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TRIM22 inhibits endometrial cancer progression through the NOD2/NF-κB signaling pathway and confers a favorable prognosis

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Abstract. Endometrial cancer (EnC) is a malignant gynecological tumor commonly observed in developed countries, specifically among post-menopausal women. Although numerous patients with EnC receive promising prognoses, those with advanced or metastatic disease often have a poor prognosis and an impaired quality of life. Tripartite motif-containing 22 (TRIM22) has been confirmed to play many crucial roles in different biological processes, from inflammatory to tumorigenesis. However, the multifaceted roles of TRIM22 in EnC remain uncharacterized. Herein, comparing normal endometrial tissues with tumor tissues obtained from patients, it was concluded that TRIM22 expression was decreased in tumor tissues. However, the overexpression of TRIM22 served to inhibit the migratory, invasive, proliferative and cell cycle activity of EnC cells. Moreover, the knockdown of TRIM22 increased the migratory, invasive, and proliferative activity of the EnC cells. Furthermore, it was found that TRIM22 effectively suppressed EnC progression through the nucleotide binding oligomerization domain containing 2 (NOD2)/nuclear factor (NF)-κB pathway. The data also demonstrated that TRIM22 functions as an inhibitor of EnC tumor xenograft growth *in vivo*. Overall, the findings of the present study define a novel regulatory role for TRIM22 in EnC progression. Moreover, TRIM22 may serve as an important prognostic predictor for EnC.

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Introduction

Endometrial cancer (EnC), originates from the endometrium and thus, is a gynecological malignancy, commonly observed in developed nations (1-3), where post-menopausal women account as the most vulnerable population. Endometrial adenocarcinoma is the most common clinicopathological type. Currently, treatment for patients with EnC relies primarily on tumor stage and histopathological type, while individualized therapies based on genetic potential biology are uncommon (4-8). Although a large proportion of patients with EnC have a good prognosis, even following the implementation of clinically radical surgical interventions and advanced adjuvant therapy, some patients still have a poor prognosis, which is ascribed to the recurrence and metastasis of tumors following initial treatment (9). Thus, an improved understanding of the regulatory mechanisms associated with EnC progression is imminently required in order to improve diagnostics and individualized treatment regimens for patients with EnC.

Tripartite motif-containing 22 (TRIM22) contains a RING finger, B-box and coiled-coil domains, and is a member of the tripartite motif (TRIM) family of proteins. It functions as an E3 ubiquitin ligase, and as a transcriptional regulator involved in various biological processes (10). Furthermore, a previous clinical study found that TRIM22 functioned as an oncogenic gene that was highly expressed in non-small cell lung cancer (NSCLC) tissue, promoting NSCLC progression and conferring a poor prognosis (11). Conversely, other studies have demonstrated that TRIM22 expression is downregulated in tumor tissue compared to adjacent healthy tissue; this decreased expression of TRIM22 is associated with high-grade malignancy and a poor prognosis (12-14). Similarly, TRIM22 regulates multifarious signaling pathways associated with the expression of different oncogenic and antioncogenic molecules to subsequently promote or suppress tumor progression (11-13,15). Furthermore, TRIM22, as a progestin target gene, has been reported to improve the prognosis and overall treatment efficacy of patients with EnC (15). Although tumor-promoting and tumor-suppressing roles have been described for TRIM22, its role in EnC remains uncharacterized.

The nuclear factor-κB (NF-κB) pathway is a major pro-inflammatory signaling pathway. Evidence suggests that it plays a vital role in carcinogenesis, protecting cells against apoptosis and promoting resistance to various cancer drugs (16). Following the degeneration of phosphorylated IκBα, NF-κB, which is located in the cytoplasm, can readily translocate to the nucleus to regulate the expression of genes associated with proliferation, migration and invasion (17). TRIM22 has been described as a crucial anti-inflammatory cytokine with multi-effect functions (18-20). Moreover, it has been reported that TRIM22 can regulate anti-inflammatory responses by disrupting LTR-driven transcription, which is independent of NF-κB. However, studies have failed to provide evidence of a role for TRIM22 in the regulation of tumor growth via the NF-κB signaling pathway. Nevertheless, as inflammation plays a significant role in tumorigenesis and malignant progression, the NF-κB signaling pathway is likely related to TRIM22-mediated tumor control. Hence, a growing need exists for the elucidation of the specific underlying molecular mechanisms associated with TRIM22-mediated malignant tumor regulation, particularly as it pertains to EnC.

Herein, it is demonstrated that TRIM22 expression is decreased in the tumor tissues of patients with EnC, and to be associated with tumor stage. Furthermore, the present study comprehensively analyzed the potential mechanisms of TRIM22 downregulation, as well as the regulatory function of TRIM22 in EnC cell migration, invasion and proliferation both *in vitro* and *in vivo*. In addition, the data of the present study reveal that TRIM22 is an important prognostic predictor and a promising therapeutic target for EnC.

Materials and methods

Patient sample analyses. Ethics committee approval was obtained from the Reproductive Center of Provincial hospital affiliated to Shandong University [approval no. (2019) Ethics Approval No. 32]. The experiments were conducted according to the regulations set out by the Declaration of Helsinki. All samples were collected from the Provincial Hospital affiliated to Shandong University (Jinan, China) from November, 2012 to June, 2016. The clinical specimens were used in the experiments as follows: Eight pairs of tumor endometrial tissues and non-tumor endometrial tissues (adjacent normal tissues and normal endometrial tissues) (matched for each patient) were used for western blot analysis; 25 normal endometrial tissues and 74 tumor endometrial tissues were used for immunohistochemistry (IHC). The patient characteristics presented in Table I. The specimens were collected after obtaining informed consent from the patients. All EnC specimens were diagnosed and assessed in accordance with the International Federation of Gynecology Oncology (FIGO) criteria (2009). All the EnC samples must have been diagnosed as endometrial adenocarcinoma and underwent initial surgery. All the normal endometrial tissues were collected from patients diagnosed as having uterine fibroids following hysterectomy. None of the patients had been administered any pre-operative medical treatments.

Cells and cell culture. As described in a previous study (21), KLE (GCC-UT0008RT/GCC-UT0008CS, http://www.taogene.com/emkt.htm#/PcMerchandises?id=f9c8f996-7ca3-473c-bd2e-8d7525340d03&categoryId=6), Ishikawa (GCC-UT0004RT/GCC-UT0004CS, http://www.taogene.com/emkt.htm#/PcMerchandises?id=89692be1-e99d-4a6f-a22d-11441b94b506&categoryId=6) and RL-952 (GCC-UT0006RT/GCC-UT0006CS, http://www.taogene.com/emkt.htm#/PcMerchandises?id=4d36c08c-34cd-4ce4-93a3-1cbdf6c3ce3d&categoryId=6) EnC cell lines were purchased from GeneChem Co., Ltd. Twenty short tandem repeat loci plus the gender determining locus, Amelogenin, were amplified using the PowerPlex® 21 System from Promega Corp., which tested and authenticated these three EnC cell lines. All cells were cultured in HyClone™ Dulbecco's modified Eagle's medium:nutrient mixture F-12 (DMEM/F-12) (HyClone; GE Healthcare Life Sciences) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Biological Industries, Israel), 1% penicillin and streptomycin (P/S) (HyClone; GE Healthcare Life Sciences) at 37˚C in a humidified 5% CO_2 atmosphere.

Immunohistochemical analysis. Paraffin-embedded sections (4-μm-thick) were dewaxed, hydrated, boiled in citric acid buffer for antigen retrieval, steeped in 0.3% hydrogen peroxide to interdict the activity of endogenous peroxidase, blocked with 3% bovine serum albumin and incubated with primary antibodies at 4˚C overnight. The following day, the sections were incubated with secondary antibodies (rabbit, PV-9001, ZSGB-Bio) at room temperature for 20 min according to the protocol of the PV-9001 Immunohistochemical kit (ZSGB-BIO). Primary antibodies were used at the following dilutions: TRIM22 antibody (1:350, NBP1-81795, Novus Biologicals, LLC), nucleotide binding oligomerization domain containing 2 (NOD2) antibody (1:250, NB100-524, Novus Biologicals, LLC), Ki-67 antibody (1:200, RB-9043-P1, eBioscience; Thermo Fisher Scientific, Inc.). These sections were then stained with hematoxylin in room temperature for 3 min, and were then cleared, dehydrated, hyalinized and mounted. Tissues staining brown in the cytoplasm or nucleus were regarded as positive. Five fields in each section were selected for further analysis using a fluorescent microscope (Olympus Corp.). The quantification of protein expression was presented with integrated optical density (IOD), using Image-Pro Plus 6.0 software. The final mean optical density was determined according to the following equation (equation 1): MOD=(IOD SUM)/(area SUM), where MOD represents the mean optical density, IOD SUM is the sum IOD of all selected fields in one image and the area SUM refers to the sum area of all selected fields.

Immunofluorescence. Paraffin-embedded sections (4-μm-thick) were dewaxed, hydrated, boiled in citric acid

Table I. Distribution, tissue characteristics and TRIM22 expression in patients with EnC.

Characteristics	Female			Trim22, means ± SD	P-value
	Case (n=74)	Controls (n=25)	Totals (n=99)		
Age (years)					0.0003
11-20	-	-	-	-	
21-30	-	1	1	-	
31-40	1	2	3	43.91±17.20	
41-50	12	21	33	38.88±16.01	
51-60	36	1	37	26.17±11.09	
61-70	22	-	22	24.22±11.93	
71-80	3	-	3	22.59±11.00	
Median (years)	57.2	44.88	51.04		
Mini-Maxi (years)	38-76	22-51	22-76		
Histological grade					<0.0001
I	25		25	31.12±7.03	
II	36		36	25.20±7.65	
III	13		13	20.95±5.69	
TNM stage					<0.0001
I	26		26	32.22±5.60	
II	26		26	29.31±5.16	
III	19		19	18.42±4.96	
IV	3		3	9.84±3.35	
Menstrual cycle phase					0.0056
Proliferative phase		15	15	42.20±12.45	
Secretory phase		10	10	56.51±10.92	

SPSS software (20.0) was used to analyze the data. Data are presented as the means ± SEM. The Student's unpaired t-test (2-tailed) was used for comparisons of TRIM22 expression between the proliferative phase and secretory phase. One-way ANOVA followed by Tukey's post-hoc test was applied for comparisons of TRIM22 expression among ≥3 groups (Histological grades, I, II, III and TNM stages, and different ages). Results indicate that TRIM22 expression was associated with age, histological grade, clinical stage and menstrual cycle phase. P<0.05 was considered to indicate a statistically significant difference. TRIM22, tripartite motif-containing 22; EnC, endometrial cancer.

buffer for antigen retrieval, blocked with 3% bovine serum albumin (Servicebio) and incubated with primary antibodies TRIM22 (1:200, NBP1-81795, Novus Biologicals, LLC) and NOD2 (1:100, NB100-524, Novus Biologicals, LLC) at 4˚C overnight. The following day, the sections were incubated with corresponding secondary fluorescent-conjugated antibodies (1:300, GB21303, rabbit, Servicebio) in the dark and at room temperature for 45 min. The sections were counterstained with 4',6'-diamidino-2-phenylindole (DAPI) (Servicebio, China) in the dark and at room temperature for 10 min, and images were acquired using a confocal laser scanning microscope (Nikon Copr.). The staining for TRIM22 was red, that for NOD2 was green, and the nuclei were stained blue.

Lentivirus infection. Ubi-TRIM22-3FLAG-SV40-EGFP-IRES-puromycin lentiviral vector (GV358) was constructed by GeneChem Co., Ltd. The primer sequences were as follows: Forward, 5'-GAGGATCCCCGGGTACCGGTCG CCACCATGGATTTCTCAGTAAAGGTAGACATAG-3' and reverse, 5'-TCCTTGTAGTCCATACCGGAGCTCGGTGGG CACACAGTCATG-3'. The TRIM22 gene was used from NCBI (NM_006074). According to the manufacturer's instructions, the lentiviral vector was transfected into the Ishikawa and KLE cells using Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) (these cells were termed TRIM22 OE). The Ubi-3FLAG-SV40-EGFP-IRES-puromycin lentiviral vector was used as a control (vector control). The cellular infection rate and GFP-positive cell number were detected by fluorescence microscopy at 72 h following infection. Stably transfected clones of TRIM22 were detected by western blot analysis.

Western blot analysis. Cells were harvested at predetermined times when the cells spread out in the dish (approximately 80%) and rinsed twice with PBS. The tumor tissues were harvested from the mice. Cell sediments and the tumor tissues were treated with SDS lysis buffer (Beyotime Institute of Biotechnology), and centrifuged at 14,000 x g for 10 min at 4˚C. Additionally, extracting the cytoplasmic and nuclear protein was performed according to the instructions of NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Inc.). Protein samples (20-40 μg) were electrophoresed through

10% polyacrylamide gels and transferred onto 0.45 μm PVDF membranes (Merck Millipore). The membranes were blocked with 5% skim milk for 1 h at room temperature, cleared thrice with 1X Tris-buffered saline and Tween-20 (TBST) (15 min/time), incubated with primary antibodies overnight at 4˚C, washed with 1X TBST thrice and incubated with secondary antibodies for 1 h at room temperature, and washed thrice with 1X TBST, developed with Immobilon Western HRP (ECL; Merck Millipore). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; ProteinTech Group, Inc.) and Lamin B1 (Abcam) were used as reference controls. Secondary antibodies (ZSGB-BIO) were accompanied with IR dyes. Blots were detected with the ChemiDoc MP Imaging System (Bio-Rad Laboratories, Inc.). Primary antibodies were used at the following dilutions: TRIM22 rabbit polyclonal antibody (1:200, NBP1-81795, Novus Biologicals, LLC), NOD2 antibody (1:1,000, NB100-524, Novus Biologicals, LLC), IκBα(E130) antibody (1:1,000, ab32518, Abcam), p-IκBα (Ser36) [EPR6235 (2)] antibody (1:10,000, ab133462, Abcam), NF-κB p65 (E379) antibody (1:50,000, ab32536, Abcam), p-p65(Ser536) (EP2294Y) antibody (1:10,000, ab76302, Abcam), GAPDH (AG0766) antibody (1:10,000, 60004-1-Ig, Proteintech) and LaminB1 antibody (1:5,000, ab16048, Abcam) and secondary antibodies (rabbit, mouse, ZB-2301, ZB-2305, ZSGB-Bio) were used at 1:5,000.

EdU incorporation assay. Cells ($1x10^4$ cells/well) were seeded in a 96-well plate in triplicate and incubated at room temperature for 24 h. Subsequently, the cells were incubated with medium containing 50 μM 5-ethynyl-2'-deoxyuridine (EdU) for an additional 2 h. The absolute ethyl alcohol (95% ethyl alcohol) was used to immobilize the cells at 4˚C. Cell proliferation was assessed using a Cell-Light™ EdU Cell Proliferation Detection kit according to the manufacturer's instructions. DNA was stained with Hoechst 33342 at room temperature for 30 min and observed using an inverted fluorescence microscope (Olympus Corp.). For each EdU test, 5 fields were randomly selected to image at x50 magnification. The absolute ethyl alcohol can accelerate the GFP to disappear out of the cells by enhancing the membrane permeability and, as a result, GFP has minimal influence on the EdU. The number of EdU-positive cells was determined according to Hoechst nuclear staining and was reported as a percentage of the total number of cells in each field.

Transwell migration and invasion assay. Cell migration assay was performed using a Transwell chamber (pore size, 0.8 μm; Merck Millipore), while the invasion assay was performed using a Transwell chamber coated with Matrigel (BD Biosciences). Endometrial cells ($6x10^4$ cells for migration assay and $4x10^4$ cells for invasion assay) in 100 μl serum-free DMEM-F12 were placed in the Transwell chamber and 700 μl DMEM-F12 containing 20% FBS was added to the lower chamber. Following incubation for approximately 24 h at 37˚C in a humidified 5% CO_2 atmosphere, the migrating or invading cells were fixed with absolute ethyl alcohol and stained with hematoxylin in room temperature for 15 min. Cells on the upper surface of the filter were removed by wiping with a small cotton swab. Five fields of the fixed cells were imaged using a fluorescent microscope (Olympus Corp.), and cells were counted.

Cell cycle assay. Cell cycle assay was implemented using the CycletestTM Plus DNA kit (BD Biosciences). According to the instructions of the kit, cells ($1.5x10^5$) were washed with the buffered solution and resuspended in A and B solutions to damage the cell membrane structure, after which they were incubated with solution C [propidium iodide (PI)] for 10 min in the dark on ice (4˚C). Solution A contained trypsin in a spermine tetrahydrochloride detergent buffer for the enzymatic disaggregation of the solid tissue fragments and digestion of cell membranes and cytoskeletons; solution B contained trypsin inhibitor and ribonuclease A in citrate-stabilizing buffer with spermine tetrahydrochloride to inhibit the trypsin activity and to digest the RNA. Cells were counted with a flow cytometer (Bio-Rad Laboratories, Inc.) and expressed as the percentage of G1, S and G2 phase cells using ModFit LTV4.1.7 software.

Co-immunoprecipitation (Co-IP) analysis. Cell lysates (approximately 1.5 mg total protein) were collected from the TRIM22-overexpressing (TRIM22 OE) Ishikawa cells using ice-cold non-denaturation lysis buffer. co-IP was performed according to the manufacturer's protocol of the Thermo Scientific Pierce Co-IP kit (Thermo Fisher Scientific, Inc.). TRIM22 and NOD2 antibodies were fixed for 2 h with AminoLink Plus coupling resin at room temperature. The resin was then washed and incubated with the cell lysates overnight at 4˚C. The following day, the resin was again washed, and elution buffer was used to elute the protein combined with the resin. A negative control, harvested from the vector control-transfected Ishikawa cells, were treated in the same manner as the Co-IP samples, including incubation with AminoLink Plus coupling resin combined with TRIM22 and NOD2 antibodies (Novus Biologicals, LLC) overnight at 4˚C. This control allowed us to observe whether the binding protein was increased in the TRIM22 OE groups. Samples were analyzed by western blot analysis using rabbit polyclonal anti-NOD2 (Novus Biologicals, LLC) and mouse monoclonal anti-TRIM22 (Novus Biologicals, LLC) antibodies.

TRIM22-specific shRNA and NF-κB-p65-specific shRNA plasmids and transient transfection. Plasmids encoding human TRIM22 shRNA, NF-κB-p65 (RelA) shRNA and scramble shRNA were purchased from Shanghai Genechem Co., Ltd. A human NF-κB-p65 shRNA plasmid, resistant to puromycin, was selected to knockdown NF-κB-p65 expression (shR p65); the TRIM22 shRNA plasmid was then used to knockdown the expression of TRIM22 (shR TRIM22). TRIM22 OE Ishikawa cells ($2x10^5$) and cells expressing relatively high levels of TRIM22 (TRIM22 RL-952 cells; $2x10^5$) were seeded into 6-well plates and cultured at 37˚C in a humidified 5% CO_2 atmosphere overnight. The following day, the cells were incubated with the transfection medium containing the shRNA plasmid and Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) for 5-7 h, after which the transfection medium was replaced with normal growth medium for incubation of the cells for a further 48 h at 37˚C in a humidified 5% CO_2 atmosphere. The NF-κB-p65 (RelA) shRNA target sequence was: 5'-CTCCATTGCGGACATGGACTT-3'; shRNA non-target sequence: 5'-TTCTCCGAACGTGTCACGT-3'; TRIM22 shRNA target sequence: 5'-CCAGATATAGACCTC

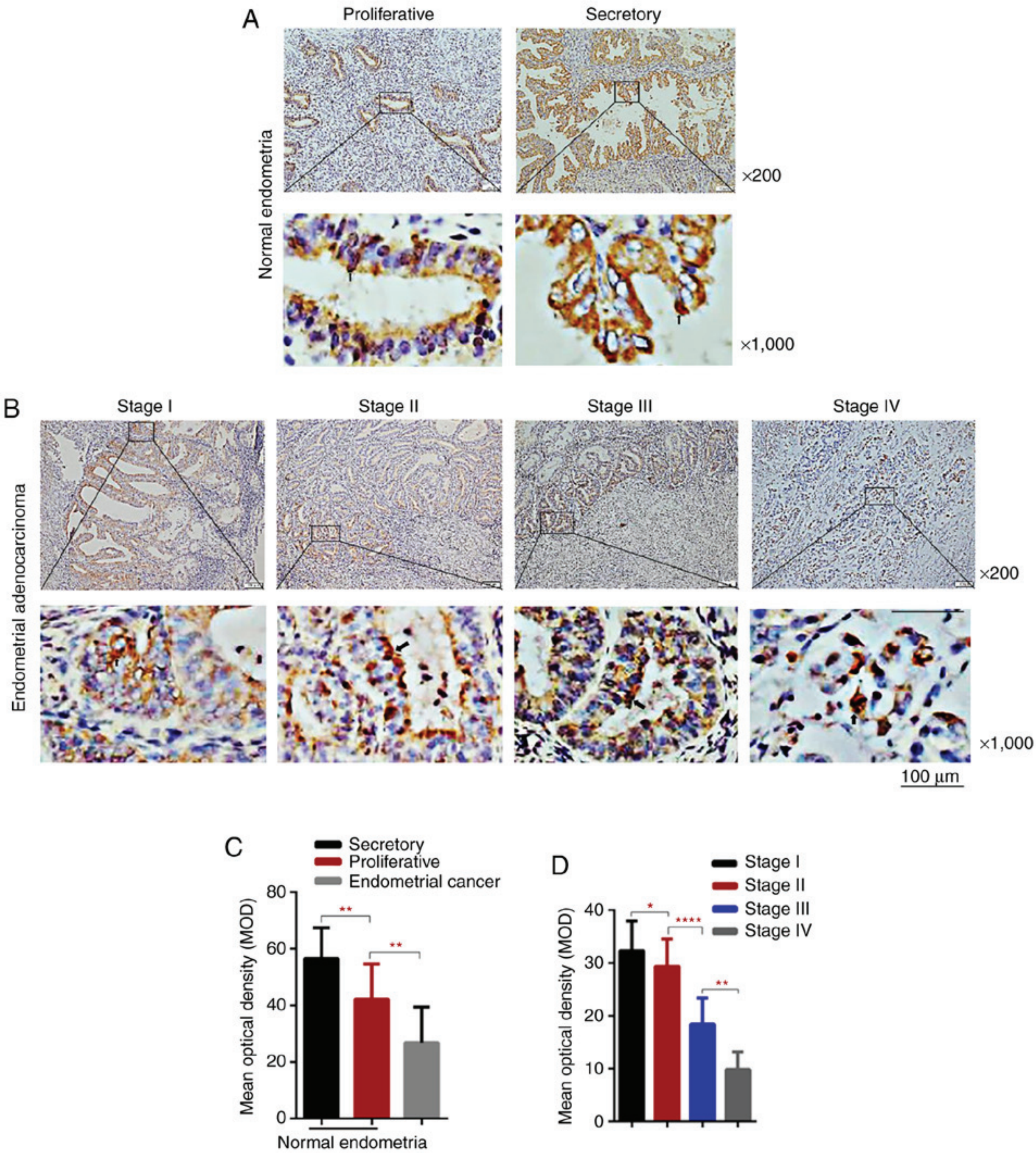

Figure 1. Low TRIM22 levels are associated with EnC prognosis and a higher overall survival rate. (A and B) Immunohistochemical assay of TRIM22 expression in normal endometrial tissues, proliferative and secretory phase, representative images (magnification, x200 and x1,000). TRIM22 expression in EnC tissues (magnification, x200 and x1,000). The cytoplasm and nucleus were stained brown with positive expression. As observed, TRIM22 levels in EnC were lower than those in normal endometrial tissue. TRIM22 expression in the secretory phase of endometrium was higher than that in the proliferative phase. (C) The IHC MOD of TRIM22 were counted in normal endometrial tissues and cancerous endometrial tissues. (D) Statistically significant differences were observed between the different stages of Enc (stage I, II, III and IV). The figure shows MOD of normal and EnC tissues stained with TRIM22 for immunohistochemistry. Image-pro Plus and SPSS software were used to measure and analyze each staining sample. Data are shown as the means ± SD. Scale bar, 100 μm..

AATA-3'; and TRIM22 shRNA non-target sequence: 5'-TTC TCCGAACGTGTCACGT-3'.

In vivo tumor xenograft measurement. Female nude mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd., a joint venture enterprise of Charles River Laboratories. The use of animals was approved by the Ethics Committee of Reproductive Hospital Affiliated to Shandong University. Their care was in accordance with institutional guidelines. A total of 18 BALB/c female nude mice which were 4-5-weeks old were kept under specific pathogen-free (SPF) conditions in the Shandong University Experimental Animal Room. Ishikawa cells infected with lentivirus (vector control and TRIM22 OE), were harvested and resuspended in 100 μl Matrigel/PBS (1:1 vol/vol; Corning, Inc.) and subcutaneously injected into the left flank of each mouse (n=9/group) with $1x10^7$ cells/inoculum(day 0). Tumors of nude mice were observed every 3 days. Tumor volume was expressed as mm^3 and

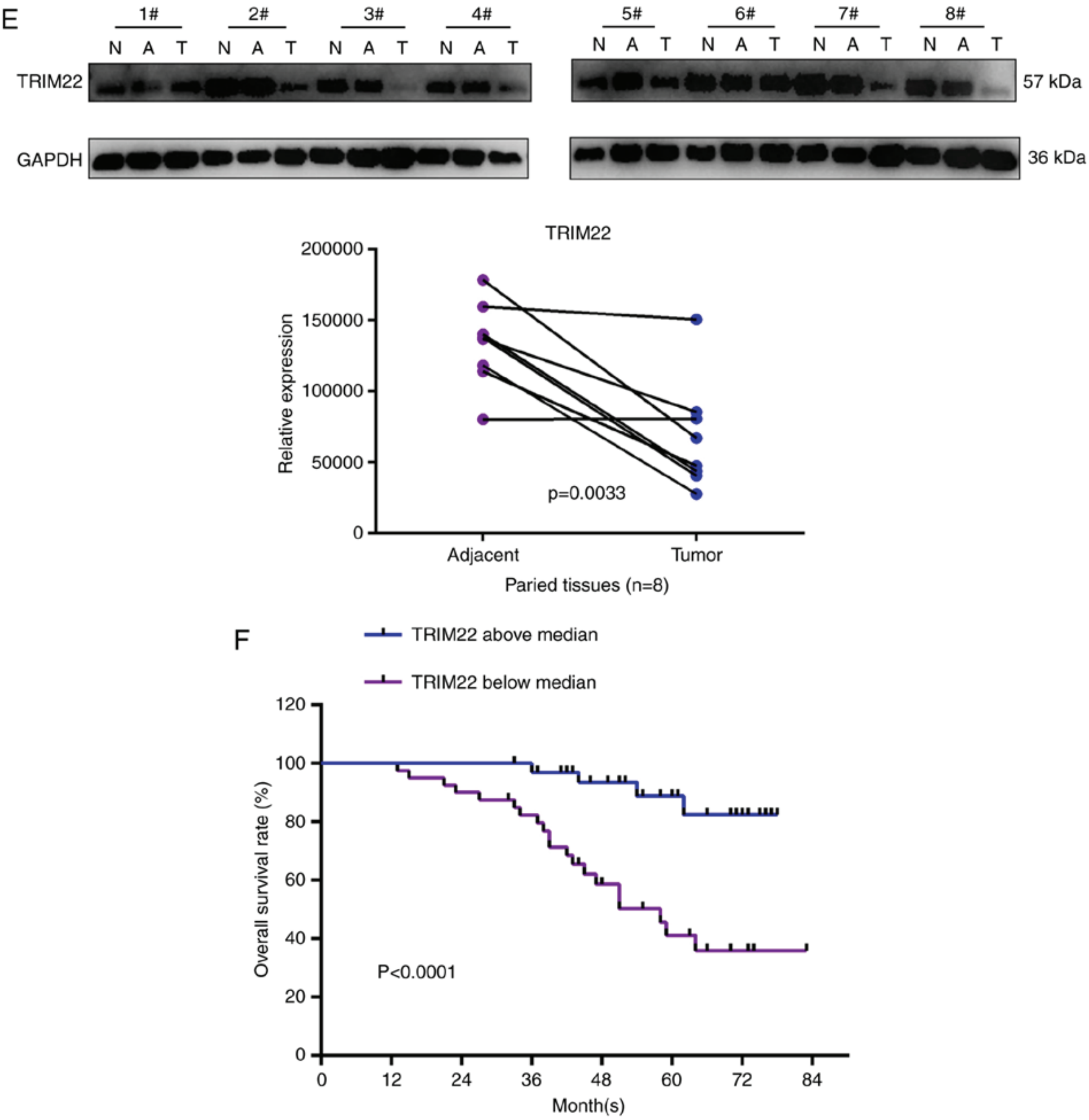

Figure 1. Continued. Low TRIM22 levels are associated with EnC prognosis and a higher overall survival rate. (E) TRIM22 protein levels in 8 pairs of EnC samples were determined by western blot analysis (N, normal endometrial tissue; A, adjacent normal tissue; T, tumor tissue). TRIM22 expression and the comparison of TRIM22 expression levels between EnC specimens and matched adjacent normal tissues in 8 pairs. (F) Kaplan-Meier analysis was used to analyze overall survival. Overall survival rate of patients with a high TRIM22 expression was significantly higher than that of patients with a low TRIM22 expression ($^{*}P<0.05$; $^{**}P<0.01$; $^{****}P<0.0001$). TRIM22, tripartite motif-containing 22; EnC, endometrial cancer.

measured using a common ruler with the traditional formula as follows: (Equation 2): $V=½ \times \text{length (mm)} \times \text{width}^2 \text{ (mm)}$.

After 27 days (day 27) (tumor volume ≤1,000 mm3), the nude mice were anesthetized with a 3-5% (v/v) mixture of isoflurane (Aerrane; isoflurane, Baxter) in synthetic air (200 ml/min), and were then sacrificed by cervical dislocation and the tumors were harvested. The tumor tissues were collected for use in western blot analysis and immobilized in 4% paraformaldehyde for IHC analysis of TRIM22 and Ki-67 expression, and for hematoxylin and eosin (H&E)-staining.

Statistical analysis. Data under normal distribution are presented as the means ± SEM. The Student's unpaired t-test (2-tailed) was used for comparisons between 2 groups. Comparisons between the tumor and the adjacent tumor samples were analyzed using a paired t-test (2-tailed), and one-way ANOVA followed by Tukey's post-hoc test was applied for comparisons among ≥3 groups. Kaplan-Meier estimation was performed to investigate the survival probability of the patients with EnC. GraphPad Prism software version 6.0 was used to visualize the results of the comparisons, as well as the P-values. A P-value <0.05 was considered to indicate a statistically significant difference.

Results

Low expression of TRIM22 in human EnC indicates malignant transformation. IHC staining was usedd to detect

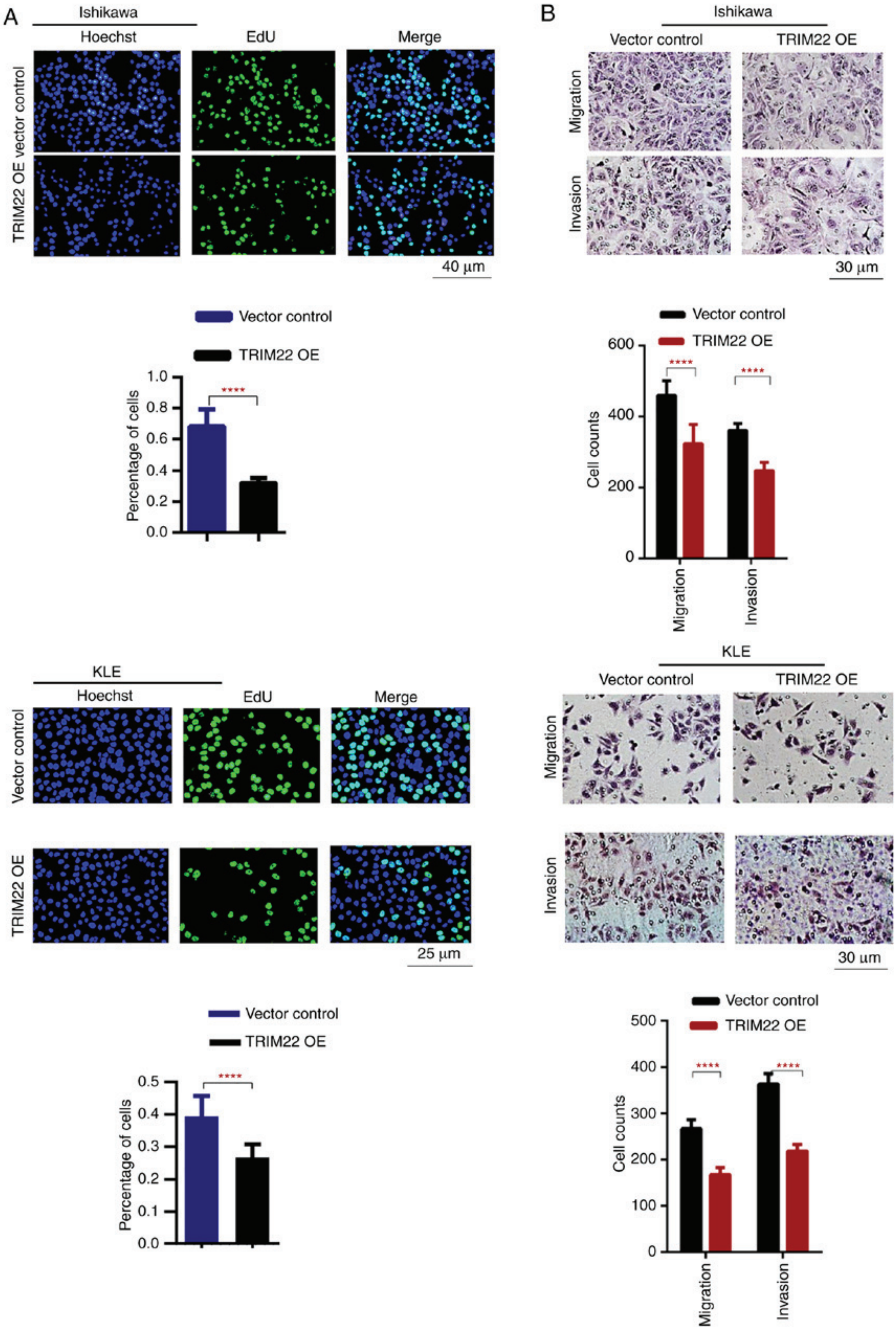

Figure 2. Functional analysis of the effects of TRIM22 on EnC cell migration, invasion, proliferation and cell cycle progression. (A) DNA synthesis of Ishikawa and KLE cells was determined by EdU assay. Green fluorescence indicated that the cells were EdU-positive. Hoechst staining presenting with blue fluorescence indicates the total number of cells. Scale bar, 50 μm. (B) Transwell Matrigel analysis revealed that TRIM22 overexpression in Ishikawa cells and KLE cells significantly suppressed the migration and invasion of tumor cells. Scale bar, 100 μm.

TRIM22 expression in endometrial tissues. IHC analysis revealed cytoplasmic and nuclear TRIM22 staining in both the normal and tumorous endometrial tissues (Fig. 1A and B). However, TRIM22 expression was lower in the EnC specimens compared to the normal endometrial tissue specimens (Fig. 1A-C). Moreover, TRIM22 expression was higher in

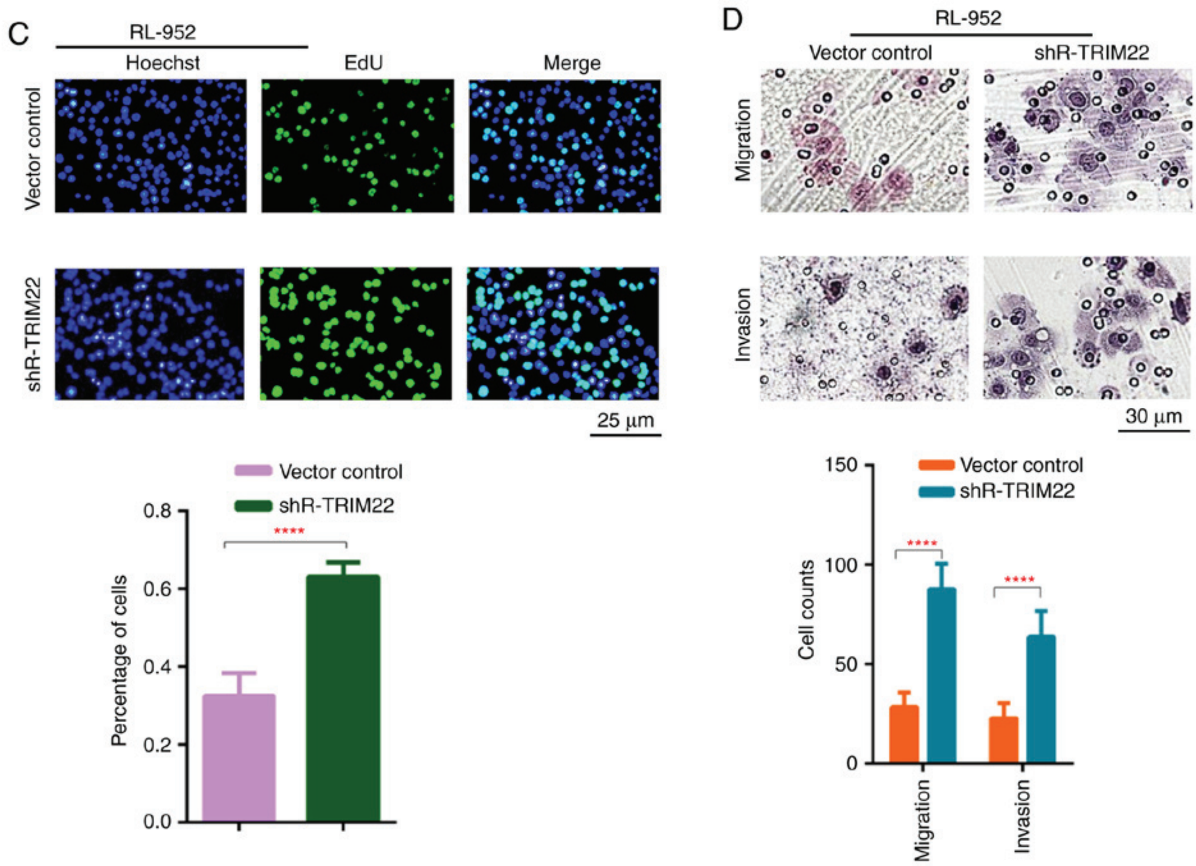

Figure 2. Continued. Functional analysis of the effects of TRIM22 on EnC cell migration, invasion, proliferation and cell cycle progression. (C) DNA synthesis of RL-952 cells was determined by EdU assay. Green fluorescence indicated that the cells were EdU-positive. Hoechst staining presenting with blue fluorescence indicates the total number of cells. Scale bar, 50 μm. (D) Conversely, reducing TRIM22 expression in RL-952 cells improved cell migration and invasion. Scale bar, 100 μm.

the secretory phase compared to the proliferative phase in normal endometrial tissues (Fig. 1A and C). An association was observed between the expression of TRIM22 and the clinical stage. Specifically, TRIM22 expression decreased with the increasing tumor stage (Fig. 1B and D, and Table I). Thereafter, western blot analysis was used to detect TRIM22 expression in 8 paired EnC samples. The results demonstrated that TRIM22 expression was decreased in the EnC tissues compared with the normal endometrial tissues and adjacent normal tissues (Fig. 1E).

To further examine the association between the TRIM22 expression level and EnC malignant conversion, Kaplan-Meier analysis was applied to analyze the results of IHC MOD and survival. These results indicated that an increased TRIM22 expression was significantly related to an improved overall survival of patients with EnC (Fig. 1F). These data support the association between the downregulated expression of TRIM22 and the malignant transformation of EnC, while suggesting that TRIM22 may be a promising prognostic predictor and therapeutic target.

TRIM22 decelerates EnC cell migration, invasion and proliferation, and inhibits cell cycle progression. The function of TRIM22 was analyzed in the KLE, Ishikawa and RL-952 EnC cell lines. Stably transfected TRIM22 OE Ishikawa and KLE cells were generated which expressed higher levels of TRIM22; in addition, shR TRIM22 RL-952 cells were generated which expressed lower levels of TRIM22. Western blot analysis confirmed that the expression of TRIM22 increased in the TRIM22 OE Ishikawa and KLE cells, and decreased in the shR TRIM22 RL-952 cells (Fig. S1A and B). Fluorescence microscopy was used to observe the transfection efficiency (Fig. S1C and D).

Furthermore, the overexpression of TRIM22 decreased Ishikawa and KLE cell proliferation, while the opposite effect was observed in the shR TRIM22 RL-952 cells (Fig. 2A and C). Moreover, the migratory and invasive abilities of the TRIM22 OE Ishikawa and KLE cells were markedly restricted; by contrast, shR TRIM22 RL-952 enhanced the cell migratory and invasive abilities (Fig. 2B and D). Simultaneously, cell cycle analysis revealed that the number of TRIM22 OE Ishikawa and KLE cells in the G1 phase increased, while that in the S phase decreased (Fig. 2E). These results indicate that TRIM22 suppresses EnC progression.

TRIM22-NOD2-NF-κB axis restricts EnC progression. As an important factor involving multiple aspects of immunity and inflammation, TRIM22 has been reported to function as a NOD2-interacting protein, and as a regulator of the NF-κB signaling pathway, dependent on NOD2 in inflammatory bowel disease (22). To determine whether the TRIM22-NOD2 interaction exists in normal endometrial tissues and endometrial tumor

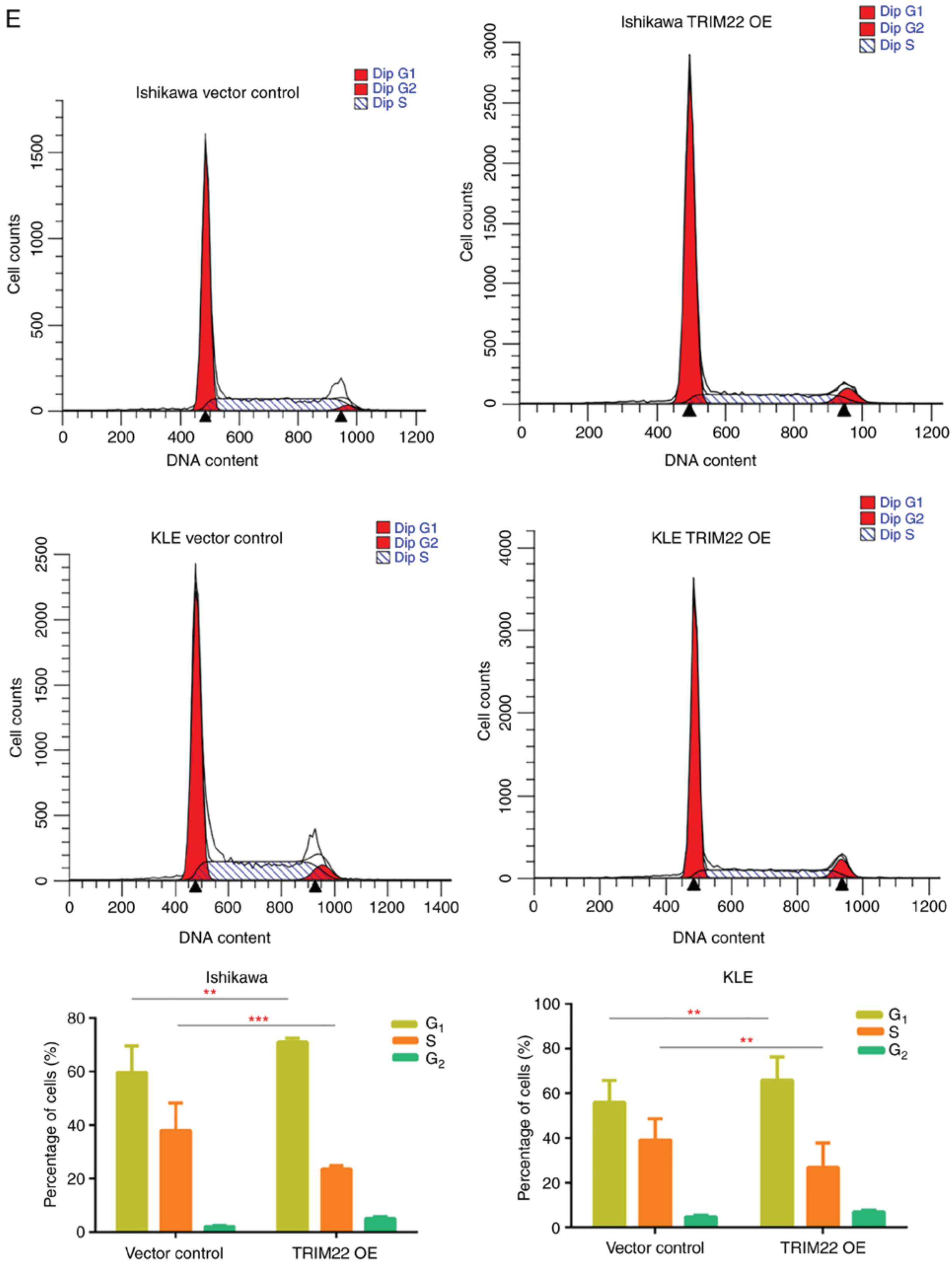

Figure 2. Continued. Functional analysis of the effects of TRIM22 on EnC cell migration, invasion, proliferation and cell cycle progression. (E) Flow cytometry was performed to analyze the PI staining lentiviral expression vector or lentiviral expression vector containing TRIM22 cDNA transfected Ishikawa and KLE cells. Cell cycle analysis indicated that the overexpression of TRIM22 in Ishikawa and KLE cells led to a higher number of cells in the G1 phase and a lower number in the S phase compared with the control cells. This confirmed that higher TRIM22 levels decreased EnC cell growth through G1 cell cycle arrest. Each experiment was conducted in triplicate (**P<0.01; ***P<0.001; ****P<0.0001). TRIM22, tripartite motif-containing 22; EnC, endometrial cancer.

tissues, immunofluorescence and co-IP were conducted. The results of immunofluorescence assay revealed that TRIM22 and NOD2 co-localized in both the normal endometrial tissues and endometrial tumor tissues (Fig. 3A). Furthermore, the expression

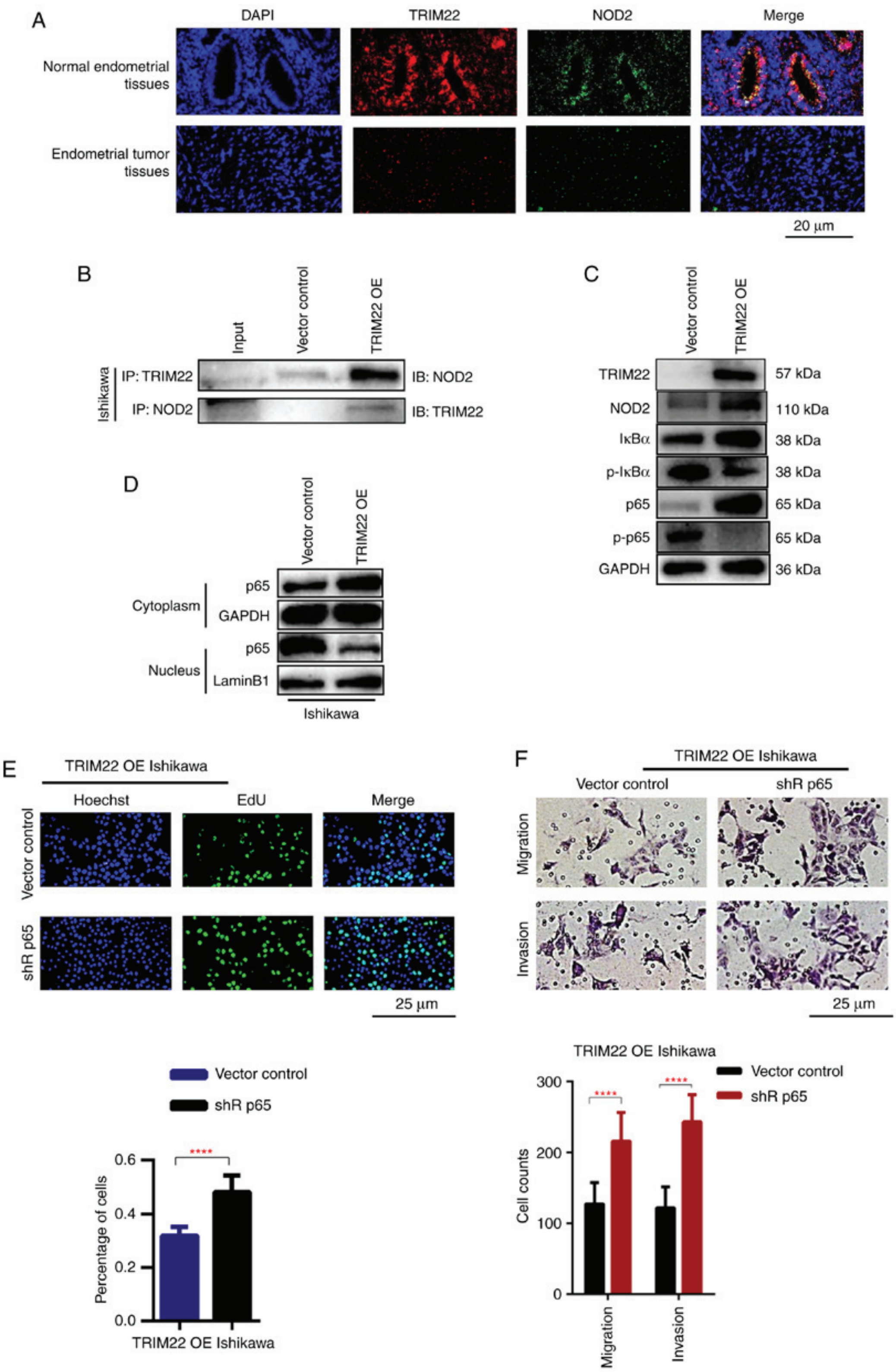

Figure 3. TRIM22 influences EnC cells by binding NOD2, and suppresses the migration, invasion and proliferation of EnC cells via the NOD2-NF-κB axis. (A) Immunofluorescence analysis of TRIM22 and NOD2 in normal endometrial tissues and endometrial tumor tissues from subjects undergoing hysterectomy and patients with EnC. TRIM22 and NOD2 colocalization was observed in the cytoplasm of both normal endometrial tissue and endometrial tumor tissue. Both TRIM22 and NOD2 colocalization and expression were decreased in EnC tissue. Scale bar, 20 µm. (B) Ishikawa cells were transfected with lentiviral construct encoding human TRIM22, and the interaction between TRIM22 and NOD2 was determined through co-immunoprecipitation examination. An empty vector construct was used as IP-negative controls. TRIM22 overexpression construct was examined. (C) Western blot analysis of IκBα degradation, phosphorylated IκBα (Ser 36), p65 and phosphorylated p65 (Ser 536) in Ishikawa TRIM22 OE cells. (D) Ishikawa cells were transfected with lentiviral construct encoding human TRIM22. Western blot analysis was applied to evaluate the location of p65 in Ishikawa and KLE cells. (E and F) Ishikawa cells were transfected with lentiviral construct encoding human TRIM22 at first and followed by transient transfection with shRNA-NF-κB-p65. Knockdown NF-κB-p65 attenuated the decrease in the migration, invasion and cell proliferation which was induced by the overexpression of TRIM22. The cell proliferative, migratory and invasive abilities of endometrial tumor cells increased. Scale bars: EdU, 50 µm; Transwell, 100 µm. ****$P<0.0001$. TRIM22, tripartite motif-containing 22; EnC, endometrial cancer; NOD2, nucleotide-binding oligomerization domain-containing protein 2.

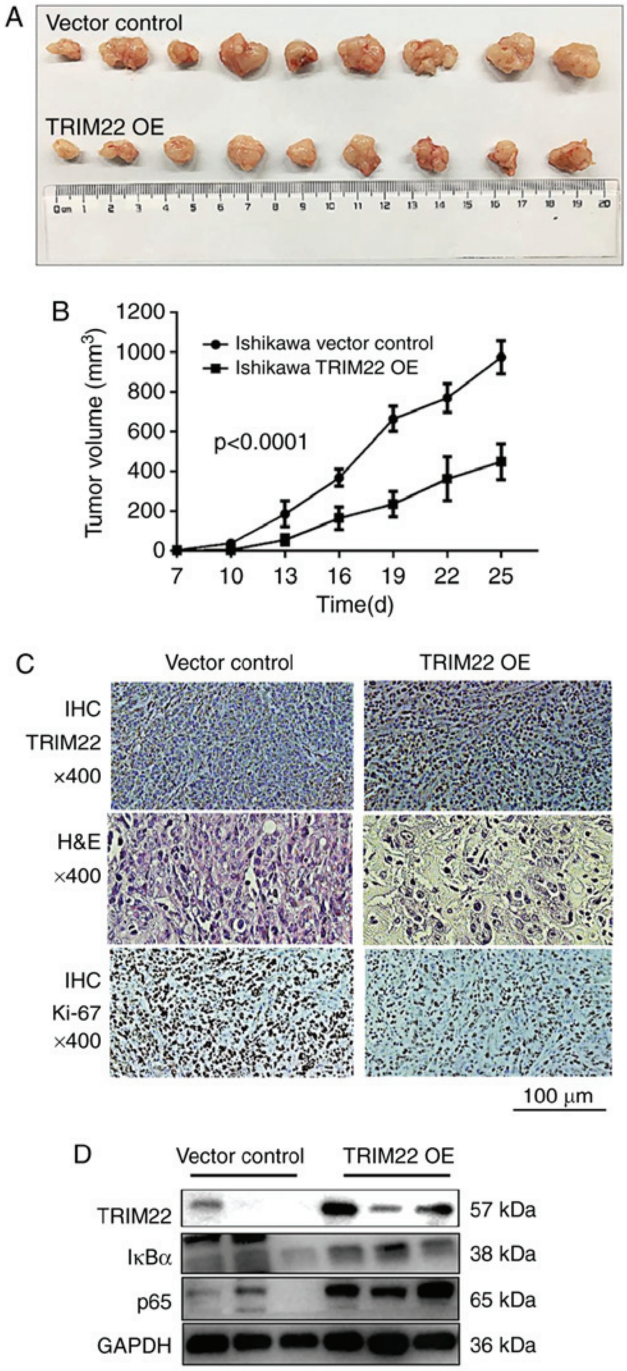

Figure 4. Overexpression of TRIM22 inhibits tumor growth *in vivo*. Ishikawa cells infected with a lentiviral construct encoding human TRIM22 (TRIM22 OE) or lentiviral construct (vector control) were subcutaneously implanted into the left flanks of female immunodeficient mice. (A) Representative images of TRIM22, Ki-67 staining and H&E staining of the xenograft tissues. Scale bar, 100 μm. (B) TRIM22 overexpression in Ishikawa cells decreased the growth of transplanted tumor in nude mice (n=9 per group). (C) Tumor volume was measured twice per week from day 7 to 25 following implantation and the curve was plotted. Data are expressed as the means ± SD (n=9 in vector control group; n=9 in TRIM22 OE group). (D) Representative western blots of the levels of TRIM22, IκBα and p65 in the tumors. TRIM22, tripartite motif-containing 22; EnC, endometrial cancer.

of NOD2 was decreased in the tumor tissues, which was consistent with TRIM22 expression (Fig. 3A). It was also found that the association between TRIM22 and endogenous NOD2 was weak in the co-IP experiments in the vector control-transfected Ishikawa cells and human endometrial tumor cell lines, and this association was enhanced in the TRIM22 OE Ishikawa cells (Fig. 3B). NOD2 binding to TRIM22 was further investigated by co-IP. It was confirmed that NOD2 was also weakly bound to the vector control-transfected Ishikawa cells, and this association increased in the TRIM22 OE Ishikawa cells (Fig. 3B). These experiments demonstrated that TRIM22 directly interacts with NOD2 protein.

Since TRIM22 co-localized and co-immunoprecipitated with NOD2, the question of whether TRIM22 affects the NOD2 signaling pathway was investigated. An alternate study reported that the role of NOD2 in regulating NF-κB, was bidirectional. Moreover, NOD2 can activate NF-κB in a number of inflammatory diseases (23-27) and can inhibit NF-κB in colorectal tumorigenesis (28-30). The expression of NOD2 and NF-κB-associated protein was detected in the vector control-transfected and TRIM22 OE Ishikawa cells, respectively, by western blot analysis. It was also found that as the expression of NOD2 increased, that of NF-κB-p65 and IκBα also increased; however, the phosphorylation of NF-κB-p65 (p-p65) and IκBα (p-IκBα) was decreased in the TRIM22 OE Ishikawa cells (Fig. 3C). This suggested that TRIM22 induced NOD2 expression and increased the expression of NOD2, subsequently decreasing NF-κB activation in Ishikawa cells. Additionally, these results suggest that NOD2 may inhibit NF-κB in EnC.

Subsequently, the regulatory role of TRIM22 in the NOD2 signaling pathway was further determined. To identify the suppressive role of TRIM22 in the translocation of NF-κB-p65, nuclear and cytoplasmic fractions were prepared from the vector control-transfected and TRIM22 OE Ishikawa cells. Western blot analysis revealed that in the cytoplasm, the expression of NF-κB-p65 in the vector control-transfected cells was lower than that in the TRIM22 OE cells; however, in the nucleus, the expression of NF-κB-p65 in the vector control-transfected cells was higher than that in the TRIM22 OE cells (Fig. 3D). These results suggest that TRIM22 inhibits NF-κB-p65 from translocating from the cytoplasm to the nucleus, and thus, it inhibits its transcriptional regulatory function. Hence, TRIM22 inhibits the activity of NF-κB-p65. NF-κB-p65 was also knocked down by transiently transfecting the shRNA-NF-κB-p65 plasmids into TRIM22 OE Ishikawa cells (shR p65). The effect of NF-κB-p65 knockdown was examined by western blot analysis (Fig. S2). The results of EdU and Transwell assays revealed that cell proliferation, migration and invasion of the shR p65 TRIM22 OE Ishikawa cells increased (Fig. 3E and F). It was thus suggested that the knockdown of NF-κB-p65 may overcome the reduced cell proliferation, migration and invasion induced by TRIM22 overexpression. These data indicate that TRIM22 suppresses the NF-κB signaling pathway by associating with NOD2.

Overexpression of TRIM22 inhibits tumor growth in vivo. Subsequently, the *in vivo* effect of TRIM22 was assessed in a BALB/c Ishikawa cell line xenograft model in nude mice. Nine mice developed palpable tumors at the injection site following the subcutaneous injection of Ishikawa cells for 7 days. The tumor size was measured twice a week for 4 weeks. TRIM22 overexpression exerted a significant inhibitory effect on tumor growth by day 25 of the study

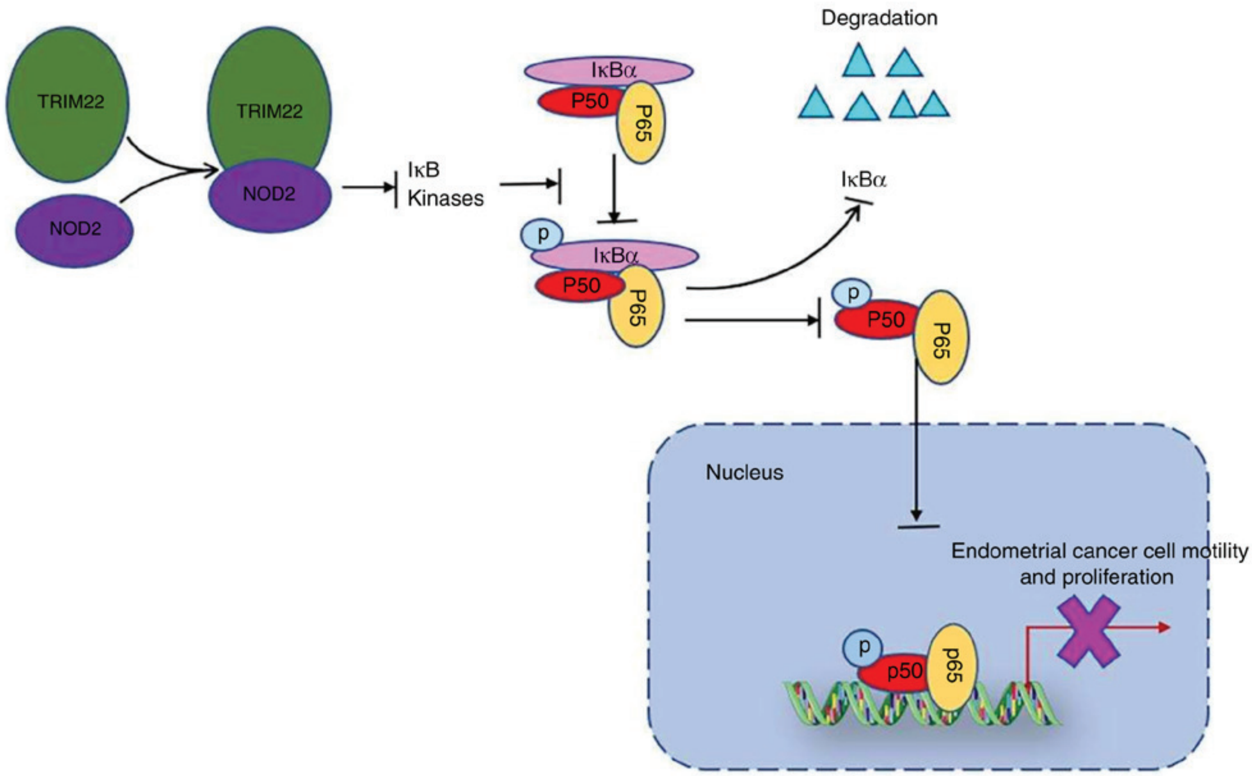

Figure 5. Schematic diagram of the model proposed to elucidate the regulatory role of TRIM22 in tumor growth and metastasis. TRIM22, tripartite motif-containing 22.

(Fig. 4). The mean tumor volume of the mice in the TRIM22 OE group from day 19 was markedly smaller than that of the vector control group (Fig. 4B).

IHC staining for Ki-67, a cell growth marker, was used to visualize xenograft tumors size as determined by the tumor cell proliferation *in vivo*. The expression of Ki-67 was significantly decreased in the malignant tumors, which were derived from the TRIM22 OE Ishikawa cells. The tumor tissues were then stained with H&E for the observation of the pathological characteristics *in vivo* (Fig. 4C).

To validate the regulatory effects of TRIM22 *in vivo*, the relevant protein expression in the tumor tissues from the nude mice were examined. The results demonstrated that the increased expression of TRIM22 also upregulated IκBα, NF-κB-p65 expression *in vivo* (Fig. 4D). Taken together, these results indicate that TRIM22 inhibits EnC development and progression by regulating the NF-κB signaling pathway.

Discussion

The multifactorial nature and complex heterogeneity of cancer contribute to the high associated global mortality rate (31). TRIM22 is a member of the TRIM family of proteins, which have been examined in the context of a myriad of inflammatory diseases, such as HIV, HBV and HCV (32-35). However, studies examining the association between TRIM22 and tumor growth are rare. A previous study demonstrated that TRIM22 expression was lower in breast cancer cell lines and tissues compared to in non-malignant mammary epithelial cell lines and normal breast tissues (12); however, TRIM22 expression has been found to be increased in NSCLC cell lines (11,36). Thus, the multi-functional role of TRIM22 in human cancer is rather complex and may be tissue-specific. Its role in EnC remains uncharacterized. Herein, it was demonstrated that TRIM22 was downregulated in both EnC samples (tumor tissues from patients with EnC) and cancer cell lines. Thereafter, it was demonstrated that a low expression of TRIM22 in EnC tissues was associated with the occurrence and a poor prognosis of malignant tumors. Moreover, the increased expression of TRIM22 inhibited the migration, invasion, proliferation and cell cycle progression of EnC cells both *in vivo* and *in vitro*. Therefore, it was hypothesized that TRIM22 overexpression contributes to improved outcomes and prognoses for patients with EnC, and may thus also be considered as a promising prognostic factor for EnC.

Tumor formation and development are derived not only from abnormal gene mutations or disorders in tumor cells, but also from complex microbial ecosystems that play a vital role in the formation of systemic innate and inflammatory responses (37). It has been revealed that TRIM proteins regulate a number of biological processes, including apoptosis, cell proliferation, innate immunity, autoimmunity, inflammatory response and tumorigenesis, through different signaling pathways (20,38). For example, TRIM19 negatively regulates NF-κB activity by translocating NF-κB to the nucleus (39). Additionally, TRIM25 regulates RIG-I-mediated antiviral activity (40); TRIM21 has also been described as playing an important role in regulating specific pro-inflammatory cytokines by modulating interferon regulatory factors (IRFs) (41,42); Lastly, TRIM22 has been reported to accelerate NSCLC progression through the AKT/GSK3β/β-catenin signaling pathway (11). However, the regulatory mechanisms employed by TRIM22 in EnC remain unclear. In the present study, it was demonstrated that TRIM22 inhibits endometrial cancer progression through the NOD2-NF-κB signaling pathway. It was also demonstrated that TRIM22 is a NOD2 interacting protein, and that the overexpression of TRIM22 induces NOD2 expression, subsequently suppressing NF-κB activation.

Mounting evidence suggests that the NF-κB signaling pathway is involved in the progression of various human tumors, including those of ovarian cancer (43), prostate cancer (44), cervical cancer (45), as well as head and neck cancer (46). A recent study demonstrated that TRIM22 negatively regulates the tumor necrosis factor receptor-associated factor 6 (TRAF6)-stimulated NF-κB pathway by binding to the TRIM22 N-terminal RING domain (47). Similarly, it was found that TRIM22 inhibits NF-κB signaling and that the overexpression of TRIM22 inhibits NF-κB-p65 translocation from the cytoplasm to the nucleus. However, the knockdown of NF-κB-p65 attenuated the effects of TRIM22 on the cells. However, a previous meta-analysis revealed that the inflammatory cytokine network is complex, with extensive interactions. For example, activated NF-κB secretes TNFα, while TNFα may increase TRIM22 expression, and activate NF-κB (48). A previous study demonstrated that TRIM22 induced the activation of NF-κB independently of other transcription factors, such as IRFs, combining with the C-terminal SPRY domain of TRIM22 (49). Possibly, the TRIM22 binding site induction of NF-κB activation in patients with EnC was caused by a deletion mutant. In addition, the role of TNFα in regulating TRIM22 expression requires further investigation.

NOD2, the cytosolic NOD-like receptors (NLRs) family member, functions as a critical player in the regulation of inflammation (50). NOD2 has been shown to regulate NF-κB signaling via two distinct pathways. The first involves NOD2 acting as a sensor of muramyl dipeptide (MDP), a composition of peptidoglycan present in gram-positive and gram-negative bacteria; MDP then induces the activation of NF-κB through NOD2, and thus, NOD2 activates the NF-κB signaling pathway (51). The second pathway requires NOD2 to be stimulated by TLR, causing the induction of IRF4, which subsequently inhibits the activation of NF-κB through interaction with MyD88 and TRAF6, effectively allowing NOD2 to suppress the NF-κB signaling pathway (52,53). The present study demonstrated that the overexpression of TRIM22 induced the expression NOD2, which subsequently inhibited the activation of NF-κB. However, it remains unclear as to how NOD2 suppresses NF-κB via TLR and thus, the specific mechanisms of the TRIM22 and NOD2 interaction warrant further investigation.

The present study demonstrated that the overall survival rate of patients with a high TRIM22 expression was significantly higher compared with that of patients expressing low levels of TRIM22. TRIM22 expression was associated with the clinical stage. It was also demonstrated that TRIM22 expression was higher in the secretory phase than in the proliferative phase, which suggests that TRIM22 expression may be related to progestin, and progestin can induce the increased expression of TRIM22, as has been previously reported (15). Moreover, progestin is commonly used as an auxiliary treatment for patients with EnC; TRIM22 has been reported as a future prognostic predictor in certain types of cancer (11,14). Although it is commonly considered that the clinical stage has a minimal association with the biological malignancy of EnC, it was thus hypothesized that TRIM22 may be a potent prognostic and diagnostic factor in patients with EnC; however, TRIM22 has been poorly studied in human tumors and the precise mechanisms between TRIM22 and progestin remain unclear. Hence, more stringent selective criteria should be employed to identify the diagnostic efficiency and true prognostic value of TRIM22. Additionally, further studies are required to elucidate the mechanisms between TRIM22 and progestin.

Certain limitations should be noted throughout the present study. Firstly, the sample size was small. Although the results of the clinical samples corresponded to TCGA data that has been previously reported (54), further studies with larger sample sizes are required in the future; secondly, the present study only selected three endometrial cancer cells, Ishikawa, KLE and RL-952 cells, which may limit the results. Thus, further studies using more EnC cells are warranted in the future.

In conclusion, the present study demonstrates that TRIM22 is downregulated in EnC and is associated with clinical treatment efficacy. Moreover, the present study highlights the function of TRIM22 in inhibiting EnC cell migration, invasion, growth and cell cycle progression. It also establishes that TRIM22 directly inhibits NF-κB activity by binding to NOD2 in EnC cells (Fig. 5). As a result, TRIM22 may serve as an effective future prognostic indicator, and the association between progestin and TRIM22 may be a potential basis for the progestin treatment of patients with EnC.

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Availability of data and materials

The data associated with the current study are available from the corresponding author on reasonable request.

Authors' contributions

LipingZ was involved in the investigative aspects of the study, as well as in the study methodology, data collection, data analysis, and in the writing of the manuscript. BZ was involved in data collection and data analysis. MW and ZX designed part of the study and supervised the study. WK provided resources and collected the follow-up data of the patients involved. KD was involved in data collection and providing resources. LinZ was involved in data analysis. XX and XZ provided resources and assisted with the animal experiments. LY was involved in project development, data analysis and in the writing of the manuscript, as well as in the reviewing and editing of the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Ethics Committee approval was obtained from the Reproductive Center of Provincial Hospital affiliated to Shandong University. The present study was performed according to the Declaration of Helsinki. All animal experiments were performed following the Ethics Committee of Reproductive Center of Provincial Hospital affiliated to Shandong University. Animal care was in accordance with institutional guidelines.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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