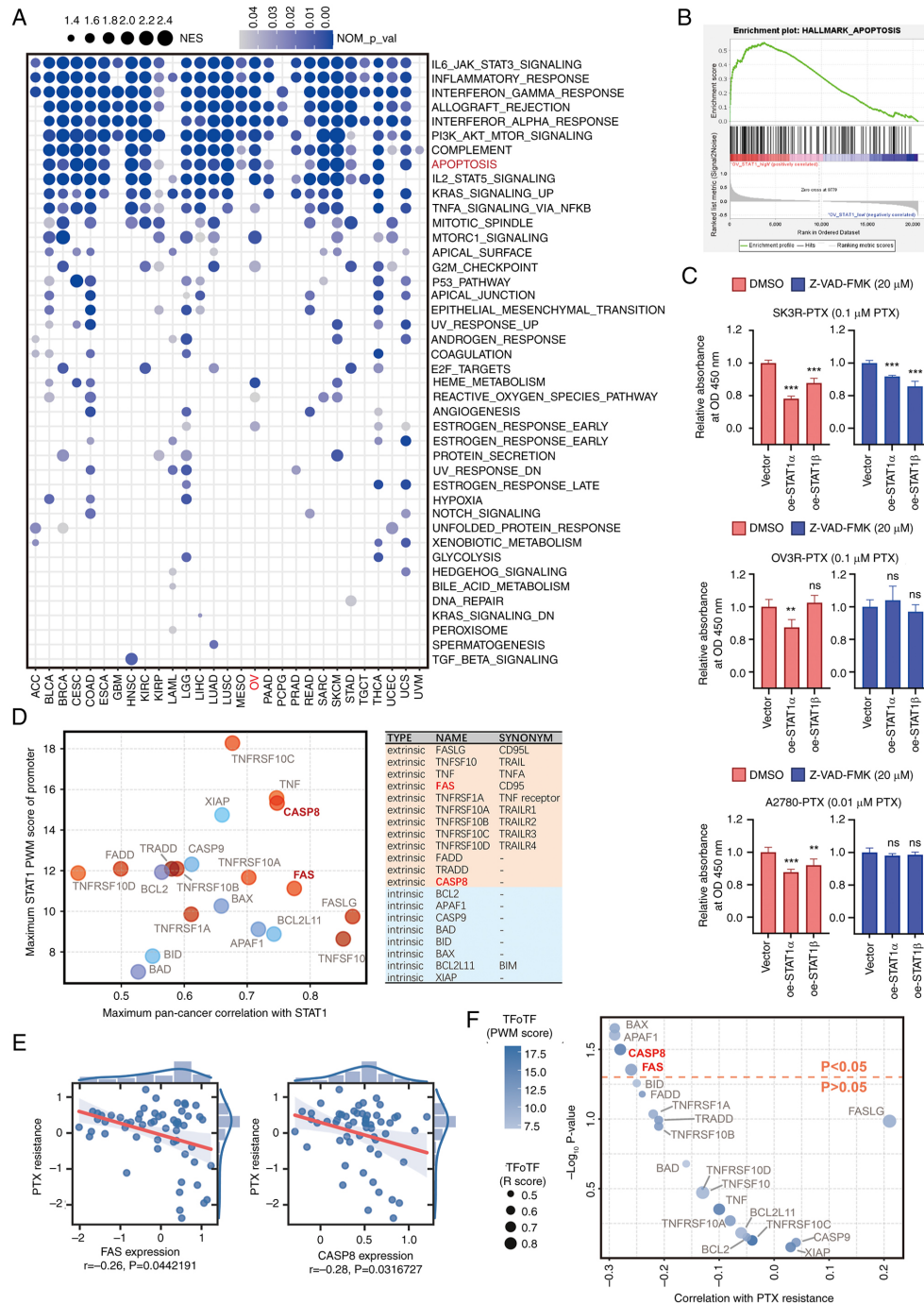


Figure 3. Association of STAT1 with PTX resistance. (A) Analysis of the biological functions of STAT1. High STAT1 expression (expression level >80th percentile) based on pan-cancer transcriptomics data was associated with enriched pathways. Horizontal coordinates indicate the different types of cancer in The Cancer Genome Atlas and vertical coordinates indicate the names of cell biological functions. The significance of each enrichment result was determined using the default setting. (B) Gene Set Enrichment Analysis of 'HALLMARK_APOPTOSIS' between STAT1 high and low expression samples of ovarian cancer (expression level >80th and <20th percentile, respectively). (C) Effect of apoptosis inhibitor Z-VAD-FMK in STAT1-overexpressing PTX-resistant cells. SK3R-PTX, OV3R-PTX and A2780-PTX cells were stably infected with empty vector, STAT1 α or STAT1 β plasmids in the presence of PTX, followed by 20 μ M Z-VAD-FMK or solvent DMSO treatment for 48 h. Cell viability was detected using a Cell Counting Kit-8 assay. Data were normalized using reads from the empty vector group. Differences among multiple groups were analyzed by one-way ANOVA followed by Tukey's Honestly Significant Difference test. Data are presented as the mean $\pm$ SD (n=5). **P<0.01; ***P<0.001 (oe-STAT1 α/β vs. Vector). (D) Analysis of genes closely related to apoptotic pathways obtained after TFoTF prediction and screening of the apoptosis gene set ('HALLMARK_APOPTOSIS'). Information about these genes is shown in the table on the right. The scatterplot on the left shows the visualization of the TFoTF prediction outcomes for these genes. The horizontal coordinate indicates the R score based on TFoTF and the vertical coordinate indicates the PWM score (k_{max}). The red circles denote genes in the extrinsic apoptosis pathway, and the blue circles denote genes in the intrinsic apoptosis pathway. (E) Correlation of CASP8 and FAS expression with cellular PTX resistance. The significance of each regression coefficient was determined using the Wald test with a t-distribution using the SciPy Python package. (F) Analysis and screening of STAT1-targeted PTX resistance-associated apoptotic genes. In the bubble plot, the horizontal coordinate indicates the Pearson correlation coefficient between the target gene of STAT1 and PTX resistance. The vertical coordinate indicates the statistical significance of the correlation. The red horizontal dashed line indicates the position of the regression P-value of 0.05. The size of each bubble indicates the R score based on TFoTF. The color depth of each bubble indicates the PWM score based on TFoTF (k_{max}). CASP8, caspase-8; FAS, Fas cell surface death receptor; NES, normalized enrichment score; NOM_p_val, normalized P-value; ns, not significant; OD, optical density; oe, overexpression vector; PTX, paclitaxel; PWM, position weight matrix; r, R-value; TFoTF, Target Finder of Transcription Factor.

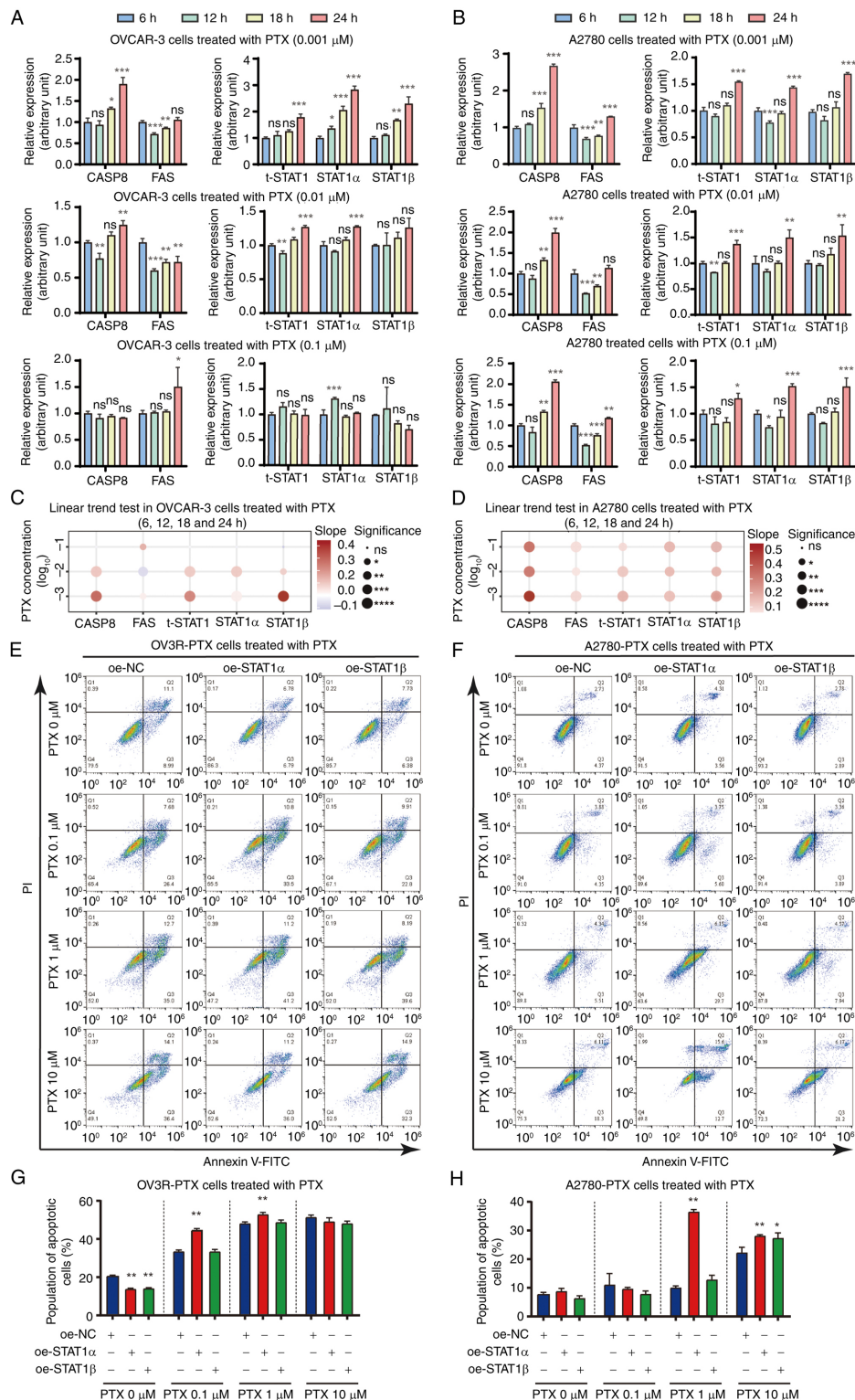


Figure 4. Effect of PTX on CASP8, FAS and STAT1 expression in time-course and dose-dependent experiments, and effect of STAT1 on apoptosis. mRNA expression levels of CASP8, FAS, t-STAT1, STAT1 α and STAT1 β were detected by reverse transcription-quantitative PCR in (A) OVCR-3 and (B) A2780 cells after treatment with 0.001, 0.01 and 0.1 μ M PTX for 6, 12, 18 and 24 h. Expression values for each group were normalized using β -actin as an internal reference. Differences among multiple groups were analyzed using one-way ANOVA followed by Tukey's Honestly Significant Difference test. Data are presented as the mean $\pm$ SD (n=3). (C) Results of linear trend test for changes in (A). The vertical coordinate indicates the concentration of PTX, and the horizontal coordinate indicates the genes. The color of the bubble represents the slope of the change trend, while the size of the bubble represents the statistical significance. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001. (D) Results of linear trend test for changes in (B). The vertical coordinate indicates the concentration of PTX, and the horizontal coordinate indicates the genes. The color of the bubble represents the slope of the change trend, while the size represents the statistical significance. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001. Detection of apoptosis in (E) OV3R-PTX and (F) A2780-PTX cells using flow cytometry. OV3R-PTX and A2780-PTX cells were infected with either oe-STAT1 α or oe-STAT1 β in the presence or absence of 0.1, 1 and 10 μ M PTX. Quantification of apoptotic cells based on flow cytometry in STAT1 α/β -overexpressing (G) OV3R-PTX and (H) A2780-PTX cells in the presence or absence of PTX. Data were evaluated and analyzed using ModFit software and are presented as the mean $\pm$ SD (n=3). *P<0.05; **P<0.01; ***P<0.001 vs. 6 h in (A) and (B) and vs. oe-NC in (G) and (H). CASP8, caspase-8; FAS, Fas cell surface death receptor; NC, negative control; ns, not significant; oe, overexpression vector; PTX, paclitaxel; t-STAT1, total STAT1.

with oe-STAT1 α and oe-STAT1 β lentivirus, respectively (Fig. S6A-C). The overexpression of STAT1 α and STAT1 β significantly enhanced the mRNA expression levels of CASP8 and FAS detected by RT-qPCR in most PTX-resistant cells, except A2780-PTX cells, in which no significant change in CASP8 expression was found after STAT1 lentiviral infection (Fig. 5A and B). Finally, the protein expression levels of CASP8 and FAS were evaluated by western blotting (Fig. 5C and D) and immunofluorescence staining (Fig. 5E and F). Despite some cell line-specificity, these data indicated that CASP8 and FAS expression could be upregulated by STAT1.

STAT1 directly binds to the promoter regions of CASP8 and FAS. ChIP-seq data of STAT1 were obtained from GEO (GSM320736). Data analysis revealed the potential binding sites of multiple genome-associated fragments of CASP8 and FAS with STAT1, and some of the fragments were located in the proximal promoter regions of CASP8 and FAS (Fig. S7). The STAT1 binding sites were found to be clustered within the promoter regions of CASP8, which was predicted using PWM scoring. The motif logo was drawn, and the specific values of the PWM were calculated (Fig. 6A). This logo visualized the conservation and preference for specific nucleotides at different positions in a DNA sequence. There were four and two clusters of binding sites within the promoter region of CASP8 and FAS, respectively (Fig. 6B). Subsequently, correlation analysis revealed that the expression of CASP8 and FAS was correlated with STAT1 expression in ovarian cancer as determined by the Wald test with t-distribution using the SciPy Python package ($P < 0.001$; Fig. 6C). Subsequently, the binding sites for STAT1 were validated using dual-luciferase reporter gene assays. Based on the sequences of the predicted binding sites, plasmids of a full-length promoter and several truncated fragments of CASP8 and FAS were constructed. For CASP8, four constructs were generated. A full-length plasmid (pGL4-CASP8-2000) contained all four binding sites. The three truncated fragments, pGL4-CASP8-1557, pGL4-CASP8-1249 and pGL4-CASP8-863, contained three, two and one proximal binding sites, respectively (Fig. 6D). The relative luciferase activity in each transfection group was measured in 293T cells after co-transfection with a dual-luciferase reporter gene plasmid and a STAT1-overexpression plasmid (oe-STAT1 α , oe-STAT1 β or empty vector). For the pGL4-CASP8-2000 and pGL4-CASP8-1249 plasmids, the relative luciferase activity was enhanced in the presence of STAT1 α (Fig. 6E). For the pGL4-CASP8-1557 and pGL4-CASP8-863 plasmids, the relative luciferase activity was enhanced in the presence of STAT1 α or STAT1 β . For FAS, two constructs were generated, including a full-length plasmid (pGL4-FAS-2000) containing two binding sites and a truncated fragment plasmid (pGL4-FAS-1019) containing one proximal binding site (Fig. 6F). For the pGL4-FAS-2000 plasmid, the relative luciferase activity was enhanced in the presence of STAT1 α rather than STAT1 β (Fig. 6G). There was no change in the relative luciferase activity for the pGL4-FAS-1019 plasmid. These data indicate that STAT1 α could directly bind to the promoters of CASP8 and FAS to regulate their expression at the transcription level.

Discussion

Chemoresistance is one of the major obstacles in anticancer treatment. The present study explored the role and regulatory mechanism of STAT1 in PTX-resistant OC cells. Overexpression of STAT1 upregulated the expression levels of apoptosis-related genes *CASP8* and *FAS*, thereby enhancing the PTX sensitivity in resistant cells *in vitro* and *in vivo*.

In the present study, analyses using mRNA-sequencing revealed that STAT1 was differentially expressed between PTX-sensitive and PTX-resistant OC cells. Low levels of STAT expression were detected in PTX-resistant cells compared with PTX-sensitive cells. Overexpression of STAT1 enhanced the PTX sensitivity in resistant cells, suggesting that STAT1 is an important factor in mediating the processes of PTX resistance. It has been demonstrated that STAT1 serves a dual role in tumorigenesis, exhibiting upregulation or downregulation in other cancer types (18,37). Some unphosphorylated STAT1 molecules also exhibit transcriptional activity (38). Our previous study demonstrated that STAT1 was upregulated in OC cells compared with normal ovarian cells (39), and overexpression of STAT1 blocked the function of the TGF- β signaling pathway (20). The present study further demonstrated that STAT1 was a potential biomarker and target for OC. Notably, similar outcomes have been shown by other groups. For example, STAT1 negatively regulated the proliferative capacity in hepatocellular carcinoma cells (40) and was a favorable factor affecting the prognosis of colorectal cancer (41). Another study demonstrated that STAT1 activity was associated with the degree of disease progression from ductal carcinoma *in situ* to invasive carcinoma in both human and mouse mammary tumors (42). Furthermore, STAT1 is a promoter of leukemia progression (43). Our previous meta-analysis reviewed the clinical impact of STAT1; high STAT1 expression was associated with longer overall survival in patients with OC, rectum adenocarcinoma, sarcoma and cutaneous melanoma, but was associated with poor prognosis in patients with lung adenocarcinoma, renal cancer, brain lower-grade glioma and pancreatic cancer (44). Existing evidence indicates that STAT1 serves a dual role and has both tumor-suppressing and tumor-promoting functions (18). The functional effect of STAT1 on tumor progression is highly context-dependent, varying with disease stage, genetic backgrounds and tissue origins (44). Therefore, targeting STAT1 for cancer therapy demands a precision medicine approach, where its activity is modulated based on the specific tumor context.

STAT1 has some common characteristics of TFs that can be targeted in anticancer therapy (45). The number of oncogenes identified is much larger than the number of TF genes. Considering that TFs are the initiators of gene expression, a series of downstream functional oncogenes and tumor suppressor genes can be activated or inhibited if the activity of specific cancer-related TFs is altered (46). Nevertheless, the regulatory mechanisms are not fully understood. The biological functions of TFs rely on protein-DNA or protein-protein interactions (47,48). Compared with the more tractable enzyme or kinase interactions, inhibitors of protein-DNA or protein-protein binding are deficient, leading to the difficulty of directly targeting the functional

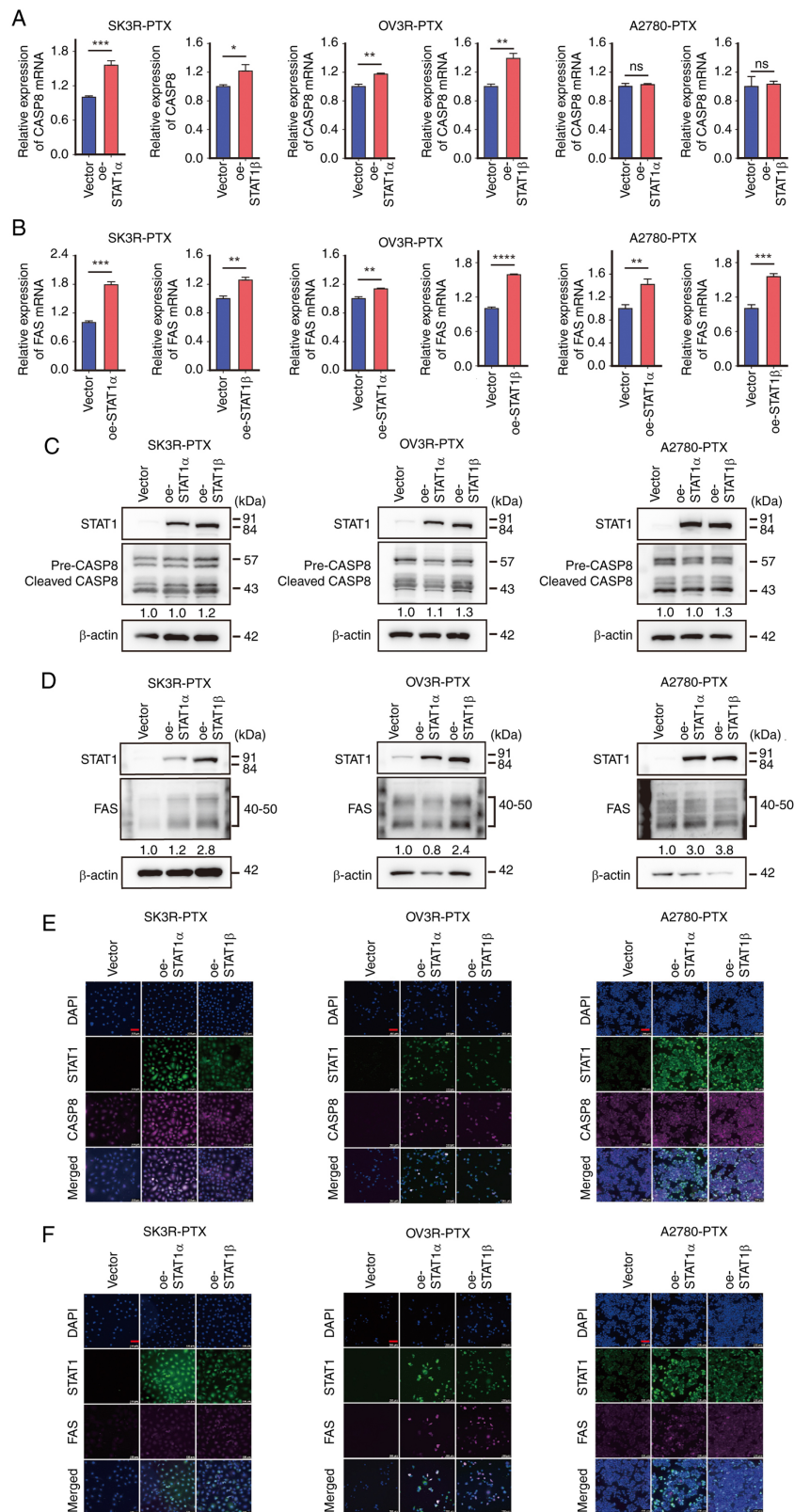


Figure 5. Effect of STAT1 on CASP8 and FAS expression in PTX-resistant cells. Detection of (A) CASP8 and (B) FAS mRNA expression in oe-STAT1α- and oe-STAT1β-lentivirus-infected SK3R-PTX, OV3R-PTX and A2780-PTX cells by reverse transcription-quantitative PCR. Expression values for each group were normalized using β-actin as an internal reference. Differences between the two groups were analyzed using Student's t-test. Data are presented as the mean ± SD (n=3). *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001. Detection of STAT1, (C) CASP8 and (D) FAS protein expression in oe-STAT1α- and oe-STAT1β-lentivirus-infected SK3R-PTX, OV3R-PTX and A2780-PTX cells by western blotting. The specific antibodies recognized a specific target. STAT1α, 91 kDa; STAT1β, 84 kDa; pre-CASP8, 57 kDa; cleaved-CASP8, 43 kDa; FAS, 40-50 kDa; β-actin, 42 kDa. The numbers under the bands indicate the densitometric value of the protein (total CASP3 and FAS) after normalization. Detection of STAT1, (E) CASP8 and (F) FAS protein expression in oe-STAT1α- and oe-STAT1β-lentivirus-infected SK3R-PTX, OV3R-PTX and A2780-PTX cells by immunofluorescence staining. The immunofluorescent signals were detected after the induction of STAT1α and STAT1β using doxycycline. DAPI was used to stain the nucleus. Red scale bar, 100 μm. CASP8, caspase-8; FAS, Fas cell surface death receptor; ns, not significant; oe, overexpression vector; pre-CASP8, caspase-8 precursor; cleaved-CASP8, cleaved caspase-8; PTX, paclitaxel.

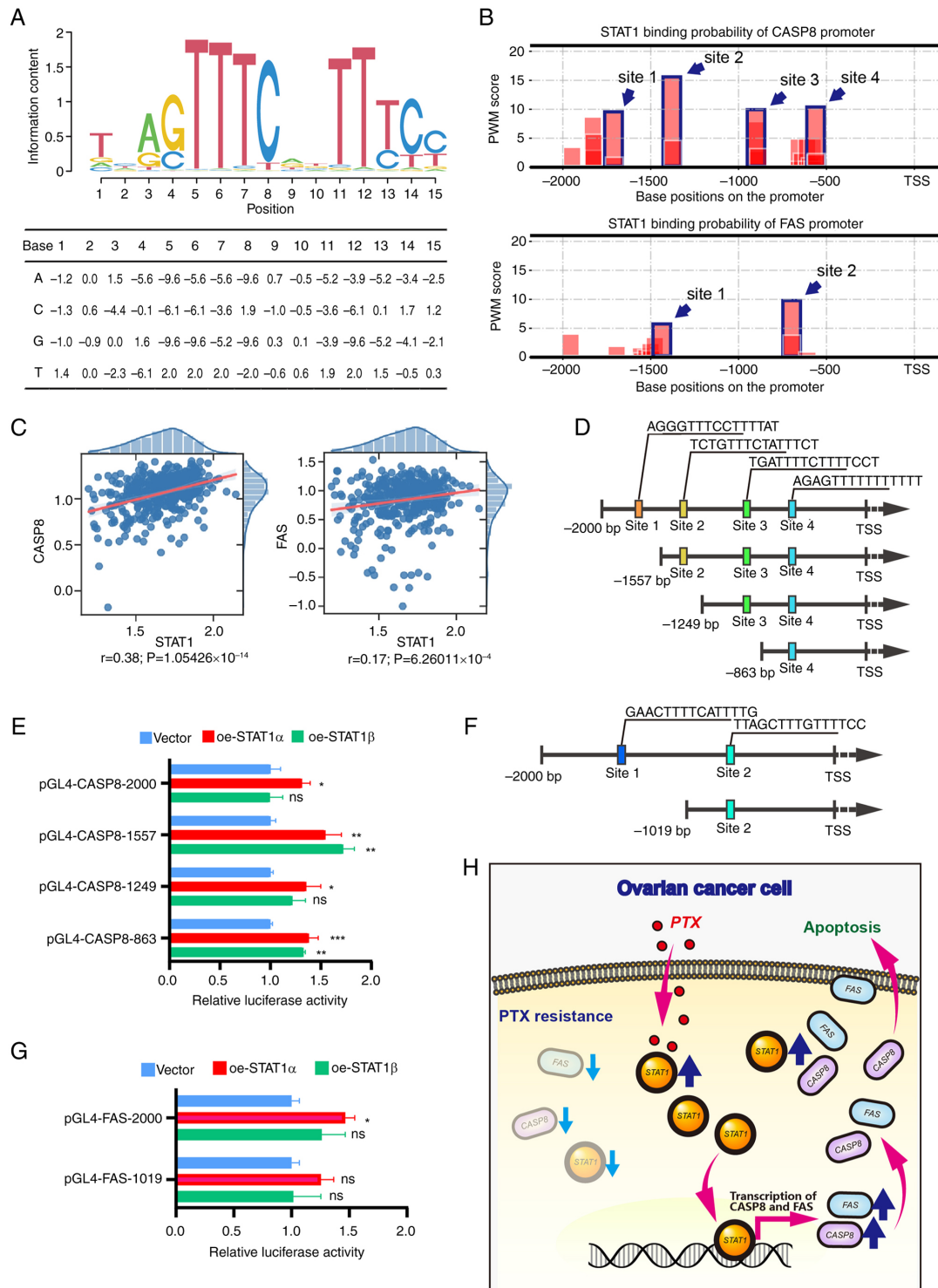


Figure 6. Detection of the interaction of STAT1 with CASP8 and FAS. (A) Prediction of STAT1 binding site sequence. The PWM of STAT1 was used to predict the binding site in the promoter region. The visualized sequence logo of the PWM is illustrated at the top, and the specific values of the PWM are shown in the table at the bottom. The x-axis represents the position in the sequence alignment. The y-axis represents the information content in bits. A higher value means that position is highly conserved. (B) Prediction of STAT1 binding sites in the CASP8 and FAS promoter regions using PWM analysis. Blue wireframes and arrows indicate high-scoring binding sites (four sites on the CASP8 promoter and two sites on the FAS promoter). (C) The correlation regression results of CASP8 and FAS with STAT1 in OC. The significance of each regression was determined using the Wald test with the t-distribution using the SciPy Python package. (D) Schematic diagram showing the full-length promoter (top) and three truncated fragments of CASP8 with colored squares indicating predicted binding sites and sequences. (E) Detection of STAT1 binding to CASP8 promoter DNA using dual-luciferase reporter gene assays. (F) Schematic diagram showing the full-length promoter (top) and one truncated fragment of FAS (bottom) with colored squares indicating predicted binding sites and sequences. (G) Detection of STAT1 directly binding to FAS promoter DNA using dual-luciferase reporter gene assays. 293T cells were co-transfected with a dual-luciferase reporter gene plasmid and oe-STAT1 α , oe-STAT1 β or empty vector. Differences among multiple groups were analyzed using one-way ANOVA followed by Tukey's Honestly Significant Difference test. Data are presented as the mean $\pm$ standard error of the mean ($n=3$). * $P<0.05$; ** $P<0.01$; *** $P<0.001$ vs. vector. (H) Schematic illustration of the regulatory mechanism of STAT1 on the reversal of PTX resistance in OC cells. In PTX-resistant cells, the expression levels of STAT1 and apoptotic factors FAS and CASP3 were low. Administration of PTX increased STAT1 expression and the overexpression of STAT1 upregulated FAS and CASP8 at the transcriptional level, thus promoting PTX-resistant cell apoptosis. CASP8, caspase-8; FAS, Fas cell surface death receptor; ns, not significant; oe, overexpression vector; OC, ovarian cancer; PTX, paclitaxel; PWM, position weight matrix; r, R-value; TSS, transcription start site.

processes of TFs (49). In addition, it is also challenging to identify the cancer-related molecules that can be manipulated for therapeutic purposes (50) since the downstream target genes of specific TFs are numerous and may have different functions.

FAS and CASP8 are two key proteins for the regulation of the apoptosis pathway. FAS is a death receptor that can directly interact with CASP8 to form a complex and initiate programmed cell death, and PTX can induce apoptosis (51,52). In the present study, the viability of PTX-resistant cells was decreased after oe-STAT1 α infection in the presence of PTX. These effects of STAT1 α were completely abolished in OV3R-PTX and A2780-PTX cells and partially abolished in SK3R-PTX cells in the presence of the caspase inhibitor Z-VAD-FMK, whereas the effect of STAT1 β was minor. The difference among these cell types was most likely due to multiple factors, such as cell type specificity, infection efficiency and the sensitivity of the inhibitor. To the best of our knowledge, the present study was the first to demonstrate that STAT1 increased FAS and CASP8 expression in PTX-resistant cells by directly binding to their promoters to regulate them at the transcriptional level. These data suggested that STAT1 reversed PTX resistance, at least in part, through FAS/CASP8-induced apoptosis.

Our previous study demonstrated that STAT1 increased OC cell proliferation (20). STAT1 exists in two isoforms. STAT1 α is considered to be the full-length STAT1 protein, which consists of 750 amino acids and has two important phosphorylation sites located in the C-terminal transactivation domain (TAD), namely, tyrosine residue 701 (Y701) and serine residue 727. STAT1 β consists of 712 amino acids and has only one phosphorylation site, Y701 (53). It has been shown that the C-terminal TAD of STAT1 can serve an important trans-regulatory role at the transcriptional level by recruiting different cofactors (54). On the other hand, the C-terminal TAD also interacts with the histone acetyltransferase CBP/p300, which promotes the DNA-binding activity of STAT1 (55). Therefore, the functional differences between STAT1 α and STAT1 β may partially be due to their sequence and structural differences in the C-terminal TAD. The present study demonstrated that overexpression of STAT1 α decreased PTX-resistant OC cell viability and inhibited tumor formation in xenograft mice. Our previous work also revealed that STAT1 α was the main STAT1 isoform expressed in OC cells (20,38). However, STAT1 β may also serve a role in PTX-resistant cells. For example, the protein levels of FAS were higher after STAT1 β overexpression than after STAT1 α overexpression in SK3R-PTX cells as detected by western blotting, and STAT1 β had a stronger transcription-promoting activity in the pGL4-CASP8-1557 group in the dual luciferase reporter gene assay compared with STAT1 α . These data suggest that the two isoforms of STAT1 have certain respective functions, depending on the targets and cancer types.

The present study had some limitations that require further exploration. First, the current study lacked clinical validation. Future studies should include prospective data as well as retrospective data analyses to evaluate the role of STAT1. The collection of patient samples is most useful to examine the expression of STAT1 associated with PTX resistance. Second, the role of STAT1 β in PTX-resistant cells is not fully understood and requires further investigation. Future studies

should investigate the upstream molecular mechanisms that regulate STAT1. For example, post-translational modifications might switch STAT1 β activation. Specific signals such as TGF- β or IL could be controlling STAT1 isoforms in PTX-resistant cells. Third, whether STAT1-influenced PTX resistance was involved in the intrinsic apoptotic pathway is unknown and would be interesting to investigate in future studies. To strengthen the effect of STAT1, a potential strategy would be targeting it in combination with PTX to overcome resistance and improve treatment efficacy. Since low STAT1 expression is a marker of PTX resistance, raising the STAT1 level in PTX-resistant patients may be a potential treatment option. For example, to re-sensitize OC to PTX, STAT1 could be introduced using viral vectors such as adenoviruses to deliver a healthy copy of the gene encoding STAT1 directly into OC cells as a gene therapy, thereby reversing their drug resistance or re-sensitizing them to PTX. Furthermore, high levels of STAT1 in tumors could predict a better response to PTX therapy. Therefore, STAT1 can be utilized as a biomarker to predict how a patient will react to PTX treatment.

In conclusion, the present study demonstrated that STAT1 expression was downregulated in PTX-resistant OC cells. Overexpression of STAT1 upregulated the key apoptotic factors FAS and CASP8 at the transcriptional level, thereby triggering the apoptotic pathway to reverse PTX resistance (Fig. 6H). These findings may provide a potential therapeutic strategy to reverse PTX resistance in OC patients by targeting STAT1.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

FW contributed to experiments, data analyses, figure generation and manuscript drafts. XX and BG contributed to the bioinformatics analysis and *in vitro* experimental validation. XL, JY, WG, JC, JF and QL contributed to the primer design, and animal and cellular experiments. GX contributed to the supervision, conceptualization, funding acquisition and project administration, and wrote and edited the final manuscript. FW and GX confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The animal studies were approved by the Ethics Committee of Jinshan Hospital, Fudan University (approval no. JIEC-2023-S68; Shanghai, China) and the Laboratory Animal Welfare and Ethics Committee of the Shanghai Public Health Clinical Center, Fudan University (approval no. 2020-A027-01; Shanghai, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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