TRIM27 regulates the expression of PDCD4 by the ubiquitin-proteasome pathway in ovarian and endometrial cancer cells

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Abstract. Programmed cell death 4 (PDCD4) is regarded as an important tumor suppressor that is lowly expressed or deleted in numerous human types of cancer, including ovarian and endometrial cancer. Tripartite motif-containing 27 (TRIM27) is closely related to the occurrence and development of tumors and is highly expressed in numerous types of cancer such as ovarian and endometrial cancer. PDCD4 can be degraded through ubiquitination, while TRIM27 has the E3 ubiquitin ligase activity. However, whether TRIM27 may regulate the expression of PDCD4 by ubiquitination effect remains unclear. In the present study, the expression of PDCD4 and TRIM27 in different ovarian and endometrial cancer cell lines was detected by reverse transcription-quantitative PCR (RT-qPCR), western blotting and immunocytochemistry. The impact of TRIM27 overexpression and knockdown on PDCD4 expression and the effective mechanism of TRIM27 regulating PDCD4 expression were also investigated *in vitro* by RT-qPCR, western blotting, co-immunoprecipitation assay, Transwell migration and Matrigel invasion assays. The results showed that the expression of TRIM27 and PDCD4 had a negative association at the protein level, and the distribution of TRIM27 and PDCD4 proteins had a phenomenon of co-localization in different ovarian and endometrial cancer cell lines. TRIM27 promoted the degradation of PDCD4 through the ubiquitin-proteasome pathway. To sum up, TRIM27 could increase the migration and invasion of ovarian and endometrial cancer cells by promoting the ubiquitination and degradation of PDCD4. The present findings may provide a new target for the treatment of ovarian and endometrial cancer.

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Introduction

Ovarian, endometrial and cervical cancer are the most common malignant tumors in the female reproductive system, and they seriously threaten the mental and physical health of women. Among the three cancers, endometrial cancer makes up 30% of sarcomas of the female genital tract (1), and its incidence is rising (2). However, the cause and pathogenesis of endometrial cancer are not very clear. Most endometrial cancers are estrogen-dependent, such as endometrioid adenocarcinoma; the others are estrogen-independent, such as clear cell tumor. Ovarian cancer is less common than endometrial and cervical cancer, but its mortality is the highest (3). The clinical symptoms of ovarian cancer are so atypical that its diagnosis is usually in the terminal stages, leading to the 5-year survival rate reducing to 15-30% (2). Therefore, it is a hot topic about the research on the etiology and pathogenesis of ovarian and endometrial cancer.

Programmed cell death 4 (PDCD4) was initially associated with cell apoptosis (4). Previously, PDCD4 was regarded as an important tumor suppressor that was lowly expressed or deleted in numerous human types of cancer, including ovarian and endometrial cancer (5), and the rate of protein downregulation or loss is higher than that of mRNA decrease or deletion in certain tumors. The low expression of PDCD4 is related to the differentiation degree of tumor cells and prognosis of some patients with tumors, suggesting that PDCD4 may be involved in the process of tumor development. It has been reported

that PDCD4 overexpression could effectively suppress the migration and invasion, as well as the proliferation of cells in numerous types of cancer (6), such as breast (7), ovarian (8-10) and endometrial cancer (11). The expression level of PDCD4 is also related to the sensitivity of tumor cells to chemotherapeutic drugs. Upregulation of PDCD4 may increase the sensitivity of tumor cells to chemotherapeutic drugs (12). However, the reason for the low expression of PDCD4 in human types of cancer remains unclear. Previous studies reported that the downregulation of PDCD4 at the mRNA level was relevant to the 5'CpG island methylation (13), and the decreased expression of PDCD4 at the protein level was related to post-transcriptional processing, such as the regulation of microRNA, the phosphorylation and ubiquitination of protein (14-16).

TRIM27 is a member of the tripartite motif (TRIM) family. Since TRIM27 was originally found as the N-terminal fusion partner with the RET tyrosine kinase proto-oncogene, it was also called RET finger protein (RFP) (17). The TRIM family members have three kinds of characteristic domains, a Ring finger domain, one or two B-box zinc finger domain and a coiled-coil domain (18). Ring finger domain can mediate the ubiquitin transfer between the proteins themselves or different substrates, thus it is a characteristic marker of numerous E3 ubiquitin ligases (19). TRIM27 protein can be localized in the nucleus, cytoplasm and cell membrane depending on different cells, and its nuclear translocation is regulated by certain signaling pathways (20). Functionally, TRIM27 is involved in transcriptional regulation, cell apoptosis, cell differentiation, inflammatory response and cell cycle. TRIM27 is closely related to the occurrence and development of tumors, and is highly expressed in numerous types of cancer such as lung (21), breast (22), ovarian (23) and endometrial cancer (24). It has been found that TRIM27 can promote the migration and invasion of cancer cells. In some tumors, TRIM27 knockdown can increase the drug-induced apoptosis (25). In addition, TRIM27, as an E3 ubiquitin ligase, downregulates the expression of other proteins through the ubiquitination and plays an important role in the regulation of antiviral natural immune responses and $CD4^+$ T cell-mediated immune responses (26,27).

Based on the aforementioned and previous studies, it was found that the levels of PDCD4 and TRIM27 have a negative relationship in multiple tumors, including ovarian and endometrial cancer. In function, both PDCD4 and TRIM27 are involved in tumorigenesis and progression, but the two proteins have opposite roles. Moreover, PDCD4 can be degraded through the ubiquitination, while TRIM27 has the E3 ubiquitin ligase activity. Therefore, it was hypothesized that TRIM27 may regulate the expression of PDCD4 by the ubiquitination effect. In the present study, the expression of PDCD4 and TRIM27 in ovarian and endometrial cancer cell lines was detected. The impact of TRIM27 overexpression and knockdown on PDCD4 expression and the effective mechanism of TRIM27 regulating PDCD4 expression were also investigated *in vitro*. The results confirmed that TRIM27 could increase the migration and invasion of ovarian and endometrial cancer cells by promoting the ubiquitination and degradation of PDCD4. The present findings may provide a new target for the treatment of ovarian and endometrial cancer.

Materials and methods

Antibodies and reagents. Rabbit monoclonal antibody against PDCD4 (cat. no. 9535) was purchased from Cell Signaling Technology, Inc. Rabbit polyclonal antibody against TRIM27 (cat. no. 12205-1-AP) was purchased from ProteinTech Group, Inc. Antibodies against HA (cat. no. H6908) and FLAG (cat. no. F1804) were purchased from Sigma-Aldrich; Merck KGaA. The β-actin antibody (cat. no. TA-09) was purchased from ZSGB-BIO. Protein synthesis inhibitor (cycloheximide, CHX) was purchased from Beyotime Institute of Biotechnology. Proteasome inhibitor (MG132) was purchased from MedChemExpress.

Cell culture. Human endometrial cancer cells (Ishikawa) and ovarian cancer cells (A2780 and SKOV3) were donated by Qilu Hospital of Shandong University. Ishikawa cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone; Cytiva), which was supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), penicillin (100 U/ml) and streptomycin (100 μg/ml). A2780 and SKOV3 cells were cultured in RPMI-1640 medium (Hyclone; Cytiva) containing 10% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml). Human endometrial cancer cells (HEC-1-A) were purchased from the China Center for Type Culture Collection. HEC-1-A cells were cultured in McCoy's 5A medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml). The cells were cultured in an incubator containing 5% CO_2 at 37˚C.

The expression vector and small interference RNA (siRNA). The expression plasmids of PDCD4, TRIM27 and ΔRingTRIM27-flag were constructed by Shanghai Genechem Co., Ltd. The expression plasmids of ubiquitin (UB)-HA, K63-HA and K48-HA were donated by Dr Wei Zhao (Department of Immunology, School of Basic Medical Sciences, Shandong University). A total of 3 different TRIM27-specific siRNAs and PDCD4-specific siRNAs were synthesized by Shanghai GenePharma Co., Ltd. The TRIM27 lentiviral expression vector and lentiviral short hairpin RNAs (shRNAs) were also constructed by Shanghai Genechem Co., Ltd. Specific siRNA sequences for PDCD4, TRIM27 and control siRNAs sequences are listed in Table SI. Specific shRNA sequences for TRIM27 and control short hairpin (sh) RNA sequences are listed in Table SII. Transfection of expression plasmids (4 μg) or siRNAs (4 μl) was performed using 10 μl Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol for 24 or 48 h at 37˚C, RT-qPCR and western blotting were used to detect corresponding mRNA and protein. TRIM27 lentiviral expression vector (Multiplicity of infection, MOI=5) or lentiviral shRNA specific for TRIM27 (MOI=20) was respectively transfected to A2780 or Ishikawa cells using LipofiterTM liposome transfection reagent according to the manufacturer's protocol (Hanbio Biotechnology Co., Ltd.). The transfection efficiency was detected by RT-qPCR and western blot analysis.

RNA extraction and RT-qPCR. Total RNA of all cells was extracted by RNA fast 2000 (Feijie *Biological Technology*), and

then reversely transcribed to cDNA using Reverse-Transcribe kit according to the manufacturer's protocol (Tiangen Biotech Co., Ltd.). qPCR was performed with UltaSYBR Mixture (Kangweishiji Biotech Co., Ltd.) and different primers. The primer sequences are listed in Table SIII. The thermocycling conditions were as follows: first, pre-denaturation at 95˚C for 10 min, followed by 39 cycles of amplification at 95˚C for 15 sec, 60˚C for 1 min and 65˚C for 5 sec. GAPDH was used as the reference gene. Each experiment was repeated at least three times. The data analysis was according to the $2^{-\Delta\Delta Cq}$ method (28).

Western blot analysis. All cells were washed by PBS and lysed by RIPA buffer (Beyotime Institute of Biotechnology) for protein extraction. A bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc.) was used to detect the protein concentration. The same amount (25 μg) of each protein was separated using 10% SDS-polyacrylamide gel and then transferred to 0.45-μm PDVF membranes (EMD Millipore). Membranes were blocked with 5% non-fat milk 2 h at room temperature. Rabbit polyclonal antibody against TRIM27 or rabbit monoclonal antibody against PDCD4 (both at 1:1,000) were added and incubated with membranes at 4˚C overnight. Following the primary incubation, the membranes were incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody (1:5,000; cat. no. 111-035-003; Jackson ImmunoResearch Laboratories, Inc.) for 50 min at room temperature. The signals were detected using enhanced chemiluminescence detection system (Thermo Fisher Scientific, Inc.). ImageJ software (version 1.8.0.172; National Institutes of Health) was used for quantitative analysis. Each experiment was repeated at least three times.

Immunocytochemistry. The cells were cultured on a coverslip in a 24-well plate overnight at 37˚C. The cells were then washed by PBS and fixed by 4% paraformaldehyde at room temperature and blocked with 2% bovine serum albumin (Beijing Solarbio Science & Technology Co., Ltd.) at 37˚C for 1 h. Rabbit anti-TRIM27 polyclonal antibody or mouse anti-PDCD4 monoclonal antibody (1:100; cat. no. sc-376430; Santa Cruz Biotechnology, Inc.) was added into different wells and incubated overnight at 4˚C. The next day, the cells were washed by PBS three times and incubated with Alexa Fluor® 488-conjugated anti-human IgG/anti-rabbit IgG (1:200; cat. no. srbAF488-1) or TRITC-conjugated goat anti-mouse IgG (1:50; cat. no. SA00007-1) (both from ProteinTech Group, Inc.) for 1 h at 37˚C. Then the cells were washed by PBS and DAPI (5 μg/ml) was added into each well. The results were observed using a fluorescent microscope. The experiment was repeated three times. The specificity of TRIM27 and PDCD4 antibodies was confirmed in the previous study by establishing negative controls.

Immunohistochemistry (IHC). Firstly, the paraffin-embedded tissue sections from the tumor of a xenograft nude mouse model of ovarian cancer used in a previous study (23) were placed in an oven at 70˚C for 2 h. Then, the sections were dewaxed in xylene and hydrated in graded alcohol before antigen microwave retrieval. The sections were washed with 3% H_2O_2 for 10 min and blocked with 10% goat serum (ZSGB-BIO) at 37˚C for 15 min, and then incubated with the primary antibodies specific for TRIM27 (1:200) or PDCD4 (1:200) overnight at 4˚C. The next day, the sections were washed by PBS and then incubated with HRP-conjugated anti-rabbit IgG (Gene Tech Co., Ltd.) at 37˚C for 45 min followed by diaminobenzidine (Gene Tech Co., Ltd.) staining. The nuclei were counterstained with hematoxylin at room temperature for 5 min. The results were observed using a light microscope.

Co-immunoprecipitation assay (co-IP). TRIM27-specific siRNA and UB-HA plasmid were transfected into A2780 cells and TRIM27 expression plasmid and UB-HA plasmid were transfected into Ishikawa cells. A total of 24 h later, the cells were washed by PBS twice, then lysed with 400 μl of IP buffer [components: NaCl, 4.4 g; NP-40, 5 ml; Tris-HCl (pH 7.4), 50 ml; EDTA, 50 ml; ddH_2O, 395 ml]. Then the cells were lysed at 4˚C for 30 min and centrifuged at 14,000 x g for 15 min at 4˚C. Protein was divided into two groups, IP group (300 μl) and Input group (80 μl). IP group protein was incubated with 3 μl of PDCD4 antibody (1:100) overnight at 4˚C in a chromatography cabinet and Input group protein was stored at -20˚C. The next day, IP group protein was incubated with 40 μl of Protein A/G Plus-Agarose (Cell Signaling Technology, Inc.) in a chromatography cabinet at 4˚C for 8-12 h. Next, the IP group protein was washed by IP buffer and centrifuged at 1,000 x g for 5 min at 4˚C to collect the supernatant, which was repeated five times and then the loading buffer was added. Finally, the IP group protein was placed at 100˚C for 6 min. Input group protein was added the loading buffer and placed at 100˚C for 6 min. The experiment was repeated three times.

Transwell migration assay. The Ishikawa cells were silenced with the lentiviral shRNAs targeting TRIM27, and the A2780 cells were overexpressed with the lentiviral expression vector targeting TRIM27. After infection, puromycin (3 μg/ml) was used for screening. Western blot analysis was performed for identification. Then the cells were overexpressed with PDCD4 plasmid or silenced with PDCD4-specific siRNA. The cells were cultured at 37˚C for 24 h after PDCD4 overexpression and silencing. The cells ($5x10^4$/well) cultured with 1% FBS medium were placed into the upper chamber of a Transwell membrane with an 8-μm pore size (EMD Millipore). A total of 650 μl medium with 20% FBS were added into the lower chamber. After incubation at 37˚C for 24 h, the cells were washed with PBS twice, fixed with 100% methanol at room temperature for 15 min, then stained with 0.5% crystal violet (Beyotime Institute of Biotechnology) at room temperature for 30 min. A total of randomly selected 5 images for each sample were observed using a light microscope. The experiment was repeated three times.

Matrigel invasion assay. The BD Biocoat Matrigel (BD Biosciences) was used to detect the effect of TRIM27 and PDCD4 on the invasion ability of Ishikawa and A2780 cells. A total of 40 μl medium without FBS and 10 μl Matrigel were mixed and added into the chamber. After incubation at 37˚C for 30 min, the cells ($8x10^4$/well) cultured with 1% FBS medium were added into the upper chamber. A total of 650 μl medium with 20% FBS were added into the lower chamber. After incubation at 37˚C for 48 h, the cells were placed into

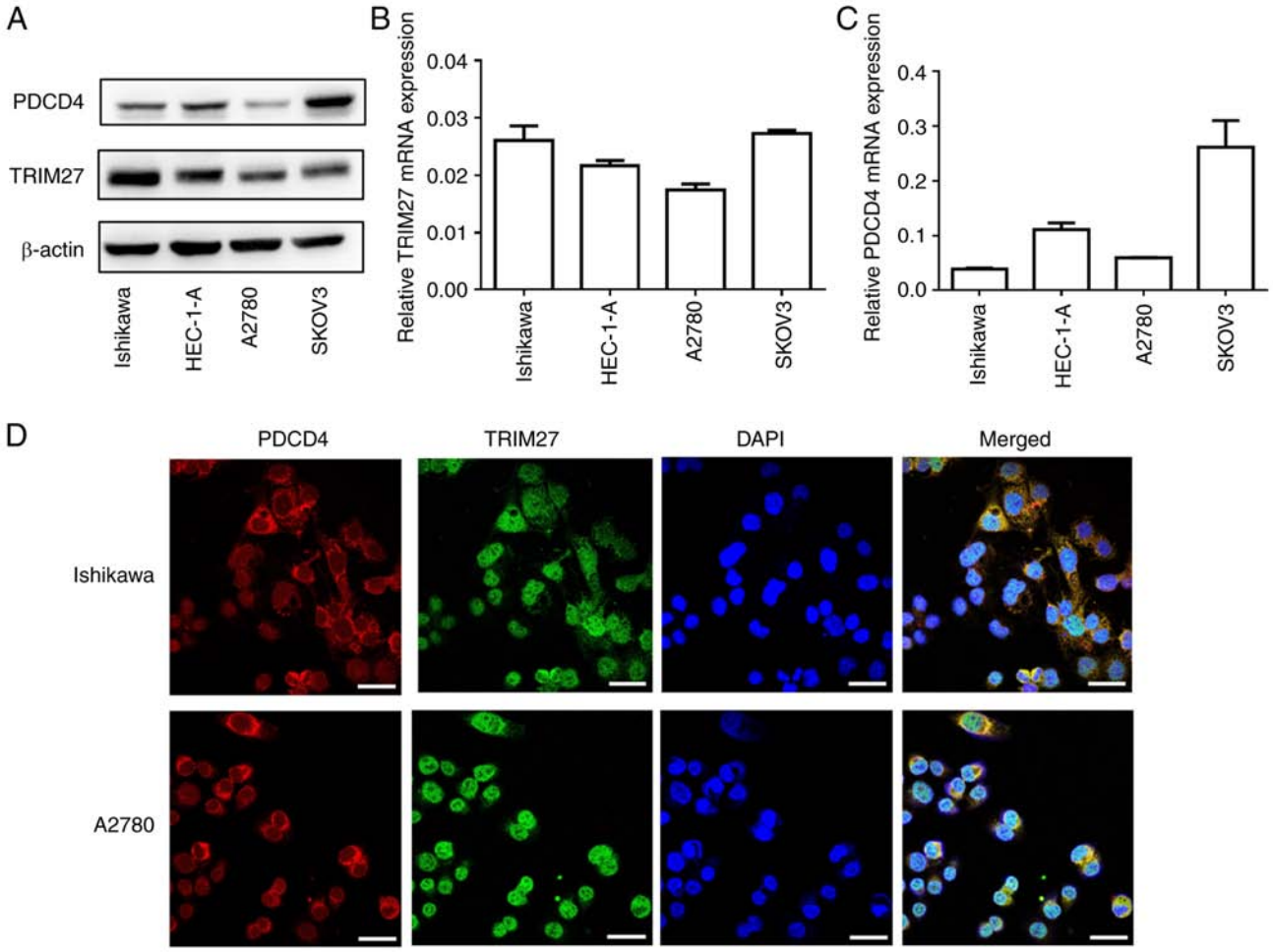

Figure 1. PDCD4 is lowly expressed while TRIM27 is highly expressed in endometrial and ovarian cancer cell lines at the protein level. (A) The expression of TRIM27 and PDCD4 in endometrial cancer cell lines (Ishikawa and HEC-1-A) and ovarian cancer cell lines (A2780 and SKOV3) was detected by western blotting. (B and C) The mRNA expression of (B) TRIM27 and (C) PDCD4 in endometrial cancer cell lines (Ishikawa and HEC-1-A) and ovarian cancer cell lines (A2780 and SKOV3) was detected by reverse transcription-quantitative PCR. (D) The distribution of TRIM27 and PDCD4 proteins had co-localization in Ishikawa and A2780 cells detected by immunocytochemistry (scale bar=25 μm). PDCD4, programmed cell death 4; TRIM27, tripartite motif-containing 27.

methyl alcohol for 15 min, and then stained with 0.5% crystal violet at room temperature for 30 min. The cells in the upper chamber were removed by cotton bud. A total of 5 randomly selected fields of the stained cells in the lower chamber were observed under a light microscope. The experiment was repeated three times.

Statistical analysis. GraphPad prism version 6 (GraphPad Software, Inc.) was used to analyze the data. The results were expressed as the mean ± SEM. One way ANOVA followed by Tukey's post hoc test was used for multiple comparisons. The unpaired Student's t-test was used to evaluate the statistical significance between two groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

PDCD4 is lowly expressed while TRIM27 is highly expressed in endometrial and ovarian cancer cell lines at the protein level. To investigate the relationship between PDCD4 and TRIM27 expression, the mRNA and protein expression of PDCD4 and TRIM27 in endometrial cancer cell lines (Ishikawa and HEC-1-A) and ovarian cancer cell lines (A2780 and SKOV3) was detected using RT-qPCR and western blotting. Among the four cell lines, a relatively low expression of PDCD4 and a relatively high TRIM27 expression was observed in Ishikawa, HEC-1-A and A2780 cells at the protein level. By contrast, PDCD4 protein expression in SKOV3 was relatively high, and TRIM27 protein expression was relatively low (Figs. 1A and S1). However, the results from RT-qPCR revealed that the expression of TRIM27 and PDCD4 had no obvious relation at the mRNA level in the aforementioned cell lines (Fig. 1B and C). The result from immunofluorescence staining indicated that PDCD4 protein was mainly distributed around the nucleus in Ishikawa and A2780 cells, and TRIM27 protein was also in the nucleus and around the nucleus. Therefore, a co-localization phenomenon of the distribution of TRIM27 and PDCD4 proteins in endometrial and ovarian cancer cell lines was identified (Fig. 1D). In addition, the results from IHC showed that TRIM27 knockdown increased the expression of PDCD4 in the tumors from a xenograft nude mouse model of ovarian cancer (Fig. S2). The results indicated that the protein expression levels of PDCD4 and TRIM27 have a negative relationship in endometrial and ovarian cancer cell lines.

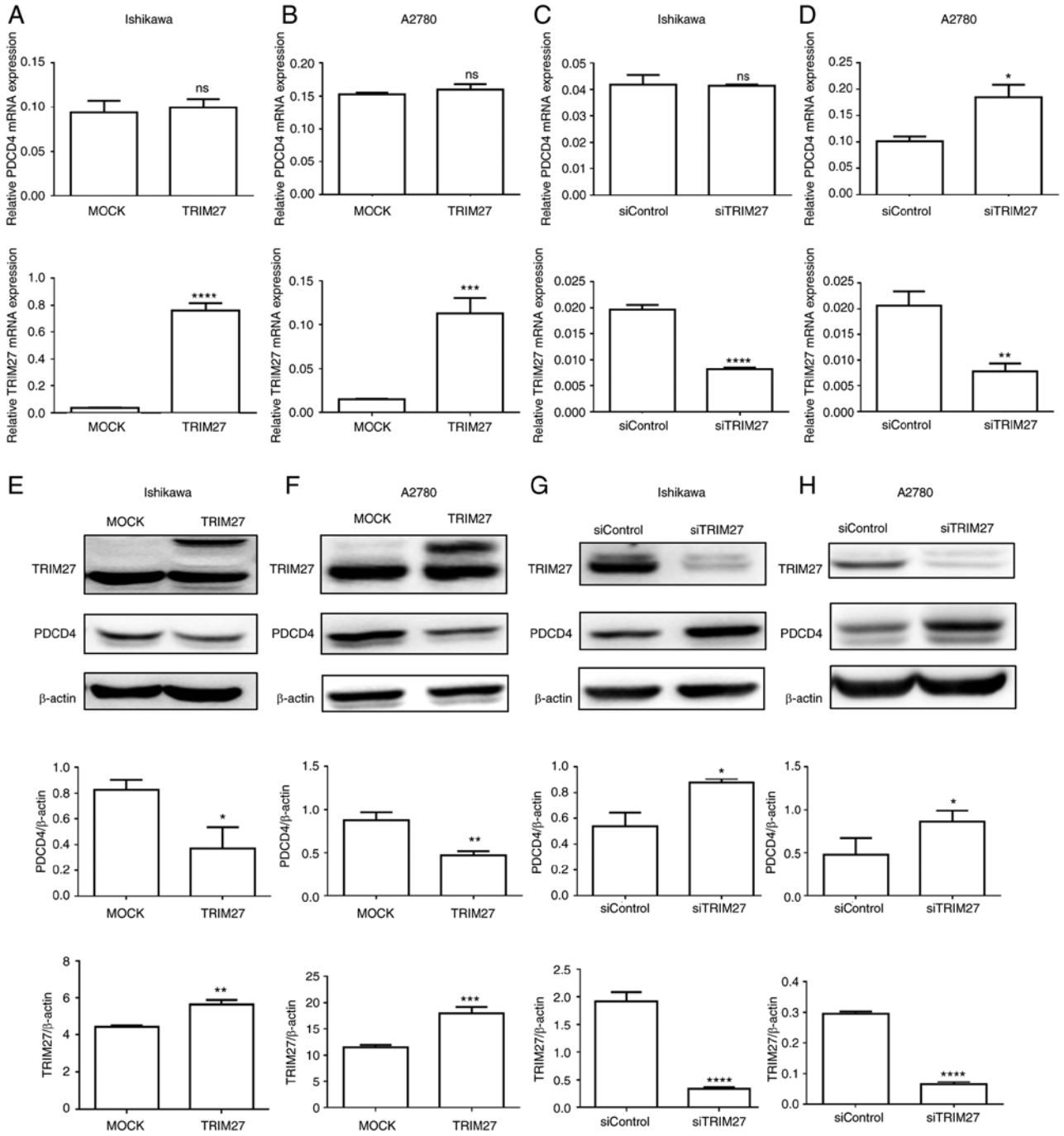

Figure 2. TRIM27 knockdown or overexpression increases or decreases the PDCD4 expression at the protein level. (A-C) PDCD4 mRNA expression had no obvious change after TRIM27 overexpression in (A) Ishikawa and (B) A2780 cells or (C) TRIM27 downregulation in Ishikawa cells. (D) Compared with the control group, PDCD4 mRNA level was higher after TRIM27 downregulation in A2780 cells. (E and F) TRIM27 overexpression significantly downregulated PDCD4 protein expression in (E) Ishikawa and (F) A2780 cells. (G and H) TRIM27 silencing obviously upregulated PDCD4 protein expression in (G) Ishikawa and (H) A2780 cells. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001. TRIM27, tripartite motif-containing 27; PDCD4, programmed cell death 4; si-, small interfering.

Silencing or overexpression of TRIM27 upregulates or downregulates the expression of PDCD4 protein. To confirm the negative association between the expression of TRIM27 and PDCD4, TRIM27 was either overexpressed or silenced in Ishikawa and A2780 cells and the mRNA and protein expression of PDCD4 was detected. RT-qPCR results revealed that PDCD4 mRNA expression had no obvious changes after TRIM27 overexpression in Ishikawa and A2780 cells or TRIM27 downregulation in Ishikawa cells (Fig. 2A-C). Compared with the control group, PDCD4 mRNA level was significantly higher after TRIM27 downregulation in A2780 cells (Fig. 2D). Furthermore, it was found that TRIM27 overexpression in Ishikawa and A2780 cells significantly downregulated the PDCD4 protein expression (Fig. 2E and F). By contrast, the level of PDCD4 protein was significantly upregulated after the TRIM27-specific siRNA was transfected into Ishikawa and A2780 cells (Fig. 2G and H). Thus, TRIM27 had a significant negative impact on the expression of PDCD4 protein.

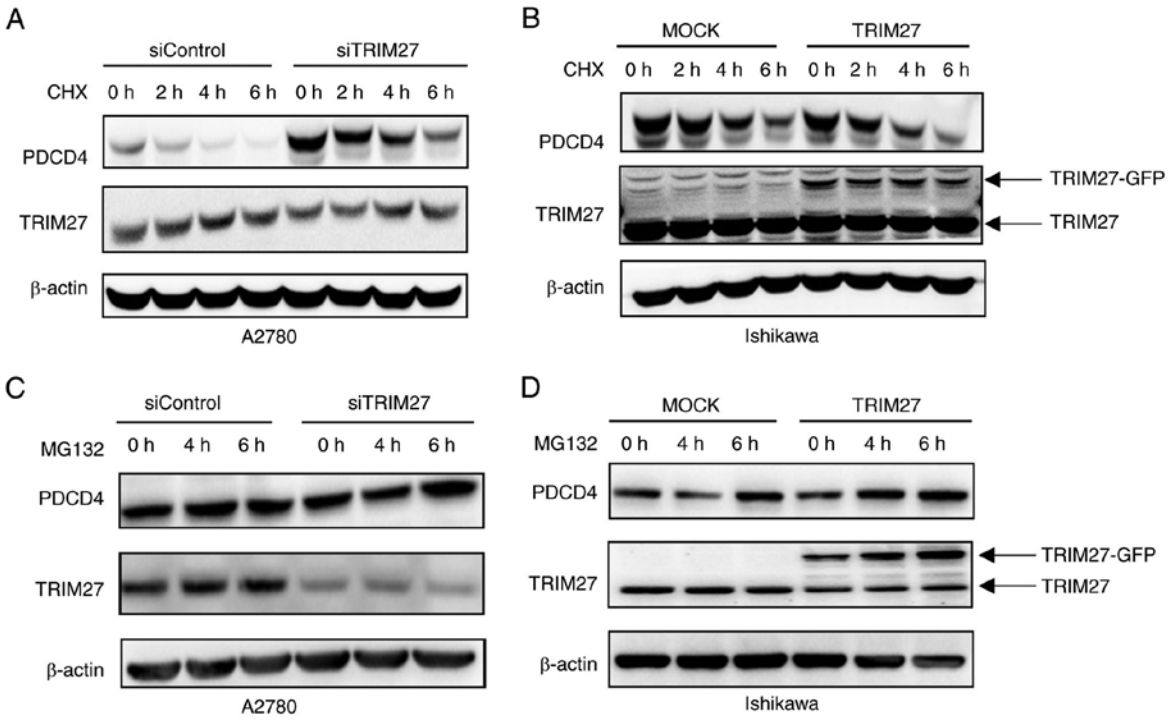

Figure 3. TRIM27 negatively regulates PDCD4 by promoting the degradation of PDCD4 via the ubiquitin-proteasome pathway. (A) The expression of PDCD4 was upregulated after TRIM27 knockdown. The degradation rate of PDCD4 in TRIM27 knockdown groups was slower than that of control groups after adding the CHX. (B) TRIM27 overexpression downregulated the protein levels of PDCD4 in Ishikawa cells, and the increasing degradation of PDCD4 protein was observed after adding CHX. (C) The expression of PDCD4 was increased after TRIM27 knockdown, and the expression of PDCD4 was decreased after MG132 stimulation for 4 h compared with the control group. (D) TRIM27 overexpression decreased the levels of PDCD4. Compared with the MG132-unstimulated group, the expression of PDCD4 was increased after MG132 stimulation for 4 and 6 h. TRIM27, tripartite motif-containing 27; PDCD4, programmed cell death 4; CHX, cycloheximide.

TRIM27 negatively regulates PDCD4 by promoting the degradation of PDCD4. To explore whether TRIM27 negatively regulated PDCD4 by promoting the degradation of PDCD4, A2780 cells were transfected with TRIM27-specific siRNA, or Ishikawa cells were transfected with a TRIM27 expression plasmid. At 0, 2, 4 and 6 h before the protein collection, the aforementioned transfected cells were added 2 μl of CHX (10 mM) and incubated at 37˚C for 0, 2, 4 or 6 h. It was found that the expression of PDCD4 was upregulated after TRIM27 knockdown in A2780 cells. The degradation rate of PDCD4 in TRIM27 knockdown groups was slower than that of control groups after adding CHX. By contrast, TRIM27 overexpression downregulated the levels of PDCD4 protein in Ishikawa cells, and the increasing degradation of PDCD4 protein was observed after adding CHX (Figs. 3A and B and S3). The results indicated that TRIM27 negatively regulates PDCD4 by promoting the degradation of PDCD4.

TRIM27 promotes the degradation of PDCD4 protein via the ubiquitin-proteasome pathway. Next, it was further confirmed that TRIM27 promoted the degradation of PDCD4 protein via the ubiquitin-proteasome pathway. A2780 cells were transfected with TRIM27-specific siRNA, or Ishikawa cells were transfected with a TRIM27 expression plasmid. At 0, 4 and 6 h before the protein collection, the aforementioned transfected cells were added 3 μl of MG132 (10 mM) and incubated at 37˚C for 0, 4 and 6 h. The results revealed that the expression of PDCD4 was increased after TRIM27 knockdown, and the expression of PDCD4 was decreased after MG132 stimulation for 4 h compared with the control group. By contrast, TRIM27 overexpression decreased the protein expression of PDCD4. Compared with the MG132-unstimulated group, the expression of PDCD4 was increased after MG132 stimulation for 4 and 6 h. These results demonstrated that the inhibitory effect of TRIM27 on PDCD4 expression disappeared after the degradation pathway of the proteasome was blocked (Figs. 3C and D and S4), suggesting that TRIM27 promotes the degradation of PDCD4 via the ubiquitin-proteasome pathway.

TRIM27 promotes the K48 and K63-linked ubiquitination of PDCD4. To investigate whether TRIM27 promoted the ubiquitination of PDCD4, A2780 cells were transfected with TRIM27-specific siRNA. After 24 h, UB-HA expression plasmid was transfected into the aforementioned cells. A total of 24 h later, the results of co-IP showed that the levels of PDCD4 ubiquitination were decreased. On the contrary, after TRIM27 and UB-HA expression plasmids were co-transfected into Ishikawa cells, the levels of PDCD4 ubiquitination increased (Fig. 4A and B). The results indicated TRIM27 promotes the ubiquitination of PDCD4. Next, the sites of ubiquitination were further detected. Ishikawa cells were respectively transfected with TRIM27 expression plasmid, K48-HA and K63-HA expression plasmids, and the proteins were collected after 24 h. TRIM27-mediated K48 and K63-linked ubiquitination of PDCD4 was detected by co-IP. The results showed that the total amount of K48 and K63-linked ubiquitination of PDCD4 was upregulated after TRIM27 overexpression, which

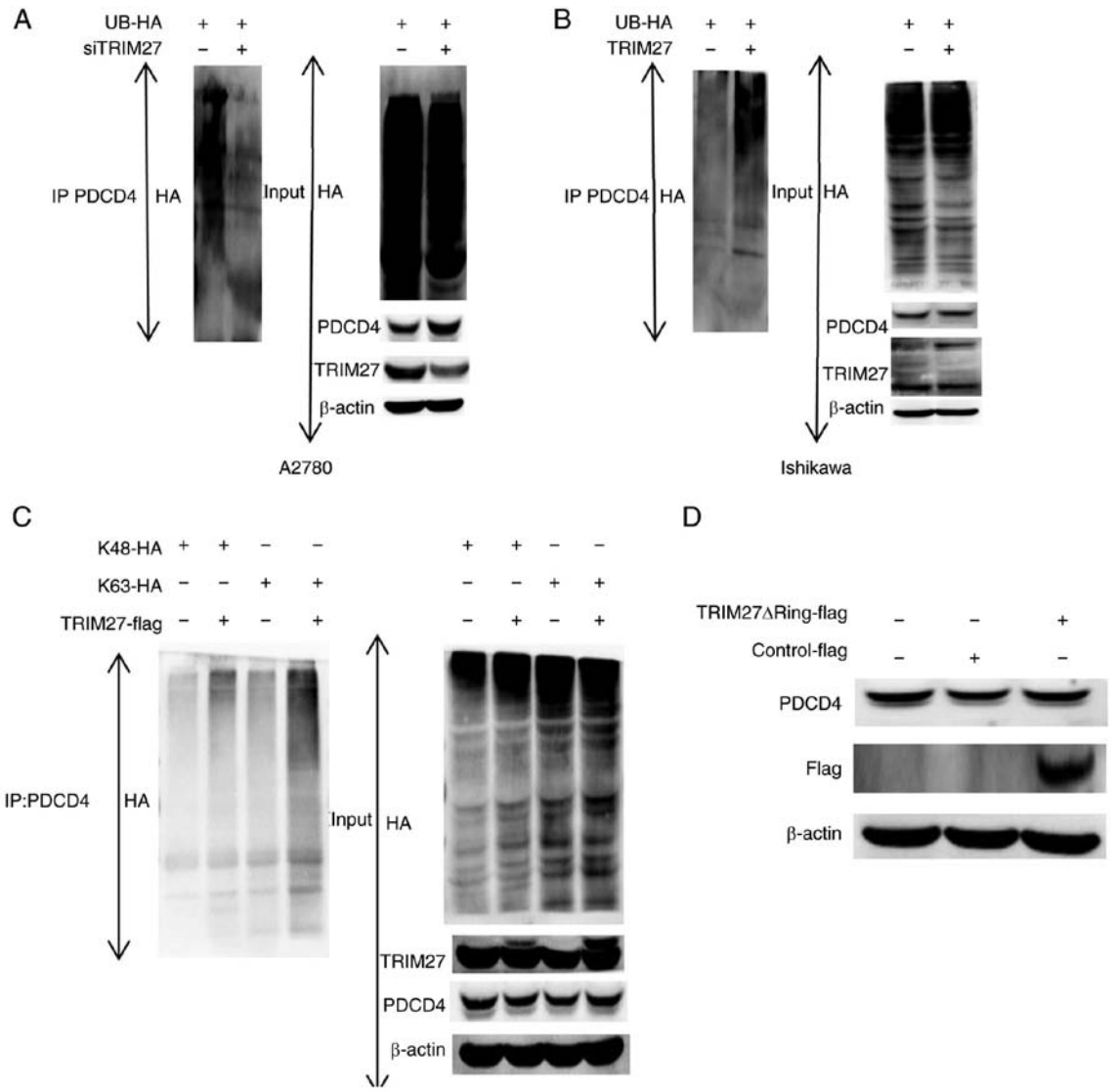

Figure 4. TRIM27 promotes K48 and K63-linked ubiquitination of PDCD4. (A) TRIM27 knockdown decreased the levels of PDCD4 ubiquitination. (B) TRIM27 overexpression increased the levels of PDCD4 ubiquitination. (C) TRIM27 promoted K48 and K63-linked ubiquitination of PDCD4. (D) The expression of PDCD4 had no change after TRIM27ΔRing-flag was transfected into Ishikawa cells. TRIM27, tripartite motif-containing 27; PDCD4, programmed cell death 4.

indicated that TRIM27 promoted the K48 and K63-linked ubiquitination of PDCD4 (Fig. 4C).

TIRM27 regulates PDCD4 expression via the Ring finger domain. The Ring finger domain of TRIM27 can mediate the ubiquitin transfer between the proteins themselves or different substrates. To confirm whether TRIM27 promoted the ubiquitination of PDCD4 via the Ring finger domain, the Ring finger domain-deleted expression plasmid (TRIM27ΔRing-flag) was constructed and transfected into Ishikawa cells. The results showed that the expression of PDCD4 had no change after TRIM27ΔRing-flag was transfected into Ishikawa cells, which indicated that TRIM27 regulated PDCD4 expression via the Ring finger domain (Fig. 4D).

TRIM27 promotes the migration and invasion of endometrial and ovarian cancer cell lines by affecting PDCD4. To confirm whether TRIM27 could enhance the migration and invasion of endometrial and ovarian cancer cells by regulating PDCD4, a lentiviral expression vector and lentiviral shRNAs specific for TRIM27 were constructed and TRIM27-overexpressed A2780 cells or TRIM27-silenced Ishikawa cells were set up. The results showed that TRIM27 overexpression significantly increased the migration and invasion ability of A2780 cells, while TRIM27 knockdown significantly decreased the migration and invasion ability of Ishikawa cells. By contrast, PDCD4 overexpression or knockdown significantly downregulated or upregulated, respectively, the migration and invasion ability of A2780 or Ishikawa cells. Furthermore, in TRIM27-overexpressing A2780 cells, PDCD4 overexpression significantly decreased the migration and invasion ability of cells. Similarly, in TRIM27-silenced Ishikawa cells, PDCD4 knockdown obviously increased the migration and invasion ability of cells (Figs. 5A-D; Figs. S5 and S6). The aforementioned results suggested that TRIM27 could promote the migration and invasion of endometrial and ovarian cancer cells by affecting PDCD4.

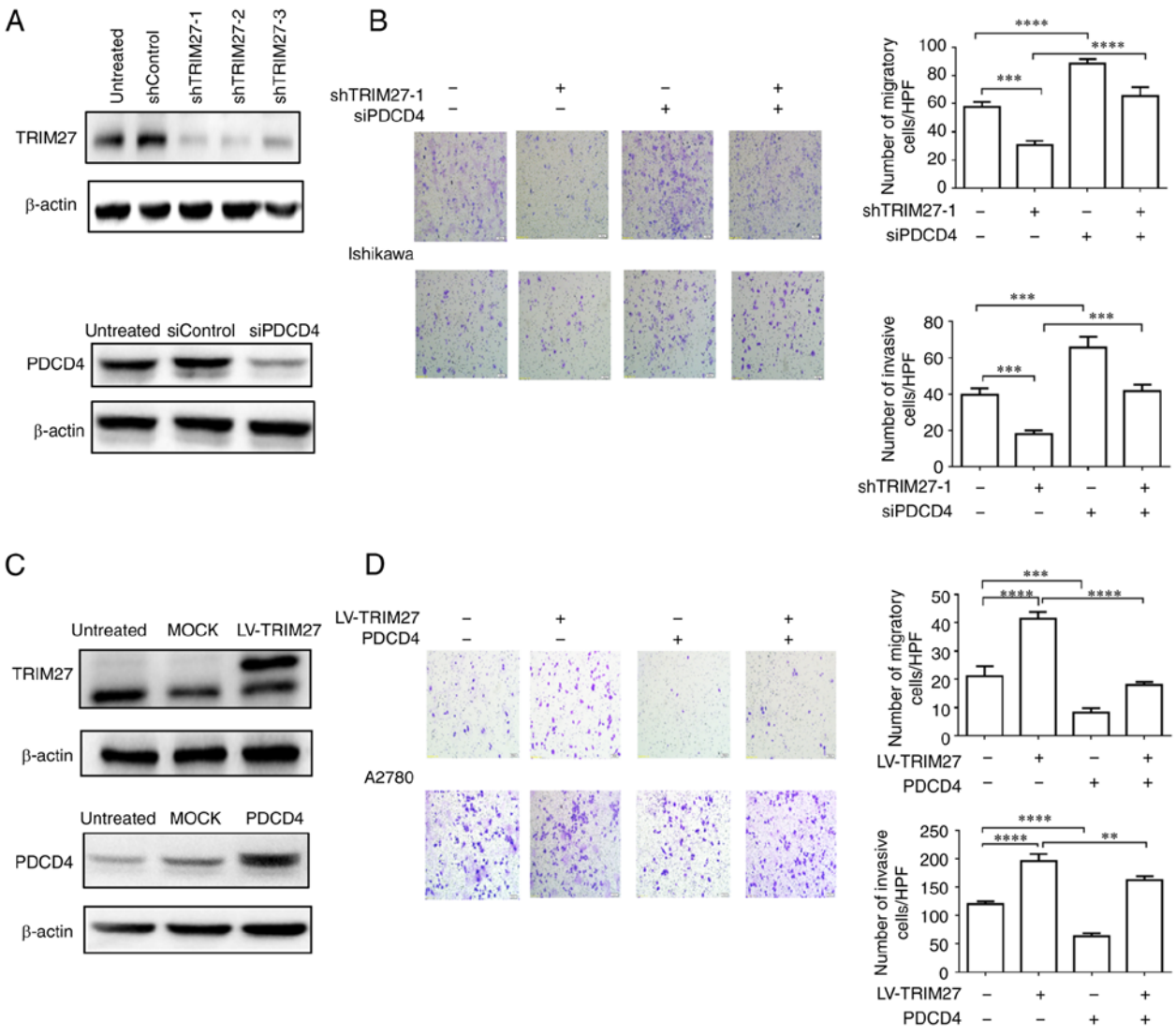

Figure 5. TRIM27 promotes the migration and invasion of endometrial and ovarian cancer cell lines by affecting PDCD4. (A) The expression of TRIM27 was detected by western blotting after transfection with three kinds of shRNAs specific for TRIM27 in Ishikawa cells. The expression of PDCD4 was detected by western blot analysis after transfection with siRNA specific for PDCD4 in Ishikawa cells. (B) In TRIM27-silenced Ishikawa cells, PDCD4 knockdown increased the migration and invasion ability of Ishikawa cells (scale bar=100 μm). (C) The expression of TRIM27 was detected by western blotting after transfection with a lentiviral TRIM27 expression vector in A2780 cells. The expression of PDCD4 was detected by western blotting after transfection with PDCD4 expression vector in A2780 cells. (D) In TRIM27-overexpressing A2780 cells, PDCD4 overexpression decreased the migration and invasion ability of A2780 cells (scale bar=100 μm). **P<0.01, ***P<0.001 and ****P<0.0001. TRIM27, tripartite motif-containing 27; PDCD4, programmed cell death 4; sh-, short hairpin; si-, small interfering.

Discussion

Previous studies showed that TRIM27 was highly expressed in ovarian serous carcinoma and endometrial cancer (23,24). Higher TRIM27 expression was significantly correlated with the metastasis and FIGO stage in patients with ovarian serous carcinoma, and an unfavorable clinical outcome in patients with endometrial cancer. PDCD4 was lowly expressed or deleted in ovarian serous cystadenocarcinomas and endometrioid endometrial carcinomas (EEC) (10,11). The loss or reduction of PDCD4 expression was significantly associated with higher pathological grade of ovarian serous cystadenocarcinomas and EEC, as well as the prognosis of patients with ovarian serous cystadenocarcinomas. The aforementioned results suggested that TRIM27 and PDCD4 have a negative relationship. The present study aimed to detect whether TRIM27 could take effect by regulating the expression of PDCD4 in endometrial and ovarian cancer cells. It was found that TRIM27 could degrade PDCD4 by the ubiquitination effect and promote the migration and invasion of endometrial and ovarian cancer cells by regulating PDCD4.

The expression of TRIM27 and PDCD4 in two kinds of endometrial cancer cell lines (Ishikawa and HEC-1-A) and two types of ovarian cancer cell lines (A2780 and SKOV3) was first detected. The four kinds of cancer cells are all epithelial-derived cells and share similar tumor biology. The results showed that TRIM27 and PDCD4 had a negative association at protein levels while they had no distinct association with each other at mRNA levels. The differences in the expression of PDCD4 and TRIM27 between normal and ovarian/endometrial cancer cells will be investigated in future studies. The immunofluorescence double staining results revealed

that the protein distribution of TRIM27 and PDCD4 existed in co-localization and were mainly distributed around the nucleus in Ishikawa and A2780 cells. It has been reported that PDCD4 and TRIM27 express in the cytoplasm and nuclei in a different situation, and contain a nuclear export sequence and shuttle between the cytoplasm and the nucleus (20,29). These results suggested that TRIM27 and PDCD4 may combine to interact.

Gene expression is regulated at multiple levels, including gene, transcriptional, post-transcriptional, translational and post-translational level. It has been reported that PDCD4 expression could be regulated at different levels (30). Gao *et al* (13) reported that the methylation of PDCD4 5'CpG islands was significantly correlated with the loss of PDCD4 mRNA expression in glioma tissue. DNA methyltransferase inhibitor 5-aza-2 deoxycytidine could block the methylation in glioma cells, restore PDCD4 expression and inhibit the proliferation of cells (13). The translation of PDCD4 mRNA is hampered by numerous miRNAs, including miR21, miR182, miR16, miR150 and miR499. The miR21 post-transcriptionally downregulates PDCD4 expression by targeting PDCD4 mRNA, inducing the increase in transformation, invasion and metastasis of cancer cells (31). PDCD4 is recognized and ubiquitinated by a ubiquitin E3 ligase, β-TRCP, consequently degraded by the proteasome after it is phosphorylated at Ser67 by p70S6K and Akt (32). The results of the present study revealed that TRIM27 overexpression downregulated the expression of PDCD4 protein, while TRIM27 knockdown had the opposite effect on PDCD4 protein expression. However, enhanced or silenced TRIM27 expression had no obvious effect on the mRNA levels of PDCD4. These results suggested that the regulation of PDCD4 expression by TRIM27 mainly exists after transcription. TRIM27 is a newly identified E3 ubiquitin ligase and degrades other proteins through the ubiquitin-proteasome pathway (26,27). In the present study, it was mainly investigated whether TRIM27 regulated the expression of PDCD4 protein via the ubiquitin-proteasome pathway.

Ubiquitination is a kind of post-translational modification. A small protein containing 76 amino acids is covalently combined with lysine residues in substrate proteins through a three-step E1, E2, and E3 enzymatic cascade. Ubiquitination has different effects on its substrates depending on the length and type of ubiquitin chains. Among them, K48-linked or K63-linked ubiquitin chains are the most common, and their functions are different. K48-linked ubiquitination mainly promotes the target proteins to be degraded by the 26S proteasome. However, K63-linked ubiquitination mostly plays an important role in altering protein-protein interactions, protein conformations, or targeting proteins for lysosomal delivery (33,34).

In the present study, it was found that the PDCD4 degradation rate was slower after stimulation with protein synthesis inhibitor CHX. Furthermore, the expression of PDCD4 was increased after proteasome inhibitor MG132 stimulation compared with the MG132-unstimulated group. These results revealed that PDCD4 could be degraded via the proteasome pathway. The present results also demonstrated that ubiquitination levels of PDCD4 were upregulated after TRIM27 overexpression, while TRIM27 knockdown inhibited the ubiquitination level of PDCD4, indicating that TRIM27 can play a role in promoting the ubiquitination of PDCD4. It has been reported that RFP (TRIM27) physically interacts with Pax7 and serves as an E3 ligase to induce ubiquitin-proteasome-dependent degradation of MyoD in muscle atrophy, which results in resistance of muscles to denervation injury (35). Cai *et al* (26) reported that TRIM27 functioned as an E3 ligase and mediated lysine 48 polyubiquitination of PI3KC2β, leading to a decrease in PI3K enzyme activity.

Similarly, Zurek *et al* (36) reported that TRIM27 mediated K48-linked ubiquitination and subsequent proteasomal degradation of NOD2 negatively influencing NOD2-mediated NF-kB activation. However, Hao *et al* (37) found that the E3 Ring ubiquitin ligase, MAGE-L2-TRIM27, facilitated K63-linked ubiquitination of WASH K220. The disruption of WASH ubiquitination impaired endosomal F-actin nucleation and retromer-dependent transport (37). The aforementioned results confirmed that TRIM27 could mediate K48 or K63-linked ubiquitination of different target proteins. Our present study found that the total amount of K48 and K63-linked ubiquitination of PDCD4 was increased after TRIM27 overexpression. Given different K48 and K63-linked ubiquitination functions, it was hypothesized that TRIM27 promotes the proteasome-dependent degradation of PDCD4 by mediating K48-linked ubiquitination of PDCD4. The role of K63-linked ubiquitination of PDCD4 needs to be further explored in the future. As aforementioned, the Ring finger is a zinc-binding domain that plays an important role in ubiquitin E3 ligase binding to UB-conjugating enzymes (E2) (38). Therefore, the Ishikawa cells were transfected with TRIM27 mutant plasmid without the Ring finger domain. The result showed that PDCD4 was not degraded, confirming that TRIM27 could degrade PDCD4 by ubiquitination via the Ring finger domain.

PDCD4 is an important tumor suppressor, which inhibits the proliferation, migration and invasion of various cancer cells. The present results demonstrated that TRIM27 promotes the ubiquitination and degradation of PDCD4. However, whether TRIM27 could enhance the migration and invasion of endometrial and ovarian cancer cells by regulating PDCD4 remains unclear. In the present study, it was found that PDCD4 overexpression decreased the migration and invasion ability of cancer cells in TRIM27-overexpressing cells, while PDCD4 knockdown increased the migration and invasion ability of cells in TRIM27-silenced cells. The aforementioned results suggested that TRIM27 could promote the migration and invasion of endometrial and ovarian cancer cell lines by affecting PDCD4.

In conclusion, the present study was, to the best of our knowledge, the first to demonstrate the negative association of TRIM27 with PDCD4. TRIM27 could degrade the PDCD4 protein level through the ubiquitin-proteasome pathway and promote the migration and invasion of endometrial and ovarian cancer cell lines by regulating PDCD4. The present results will provide important insights into the effective mechanism of TRIM27 and the reason for PDCD4 downregulation in cancers.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

ZW and XiaW designed the project and conducted the experimental study. HY performed the experiments and wrote the manuscript. LW, ZT, CY, DZ, MJ and CW participated in performing certain experiments. YL, CX and XisW were involved in data collection and statistical analysis. YS and LZ participated in designing the experiments and reviewing the manuscript. All authors read and approved the final manuscript. ZW and HY confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Visser NCM, Reijnen C, Massuger LFAG, Nagtegaal ID, Bulten J and Pijnenborg JMA: Accuracy of endometrial sampling in endometrial carcinoma: A Systematic review and meta-analysis. Obstet Gynecol 130: 803-813, 2017.
2. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, ParkinDM, Forman D and Bray F: Cancer incidence and mortality worldwide: Sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer 136: E359-E386, 2015.
3. Hinchcliff EM, Bednar EM, Lu KH and Rauh-Hain JA: Disparities in gynecologic cancer genetics evaluation. Gynecol Oncol 153: 184-191, 2019.
4. Schlichter U, Burk O, Worpenberg S and Klempnauer KH: The chicken Pdcd4 gene is regulated by v-Myb. Oncogene 20: 231-239, 2001.
5. Jansen AP, Camalier CE, Stark C and Colburn NH: Characterization of programmed cell death 4 in multiple human cancers reveals a novel enhancer of drug sensitivity. Mol Cancer Ther 3: 103-110, 2004.
6. Wang Q and Yang HS: The role of Pdcd4 in tumour suppression and protein translation. Biol Cell: May 28, 2018 (Epub ahead of print).
7. Nieves-Alicea R, Colburn NH, Simeone AM and Tari AM: Programmed cell death 4 inhibits breast cancer cell invasion by increasing tissue inhibitor of metalloproteinases-2 expression. Breast Cancer Res Treat 114: 203-209, 2009.
8. Wei N, Liu SS, Chan KK and Ngan HY: Tumour suppressive function and modulation of programmed cell death 4 (PDCD4) in ovarian cancer. PLoS One 7: e30311, 2012.
9. Wei ZT, Zhang X, Wang XY, Gao F, Zhou CJ, Zhu FL, Wang Q, Gao Q, Ma CH, Sun WS, *et al*: PDCD4 inhibits the malignant phenotype of ovarian cancer cells. Cancer Sci 100: 1408-1413, 2009.
10. Wang X, Wei Z, Gao F, Zhang X, Zhou C, Zhu F, Wang Q, Gao Q, Ma C, Sun W, *et al*: Expression and prognostic significance of PDCD4 in human epithelial ovarian carcinoma. Anti Res 28: 2991-2996, 2008.
11. Liu Y, Sun H, Mao H, Gao M, Tan X, Li Y, Li Y, Muloye GM, Zhang L, Wang X and Wei Z: Expression of tumor suppressor programmed cell death 4 in endometrioid endometrial carcinomas and clinicopathological significance. Oncol Lett 15: 9369-9376, 2018.
12. Zhang X, Wang X, Song X, Liu C, Shi Y, Wang Y, Afonja O, Ma C, Chen YH and Zhang L: Programmed cell death 4 enhances chemosensitivity of ovarian cancer cells by activating death receptor pathway in vitro and in vivo. Cancer Sci 101: 2163-2170, 2010.
13. Gao F, Wang X, Zhu F, Wang Q, Zhang X, Guo C, Zhou C, Ma C, Sun W, Zhang Y, *et al*: PDCD4 gene silencing in gliomas is associated with 5'CpG island methylation and unfavourable prognosis. J Cell Mol Med 13: 4257-4267, 2009.
14. Sun J, Yong J and Zhang H: microRNA-93, upregulated in serum of nasopharyngeal carcinoma patients, promotes tumor cell proliferation by targeting PDCD4. Exp Ther Med 19: 2579-2587, 2020.
15. Zhou XH, Chai HX, Bai M and Zhang Z: LncRNA-GAS5 regulates PDCD4 expression and mediates myocardial infarction-induced cardiomyocytes apoptosis via targeting MiR-21. Cell Cycle 19: 1363-1377, 2020.
16. Cui H, Wang Q, Lei Z, Feng M, Zhao Z, Wang Y and Wei G: DTL promotes cancer progression by PDCD4 ubiquitin-dependent degradation. J Exp Clin Cancer Res 38: 350, 2019.
17. Townson SM, Kang K, Lee AV and Oesterreich S: Novel role of the RET finger protein in estrogen receptor-mediated transcription in MCF-7 cells. Biochem Biophys Res Commun 349: 540-548, 2006.
18. Micale L, Chaignat E, Fusco C, Reymond A and Merla G: The tripartite motif: Structure and function. Adv Exp Med Biol 770: 11-25, 2012.
19. Joazeiro CA and Weissman AM: RING finger proteins: Mediators of ubiquitin ligase activity. Cell 102: 549-552, 2000.
20. Harbers M, Nomura T, Ohno S and Ishii S: Intracellular localization of the Ret finger protein depends on a functional nuclear export signal and protein kinase C activation. J Biol Chem 276: 48596-48607, 2001.
21. Iwakoshi A, Murakumo Y, Kato T, Kitamura A, Mii S, Saito S, Yatabe Y and Takahashi M: RET finger protein expression is associated with prognosis in lung cancer with epidermal growth factor receptor mutations. Pathol Int 62: 324-330, 2012.
22. Tezel GG, Uner A, Yildiz I, Guler G and Takahashi M: RET finger protein expression in invasive breast carcinoma: Relationship between RFP and ErbB2 expression. Pathol Res Pract 205: 403-408, 2009.
23. Ma Y, Wei Z, Bast RC Jr, Wang Z, Li Y, Gao M, Liu Y, Wang X, Guo C, Zhang L and Wang X: Downregulation of TRIM27 expression inhibits the proliferation of ovarian cancer cells in vitro and in vivo. Lab Invest 96: 37-48, 2016.
24. Tsukamoto H, Kato T, Enomoto A, Nakamura N, Shimono Y, Jijiwa M, Asai N, Murakumo Y, Shibata K, Kikkawa F and Takahashi M: Expression of Ret finger protein correlates with outcomes in endometrial cancer. Cancer Sci 100: 1895-1901, 2009.
25. Horio M, Kato T, Mii S, Enomoto A, Asai M, Asai N, Murakumo Y, Shibata K, Kikkawa F and Takahashi M: Expression of RET finger protein predicts chemoresistance in epithelial ovarian cancer. Cancer Med 1: 218-229, 2012.
26. Cai X, Srivastava S, Sun Y, Li Z, Wu H, Zuvela-Jelaska L, Li J, Salamon RS, Backer JM and Skolnik EY: Tripartite motif containing protein 27 negatively regulates CD4 T cells by ubiquitinating and inhibiting the class II PI3K-C2β. Proc Natl Acad Sci USA 108: 20072-20077, 2011.
27. Zheng Q, Hou J, Zhou Y, Yang Y, Xie B and Cao X: Siglec1 suppresses antiviral innate immune response by inducing TBK1 degradation via the ubiquitin ligase TRIM27. Cell Res 25: 1121-1136, 2015.
28. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
29. Böhm M Sawicka K, Siebrasse JP, Brehmer-Fastnacht A, Peters R and Klempnauer KH: The transformation suppressor protein Pdcd4 shuttles between nucleus and cytoplasm and binds RNA. Oncogene 22: 4905-4910, 2003.

30. Matsuhashi S, Manirujjaman M, Hamajima H and Ozaki I: Control mechanisms of the tumor suppressor PDCD4: Expression and functions. Int J Mol Sci 20: 2304, 2019.
31. Frankel LB, Christoffersen NR, Jacobsen A, Lindow M, Krogh A and Lund AH: Programmed cell death 4 (PDCD4) is an important functional target of the microRNA miR-21 in breast cancer cells. J Biol Chem 283: 1026-1033, 2008.
32. Dorrello NV, Peschiaroli A, Guardavaccaro D, Colburn NH, Sherman NE and Pagano M: S6K1- and betaTRCP-mediated degradation of PDCD4 promotes protein translation and cell growth. Science 314: 467-4671, 2006.
33. Mallette FA and Richard S: K48-linked ubiquitination and protein degradation regulate 53BP1 recruitment at DNA damage sites. Cell Res 22: 1221-1223, 2012.
34. Metcalf JL, Bradshaw PS, Komosa M, Greer SN, Stephen Meyn M and Ohh M: K63-Ubiquitylation of VHL by SOCS1 mediates DNA double-strand break repair. Oncogene 33: 1055-1065, 2014.
35. Joung H, Eom GH, Choe N, Lee HM, Ko JH, Kwon DH, Nam YS, Min H, Shin S, Kook J, *et al*: Ret finger protein mediates Pax7-induced ubiquitination of MyoD in skeletal muscle atrophy. Cell Signal 26: 2240-2248, 2014.
36. Zurek B, Schoultz I, Neerincx A, Napolitano LM, Birkner K, Bennek E, Sellge G, Lerm M, Meroni G, Söderholm JD and Kufer TA: TRIM27 negatively regulates NOD2 by ubiquitination and proteasomal degradation. PLoS One 7: e41255, 2012.
37. Hao YH, Doyle JM, Ramanathan S, Gomez TS, Jia D, Xu M, Chen ZJ, Billadeau DD, Rosen MK and Potts PR: Regulation of WASH-dependent actin polymerization and protein trafficking by ubiquitination. Cell 152: 1051-1064, 2013.
38. Lipkowitz S and Weissman AM: RINGs of good and evil: RING finger ubiquitin ligases at the crossroads of tumour suppression and oncogenesis. Nat Rev Cancer 11: 629-643, 2011.