



# Effects of antisense lncRNA PCBP1-AS1 on biological behaviors of vulvar squamous carcinoma cells by regulating TRAF5 and NF- $\kappa$ B expression

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**Background:** Given antisense long noncoding RNAs (lncRNAs) poly C binding protein-1 antisense RNA1 (PCBP1-AS1) was significantly co-expressed with tumor necrosis factor receptor associated factor 5 (TRAF5) mRNA in vulvar squamous cell carcinoma (VSCC). TRAF5 could mediate nuclear factor kappa B (NF- $\kappa$ B) activation and regulate biological behaviors of tumor cells. Herein, we determined whether PCBP1-AS1 could affect the tumor behaviors of VSCC by regulating TRAF5 and NF- $\kappa$ B expression.

**Methods:** The expression of PCBP1-AS1 and TRAF5 mRNA in VSCC tissues and cells was detected via quantitative real-time polymerase chain reaction, while the protein expression levels of TRAF5, p65, p-p65, c-Rel, and I $\kappa$ B $\alpha$  were examined by immunoblotting. After the overexpression of PCBP1-AS1 and RNA interference knockdown of TRAF5 in SW954 cells, NF- $\kappa$ B activation-nuclear translocation fluorescence, the CCK-8 assay, Annexin V-FITC & PI fluorescent staining, the wound healing assay, and the Transwell invasion assay were performed to evaluate the activity of NF- $\kappa$ B, cellular proliferation, apoptosis, migration, and invasion.

**Results:** PCBP1-AS1 and TRAF5 mRNA expression were downregulated in VSCC tissues and were correlated with patient clinicopathological characteristics. The protein expression of TRAF5, p65, p-p65, c-Rel, and I $\kappa$ B $\alpha$  protein in VSCC tissues was significantly decreased. We demonstrated that PCBP1-AS1 positively regulated the expression of TRAF5. PCBP1-AS1 overexpression or knockdown of TRAF5 regulated the activity of the NF- $\kappa$ B pathway. Increase of PCBP1-AS1 significantly inhibited proliferation and enhanced apoptosis via TRAF5-mediated regulation of the NF- $\kappa$ B signaling pathway. Additionally, PCBP1-AS1 can suppress migration and invasion in an NF- $\kappa$ B-independent manner.

**Conclusions:** As a tumor suppressor gene, PCBP1-AS1 regulates the proliferation and apoptosis of VSCC via TRAF5-mediated expression of the NF- $\kappa$ B.

**Keywords:** Vulvar squamous cell carcinoma (VSCC); lncRNA; poly C binding protein-1 antisense RNA1 (PCBP1-AS1); tumor necrosis factor receptor associated factor 5 (TRAF5); nuclear factor kappa B (NF- $\kappa$ B)

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## Introduction

Vulvar cancer is one of multiple malignant tumors of the female reproductive organs, the majority of which are primary vulvar squamous cell carcinomas (VSCC) (1). Recently, the incidence of VSCC has increased yearly (2), threatening the health of older women because the five-year survival rate of advanced VSCC is only 20%.

Poly C binding protein-1 antisense RNA1 (PCBP1-AS1) is an antisense long noncoding RNA (lncRNA) of PCBP1. Antisense lncRNA is an endogenous lncRNA molecule that is complementary to the sequence of other transcripts and regulates the expression of target genes at the transcriptional and posttranscriptional levels. Antisense lncRNA may play a unique role in the development of disease. Our group was the first to report a genome-wide expression profile of lncRNA molecules in VSCC tissues compared with corresponding adjacent normal vulvar tissue using microarray analysis technology (3). We found that PCBP1-AS1 and TRAF5 mRNA were significantly coexpressed in VSCC using bioinformatics analysis and target gene prediction (Correlation >0.99, P value <0.05).

Tumor necrosis factor receptor associated factor 5 (TRAF5) is an important intracellular signal transduction protein. Previous studies have shown that TRAF5 mediates activation of the nuclear factor kappa B (NF- $\kappa$ B) signal transduction pathway, playing a regulatory role in the biological behavior of tumor cells (4).

In the study, we aimed to investigate the expression of PCBP1-AS1 in VSCC tissues and analyze its clinical significance. Specifically, we determined that PCBP1-AS1 could affect the proliferation and apoptosis of VSCC SW954 cells through TRAF5-mediated regulation of the NF- $\kappa$ B signaling pathway.

## Methods

### *Tissue samples*

Nineteen pairs of VSCC and corresponding adjacent normal vulvar tissues were consecutively collected from patients undergoing surgical resection at the Department of Gynecology, Shengjing Hospital, China Medical University from January 2011 to October 2016. Patients ranged in age from 34 to 85 years, with a mean age of  $56.9 \pm 13.9$  years. All specimens were confirmed by pathological examination, and no patients underwent radiotherapy, chemotherapy, or any

other medical intervention prior to surgery. The collected tissue samples were immediately frozen in liquid nitrogen after resection and were stored in liquid nitrogen until RNA and protein extraction. Patients in this study all provided written informed consent. This study was approved by the Ethics Committee of Shengjing Hospital affiliated with China Medical University.

### *Cell lines and transfection*

The SW954 cell line was purchased from Shanghai Huiying Biological Technology co., LTD (Shanghai, China). All cells were cultured in RPMI-1640 medium (HyClone, Logan, Utah, USA) containing 20% heat-inactivated fetal bovine serum (FBS; BioInd, Kibbutz Beit Haemek, Israel) and 1% penicillin/streptomycin (Beyotime Biotechnology, Shanghai, China) in a humidified incubator containing 5% CO<sub>2</sub> at 37 °C.

SW954 cells were transfected with either a PCBP1-AS1 overexpression plasmid, a TRAF5 shRNA plasmid, or their corresponding negative control vectors (Genechem, Shanghai, China) individually or simultaneously using the Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

The cells in the blank control group and TRAF5-RNAi-0 group were treated with recombinant human TNF- $\alpha$  (Novus, Colorado, USA), an NF- $\kappa$ B activator, as the activation groups. The cells in the blank control group and PCBP1-AS1-over group were treated with BAY11-7082 (Beyotime, Shanghai, China), an NF- $\kappa$ B inhibitor, as the inhibition groups. The cells in the blank control group, PCBP1-AS1-over group, and TRAF5-RNAi-0 group were left untreated as controls. The concentrations of recombinant human TNF- $\alpha$  and BAY11-7082 were both  $2 \times 10^{-5}$  g/mL. Cells were collected for further experiments at 24 or 48 h after transfection.

### *Total RNA extraction, reverse transcription and qRT-PCR*

Total RNA was extracted from 19 pairs of VSCC and corresponding adjacent normal vulvar tissue specimens or were cultured cells using Trizol reagent (Takara, Tokyo, Japan). RNA was reverse transcribed into cDNA using a PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Tokyo, Japan). Quantitative RT-PCR was performed using a SYBR® PrimeScript™ RT-PCR Kit on a Roche LightCycler

480 II real-time PCR platform (Roche, Basel, Switzerland). Gene expression in each sample was normalized to GAPDH expression. The primer sequences used were as follows: for GAPDH, 5'-GCACCGTCAAGGCTGAGAAC-3' (forward) and 5'-TGGTGAAGACGCCAGTGGGA-3' (reverse); for PCBP1-AS1, 5'-TGCCAAGAGCCTATCCATTC-3' (forward) and 5'-TCACTCCCTTCACCCTGTCT-3' (reverse); for TRAF5, 5'-AACCTGACCCCAATAGCAGC-3' (forward) and 5'-TCAGTTAAGTCCACGGCCAC-3' (reverse). Real-time PCRs were performed in triplicate. The relative expression levels of PCBP1-AS1 and TRAF5 were calculated using the  $2^{-\Delta\Delta C_t}$  value method.

#### *Western blot assay*

Total protein samples were collected from tissue specimens and were cultured cells in RIPA lysis buffer (Beyotime, Shanghai, China) or cell lysis buffer (Beyotime, Shanghai, China) containing 1% protease phosphatase inhibitor cocktail (Beyotime, Shanghai, China). Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Massachusetts, USA). The membranes were blocked in Tween-20 containing 5% nonfat milk and were incubated with the following primary antibodies overnight at 4 °C: rabbit anti-human TRAF5 (Proteintech, Chicago, USA; 1:500), rabbit anti-human p65/RELA (Proteintech, Chicago, USA; 1:500), rabbit anti-human phosphorylated NF- $\kappa$ B p65 (pSer536) (Cell Signaling Technology, Boston, USA; 1:500), rabbit anti-human c-Rel (Proteintech, Chicago, USA; 1:500), rabbit anti-human I $\kappa$ B $\alpha$  (Cell Signaling Technology, Boston, USA; 1:500) or mouse anti-human GAPDH (Proteintech, Chicago, USA; 1:10,000). After washing with TBST, the membranes were incubated with either goat anti-rabbit IgG (H + L) (Zhongshan Golden Bridge, Beijing, China; 1:5,000) or goat anti-mouse IgG (Proteintech, Chicago, USA; 1:5,000) secondary antibody diluted with TBST, as appropriate. Proteins were visualized using an electrochemiluminescence (ECL) detection kit (Absin, Shanghai, China), and band intensities were quantified using the Azure c300 Chemiluminescent Image System (Azure Biosystems, Dublin, CA, USA). Quantitative analysis was performed using ImageJ 1.44p software (National Institutes of Health, Bethesda, Maryland, USA). TRAF5, p65, p-p65, c-Rel, and I $\kappa$ B $\alpha$  protein expression levels were normalized to those of GAPDH.

#### *NF- $\kappa$ B activation-nuclear translocation fluorescence labeling assay*

SW954 cells ( $5 \times 10^5$  cells/well) were seeded into 6-well plates and were transfected with the PCBP1-AS1 overexpression plasmid (PCBP1-AS1-over), TRAF5 shRNA plasmid (TRAF5-RNAi-0), both the PCBP1-AS1 overexpression plasmid and TRAF5 shRNA plasmid (PCBP1-AS1-over + TRAF5-RNAi-0), or the corresponding negative control vectors (PCBP1-AS1-NC + TRAF5-RNAi-NC) for 24 h. The cells were immunofluorescence labeled using a cellular NF- $\kappa$ B Activation-Nuclear Translocation Assay Kit (Beyotime, Shanghai, China) according to the manufacturer's protocol.

#### *Cell proliferation assay*

Cell proliferation was evaluated using the Cell Counting Kit-8 (CCK-8) assay (Dojindo Laboratories, Kumamoto, Japan). SW954 cells ( $2 \times 10^3$  cells/well) were plated in 96-well plates containing 100  $\mu$ L of complete medium. The cells were incubated overnight and then were transfected with different plasmids. Absorbances were measured at 450 nm using a Synergy H1 Microplate Reader (BioTek, Winooski, Vermont, USA) at each time point. Cell proliferation curves were plotted using the mean absorbance (optical density OD) at each time point. The cell proliferation inhibition rate = [(OD experimental group - OD blank group) - (OD negative control group - OD blank group)] / (OD negative control group - OD blank group)  $\times 100\%$ . The experiment was conducted using both NF- $\kappa$ B activation and inhibition groups as well as control groups.

#### *Cell apoptosis assay*

SW954 cells ( $5 \times 10^5$  cells/well) were seeded into 6-well plates and were transfected with different plasmids. Cells were collected by trypsinization, washed twice with cold PBS, and then were resuspended in  $1 \times 10^6$  cells/ml. One hundred microliters of each cell suspension were transferred to a corresponding 5-mL flow tube and was stained with Annexin V-FITC (5  $\mu$ L) and propidium iodide (5  $\mu$ L) using the Annexin V-FITC Apoptosis Detection Kit (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's protocol. The percentage of apoptotic cells was quantified using a BD FACSCalibur Flow Cytometer (BD Biosciences, California, USA).

### Wound healing assay

SW954 cells ( $5 \times 10^5$  cells/well) were seeded into 6-well plates and were transfected with different plasmids. When the cell density reached approximately 90-95% confluence, artificial and homogeneous scratch wounds were created on the confluent monolayer cells using a sterile 200- $\mu$ L pipette tip. The speed of wound healing was monitored and imaged at three different time points (0, 24, and 48 h) using an Eclipse NI microscope (Nikon, Tokyo, Japan). The percent of wound healing was calculated using ImageJ 1.44p software (National Institutes of Health, Bethesda, Maryland, USA).

### Transwell invasion assay

Twenty-four-well Transwell chambers with an 8- $\mu$ m pore size (Corning, New York, USA) were used to evaluate the cell invasion potential. SW954 cells were transfected with different plasmids. Twenty-four hours after transfection, the cells were harvested. Transfected or blank SW954 cells ( $2 \times 10^5$  cells/well) were seeded onto Matrigel-coated membranes in the upper chamber containing serum-free RPMI 1640 medium. The bottom chamber contained RPMI 1640 medium with 20% FBS. Cells were incubated at 37 °C with 5% CO<sub>2</sub> for 24 h, after which the upper layer of the chamber was scrubbed with a sterile cotton swab to remove any remaining cells, and then the bottom of the chamber was fixed with 4% paraformaldehyde and stained with 0.1% crystal violet (Beyotime, Shanghai, China). Cells that invaded through the membrane were counted and photographed in five random fields of each chamber using an Eclipse NI microscope (Nikon, Tokyo, Japan), and then the average number of invasive cells was calculated. Each experiment was performed in triplicate.

### Statistical analysis

The data are presented as means  $\pm$  standard deviation (SD). All statistical analyses were performed using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Differences among data were evaluated using ANOVA for multiple-group comparisons and Student's *t*-test for two-group comparisons, whereas the correlations between PCBP1-AS1 or TRAF5 mRNA expression and the clinicopathological characteristics of patients with VSCC were analyzed using Fisher's exact test. Graph Pad Prism<sup>®</sup> version 5.01 software (GraphPad Software Inc., San Diego, California, USA)

was used to build statistical histograms. Differences were considered statistically significant at a P value <0.05.

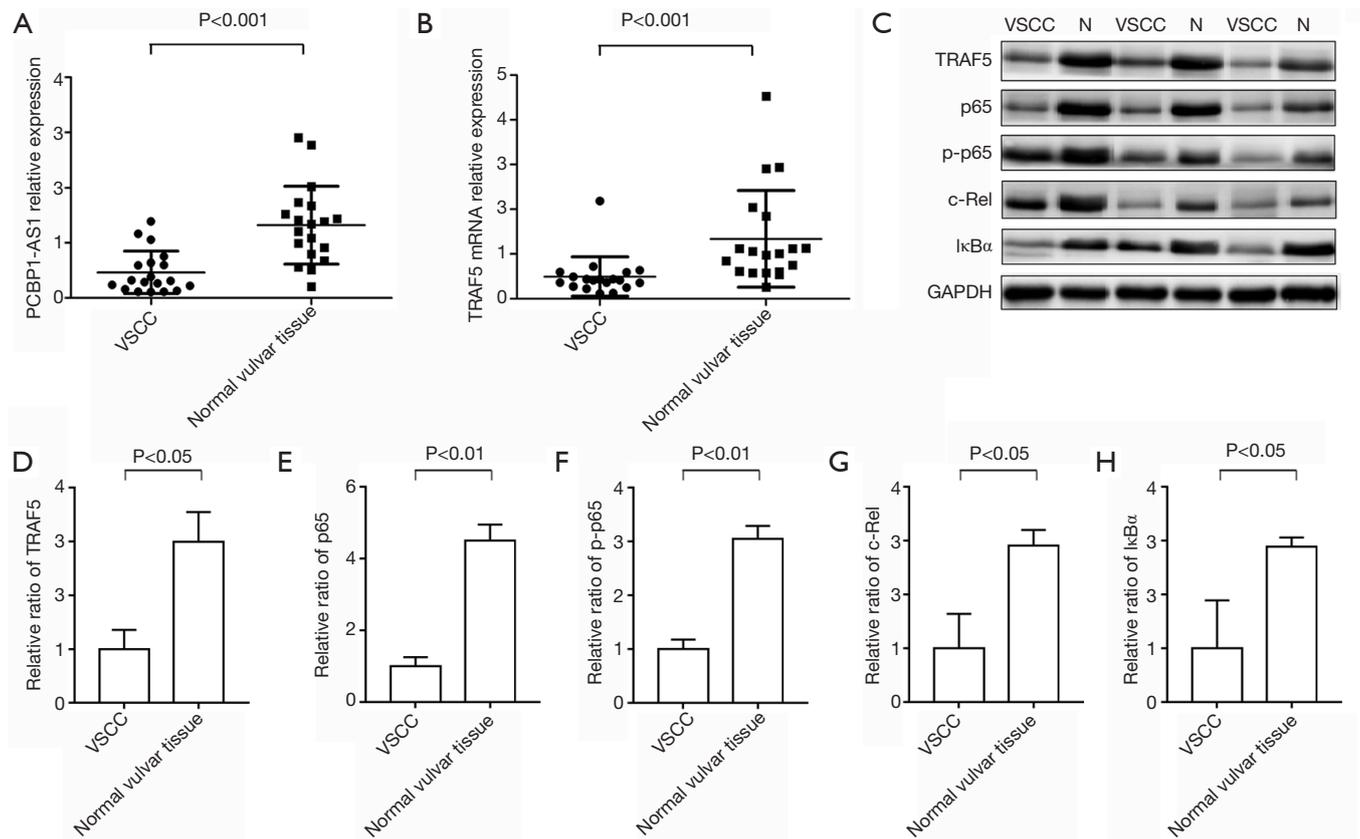
## Results

### *The expression of PCBP1-AS1, TRAF5 and NF- $\kappa$ B pathway proteins are downregulated in VSCC tissues*

To determine the levels of PCBP1-AS1, TRAF5 and NF- $\kappa$ B pathway proteins in VSCC tissues, qRT-PCR was performed to evaluate the levels of PCBP1-AS1 and TRAF5 mRNA and immunoblotting was used to evaluate the expressions of TRAF5, p65, p-p65, c-Rel, and I $\kappa$ B $\alpha$  protein in 19 paired VSCC tissue samples and their corresponding adjacent normal vulvar tissue. As shown in *Figure 1A*, the expression of PCBP1-AS1 in VSCC tissues ( $0.465 \pm 0.384$ ) was significantly downregulated compared with the corresponding adjacent normal vulvar tissue ( $1.321 \pm 0.707$ ,  $P < 0.001$ ). As shown in *Figure 1B*, the expression level of TRAF5 mRNA in VSCC tissues ( $0.495 \pm 0.439$ ) was also markedly decreased compared with the corresponding adjacent normal vulvar tissue ( $1.336 \pm 1.081$ ,  $P < 0.001$ ). Additionally, TRAF5 protein expression in VSCC tissues was significantly lower than normal vulvar tissue ( $P = 0.012$ ) (*Figure 1C,D*), which was similar with p65 ( $P < 0.01$ ) (*Figure 1E*), p-p65 ( $P = 0.008$ ) (*Figure 1F*), c-Rel ( $P = 0.016$ ) (*Figure 1G*) and I $\kappa$ B $\alpha$  proteins ( $P = 0.032$ ) (*Figure 1H*).

### *Correlation between the expression of PCBP1-AS1 and TRAF5 mRNA and clinicopathological characteristics of patients with VSCC*

To identify the clinical relevance of PCBP1-AS1 and TRAF5 mRNA expression in VSCC, the correlation between PCBP1-AS1 and TRAF5 mRNA expression and clinicopathological parameters such as age, tumor size, tumor differentiation, FIGO stage, lymph node metastasis, distant metastasis, and squamous cell carcinoma antigen (SCC-Ag) was examined. Using the results of the qRT-PCR assay to establish median values (M) of the relative expression levels of PCBP1-AS1 and TRAF5 mRNA in VSCC tissues as a boundary, we divided the 19 cases into a high expression group and low expression group. The median values were 0.309 for PCBP1-AS1 ( $M = 0.309$ ) and 0.414 for TRAF5 mRNA ( $M = 0.414$ ).  $P < 0.05$  was considered statistically significant. As shown in *Table 1*, PCBP1-AS1 downregulation in VSCC tissues was significantly correlated with the tumor size ( $P = 0.023$ ), tumor differentiation



**Figure 1** The decreased expression of PCBP1-AS1, TRAF5, and NF- $\kappa$ B pathway proteins in VSCC and normal tissues. Relative expression levels of PCBP1-AS1 (A) and TRAF5 mRNA (B) in VSCC tissue and corresponding adjacent normal vulvar tissue. (C) Immunoblot results of TRAF5, p65, p-p65, c-Rel, and I $\kappa$ B $\alpha$  protein expression in VSCC tissues and corresponding adjacent normal vulvar tissue. (D-H) Relative expression levels of TRAF5, p65, p-p65, c-Rel, and I $\kappa$ B $\alpha$  protein in VSCC tissues and corresponding adjacent normal vulvar tissues. PCBP1-AS1, poly C binding protein-1 antisense RNA1; TRAF5, tumor necrosis factor receptor associated factor 5; NF- $\kappa$ B, nuclear factor kappa B; VSCC, vulvar squamous cell carcinomas.

( $P=0.020$ ), FIGO stage ( $P=0.020$ ), lymph node metastasis ( $P=0.020$ ), distant metastasis ( $P=0.033$ ) and SCC-Ag ( $P=0.033$ ) but was not closely related to the patient's age ( $P=0.303$ ). Decreased TRAF5 mRNA expression was markedly correlated with the FIGO stage ( $P=0.020$ ) and lymph node metastasis ( $P=0.001$ ) but not with other clinical characteristics.

#### PCBP1-AS1 regulates the activity of the NF- $\kappa$ B pathway via TRAF5

To evaluate whether PCBP1-AS1 regulates the activity of the NF- $\kappa$ B pathway via TRAF5, the expression of PCBP1-AS1 and TRAF5 in SW954 cells was altered via transfection. The levels of PCBP1-AS1 and TRAF5 mRNA

were detected by qRT-PCR, and the expressions of TRAF5 protein and NF- $\kappa$ B pathway proteins, including p65, p-p65, c-Rel, and I $\kappa$ B $\alpha$  protein, were analyzed by immunoblotting. As shown in *Figure 2A*, compared with the blank control group, PCBP1-AS1 level was markedly increased ( $P < 0.01$ ) in the PCBP1-AS1-over group. Simultaneously, TRAF5 mRNA expression was significantly upregulated ( $P=0.008$ ). In the TRAF5-RNAi-0 group (*Figure 2B*), TRAF5 mRNA expression was significantly downregulated ( $P=0.004$ ), but the PCBP1-AS1 expression level was not markedly altered ( $P > 0.05$ ). In the PCBP1-AS1-over + TRAF5-RNAi-0 group, no obvious change was noted in PCBP1-AS1 expression compared with PCBP1-AS1-over group ( $P > 0.05$ ), whereas the expression of TRAF5 mRNA was significantly higher than TRAF5-RNAi-0 group ( $P < 0.01$ ).

**Table 1** The correlations between PCBP1-AS1 and TRAF5 mRNA expression and clinicopathological characteristics of patients with VSCC

Clinicopathological characteristics	Case number	Expression level of PCBP1-AS1			Expression level of TRAF5 mRNA		
		High	Low	P value	High	Low	P value
Age/year				0.303			0.582
≤50	4	3	1		1	3	
>50	15	6	9		8	7	
Tumor size (d/cm)				0.023			0.179
≤4	9	7	2		6	3	
>4	10	2	8		3	7	
Tumor differentiation				0.020			0.170
Well and moderately	11	8	3		7	4	
Poorly	8	1	7		2	6	
FIGO stage				0.020			0.020
I-II	7	6	1		6	1	
III-IV	12	3	9		3	9	
Lymph node metastasis				0.020			0.001
Yes	12	3	9		2	10	
No	7	6	1		7	0	
Distant metastasis				0.033			0.303
Yes	5	0	5		1	4	
No	14	9	5		8	6	
SCC-Ag (μg/L)				0.033			0.582
≤4	4	4	0		1	3	
>4	15	5	10		8	7	

PCBP1-AS1, poly C binding protein-1 antisense RNA1; TRAF5, tumor necrosis factor receptor associated factor 5; VSCC, vulvar squamous cell carcinomas.

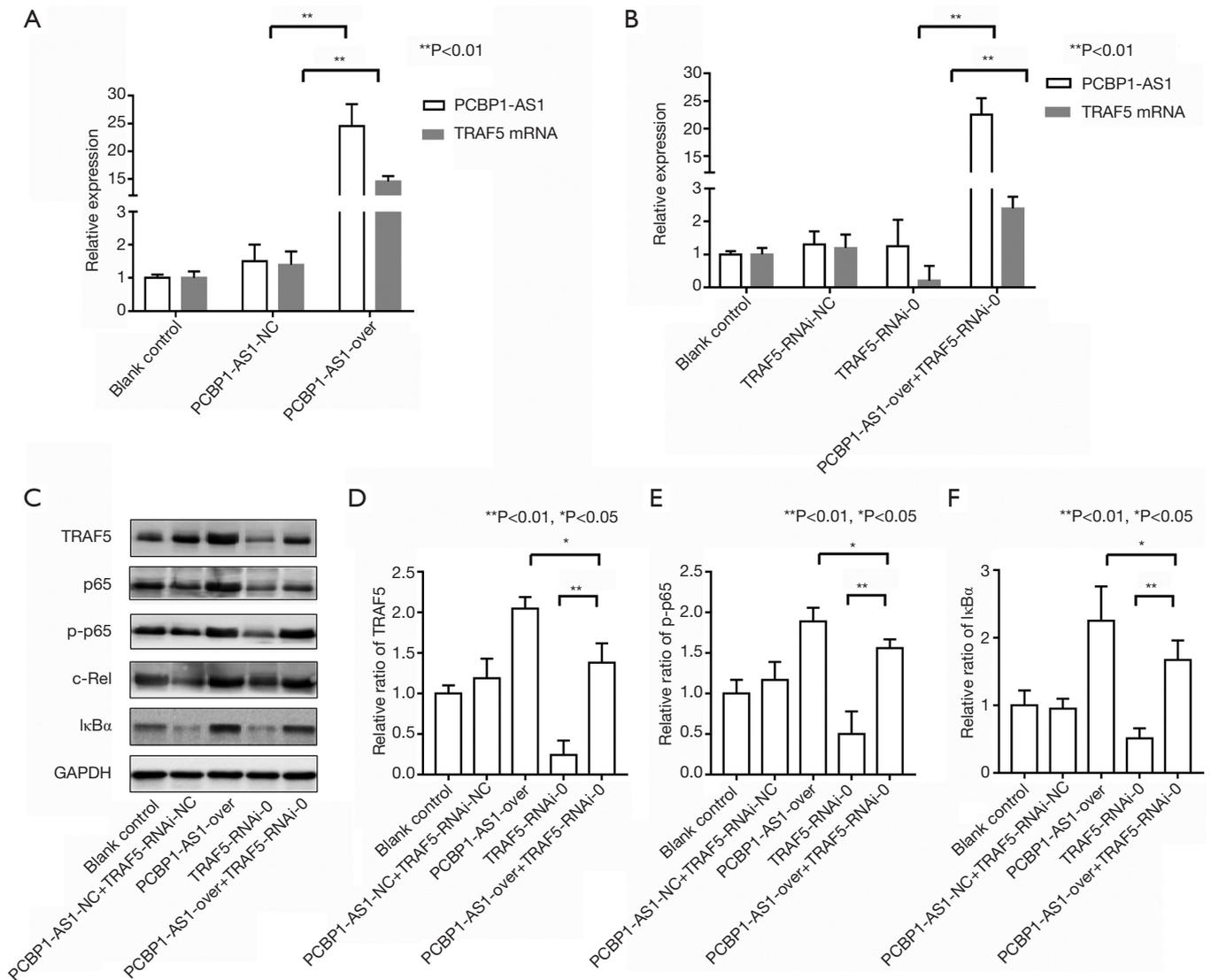
Consistently with TRAF5 mRNA alteration, the expressions of TRAF5 and NF-κB pathway proteins were changed in different transfection groups (*Figure 2C,D,E,F*).

Thus, the results indicated that there were correlation between the expressions of PCBP1-AS1, TRAF5 and NF-κB pathway proteins.

#### ***Abnormal expression of PCBP1-AS1 influences SW954 cell proliferation and apoptosis by regulating TRAF5-mediated activation of the NF-κB signaling pathway***

To investigate the biological effects of PCBP1-AS1 on VSCC, the experimental groups of SW954 cells with altered expression PCBP1-AS1 and TRAF5 were treated

with either an NF-κB signaling pathway activator (TNF-α) or inhibitor (BAY11-7082), and then the CCK-8 assay and Annexin V-FITC & PI staining were conducted to assess cell proliferation and apoptosis, respectively. As shown in *Figure 3A*, the proliferation of SW954 cells in the PCBP1-AS1-NC + TRAF5-RNAi-NC group was not significantly changed ( $P>0.05$ ) beginning at 18 h after transfection compared with blank control group. However, cell proliferation in the PCBP1-AS1-over group was significantly decreased ( $P=0.005$ ), with proliferation inhibition rates of 34.04%, 32.08%, 29.79%, and 28.85% at 18, 24, 36 and 48 h, respectively. Compared with the blank control group, the proliferation of SW954 cells in the TRAF5-RNAi-0 group was markedly accelerated

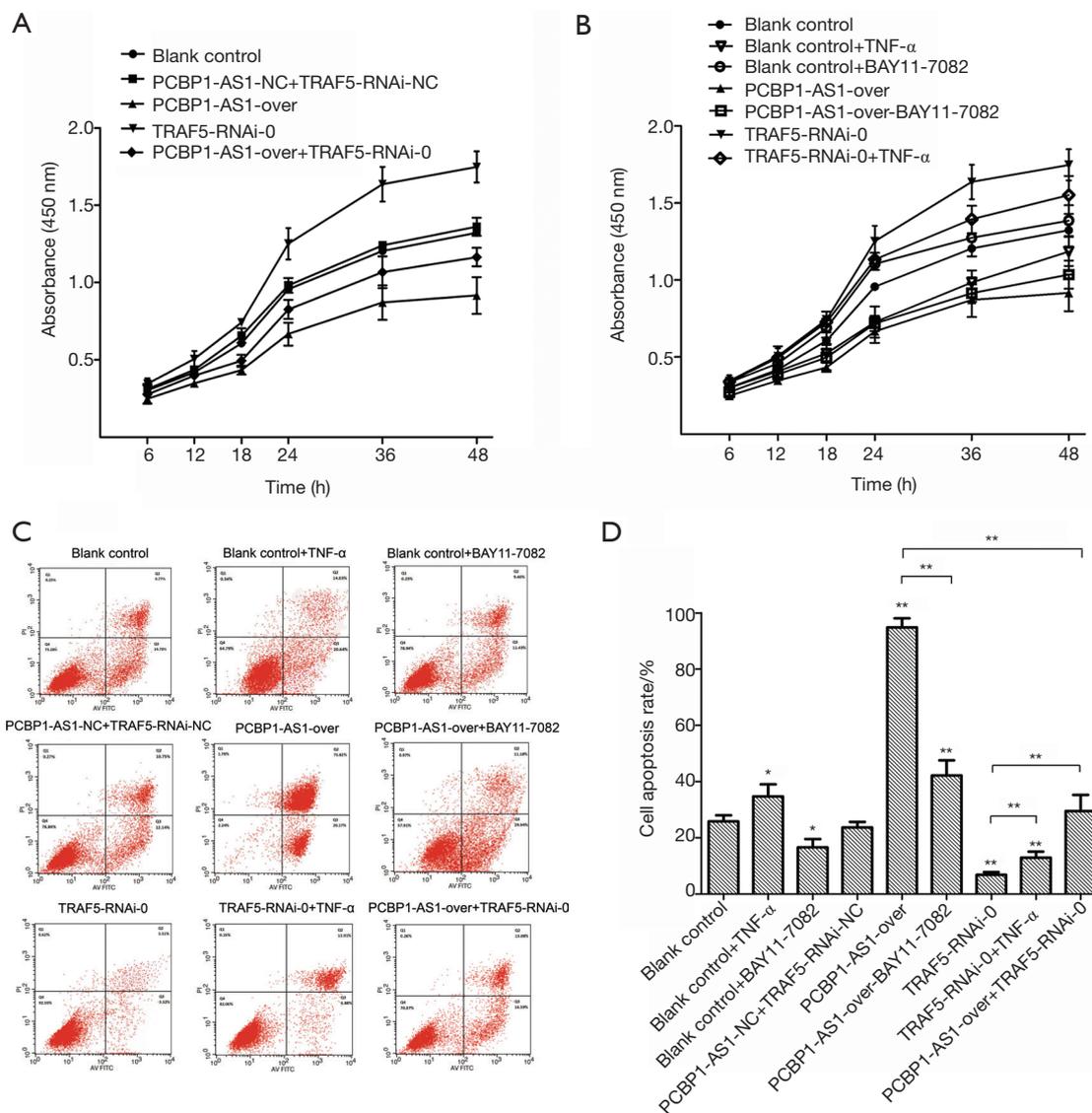


**Figure 2** PCBP1-AS1 regulates NF- $\kappa$ B pathway activity via TRAF5. (A) PCBP1-AS1 promotes expression of TRAF5 mRNA in SW954 cells. (B) TRAF5 can't regulate expression of PCBP1-AS1 in SW954 cells. (C) PCBP1-AS1 regulates NF- $\kappa$ B pathway activity via TRAF5 by immunoblot results. (D-F) Relative expression levels of TRAF5 (D), p-p65 (E) and I $\kappa$ B $\alpha$  (F) protein in blank control, PCBP1-AS1-NC + TRAF5-RNAi-NC, PCBP1-AS1-over, TRAF5-RNAi-0, and PCBP1-AS1-over + TRAF5-RNAi-0 groups. PCBP1-AS1, poly C binding protein-1 antisense RNA1; TRAF5, tumor necrosis factor receptor associated factor 5; NF- $\kappa$ B, nuclear factor kappa B.

( $P=0.005$ ); the cell proliferation inhibition rates at 18, 24, 36, and 48 h were 12.92%, 27.49%, 31.80%, and 28.34%, respectively. Compared with the PCBP1-AS1-over group, the proliferation of SW954 cells in the PCBP1-AS1-over + TRAF5-RNAi-0 group was significantly accelerated ( $P=0.009$ ) but was drastically decreased compared with the TRAF5-RNAi-0 group ( $P<0.01$ ). As shown in *Figure 3B*, compared with the blank control group, the proliferation of SW954 cells in the blank control + TNF- $\alpha$  group was

slower ( $P=0.023$ ), while that of the blank control + BAY11-7082 group was faster ( $P=0.047$ ). The proliferation of SW954 cells in the PCBP1-AS1-over + BAY11-7082 group was faster than in the PCBP1-AS1-over group ( $P=0.038$ ). Compared with the TRAF5-RNAi-0 group, the proliferation of SW954 cells in the TRAF5-RNAi-0 + TNF- $\alpha$  was slower ( $P=0.008$ ).

The rate of apoptosis in each experimental group is shown in *Figure 3C,D*. Compared with the blank control



**Figure 3** Abnormal expression of PCBP1-AS1 influences SW954 cell proliferation and apoptosis by regulating TRAF5-mediated regulation of the NF-κB signaling pathway. (A) CCK-8 assay of proliferative ability of SW954 cells in the blank control, PCBP1-AS1-NC + TRAF5-RNAi-NC, PCBP1-AS1-over, TRAF5-RNAi-0, and PCBP1-AS1-over + TRAF5-RNAi-0 groups. (B) CCK-8 assay of SW954 cells in the blank control, blank control + TNF-α, blank control + BAY11-7082, PCBP1-AS1-over, PCBP1-AS1-over + BAY11-7082, TRAF5-RNAi-0, and TRAF5-RNAi-0 + TNF-α groups. (C,D) Annexin V-FITC & PI immunofluorescence assay to detect SW954 cell apoptosis (C) and the apoptosis levels (D) in each experimental group. \*P<0.05, \*\*P<0.01. PCBP1-AS1, poly C binding protein-1 antisense RNA1; TRAF5, tumor necrosis factor receptor associated factor 5; NF-κB, nuclear factor kappa B.

group, the rate of apoptosis cells in the PCBP1-AS1-NC + TRAF5-RNAi-NC group was relatively unchanged while cells in the PCBP1-AS1-over group experienced significantly more apoptosis (P<0.01). Apoptosis was decreased in the TRAF5-RNAi-0 group compared with the blank control (P=0.007). Compared with the PCBP1-AS1-

over group, the rate of apoptosis in the PCBP1-AS1-over + TRAF5-RNAi-0 group was drastically decreased (P=0.002) but was increased compared with the TRAF5-RNAi-0 group (P=0.008). Furthermore, compared with the blank control group, the rate of apoptosis in the blank control + TNF-α cells was increased (P=0.046) but was decreased

in the blank control + BAY11-7082 cells ( $P=0.042$ ). The rate of apoptosis in the PCBP1-AS1-over + BAY11-7082 group was markedly lower than that in the PCBP1-AS1-over group ( $P=0.006$ ). Conversely, apoptosis in the TRAF5-RNAi-0 + TNF- $\alpha$  group was increased compared with that in the TRAF5-RNAi-0 group ( $P=0.004$ ).

### ***PCBP1-AS1 influences SW954 cell migration and invasion by regulating TRAF5 in an NF- $\kappa$ B-independent manner***

To evaluate the effects of PCBP1-AS1 on migration and invasion behavior of VSCC, we performed the wound healing assay and Transwell invasion assay in SW954 cells with altered expression of PCBP1 and TRAF5. As shown in *Figure 4A*, at 48 hours after *in vitro* wound induction, SW954 cells in the blank control, blank control + TNF- $\alpha$ , blank control + BAY11-7082, and PCBP1-AS1-NC + TRAF5-RNAi-NC groups had covered approximately 5/8 of the approximately 2-mm wide scratch, whereas cells in the PCBP1-AS1-over and PCBP1-AS1-over + BAY11-7082 groups covered only approximately 1/4 of the scratch. By contrast, cells in the TRAF5-RNAi-0 and TRAF5-RNAi-0 + TNF- $\alpha$  groups had nearly covered all of the scratch. Cells in the PCBP1-AS1-over + TRAF5-RNAi-0 group were covered nearly 1/2 of the scratch.

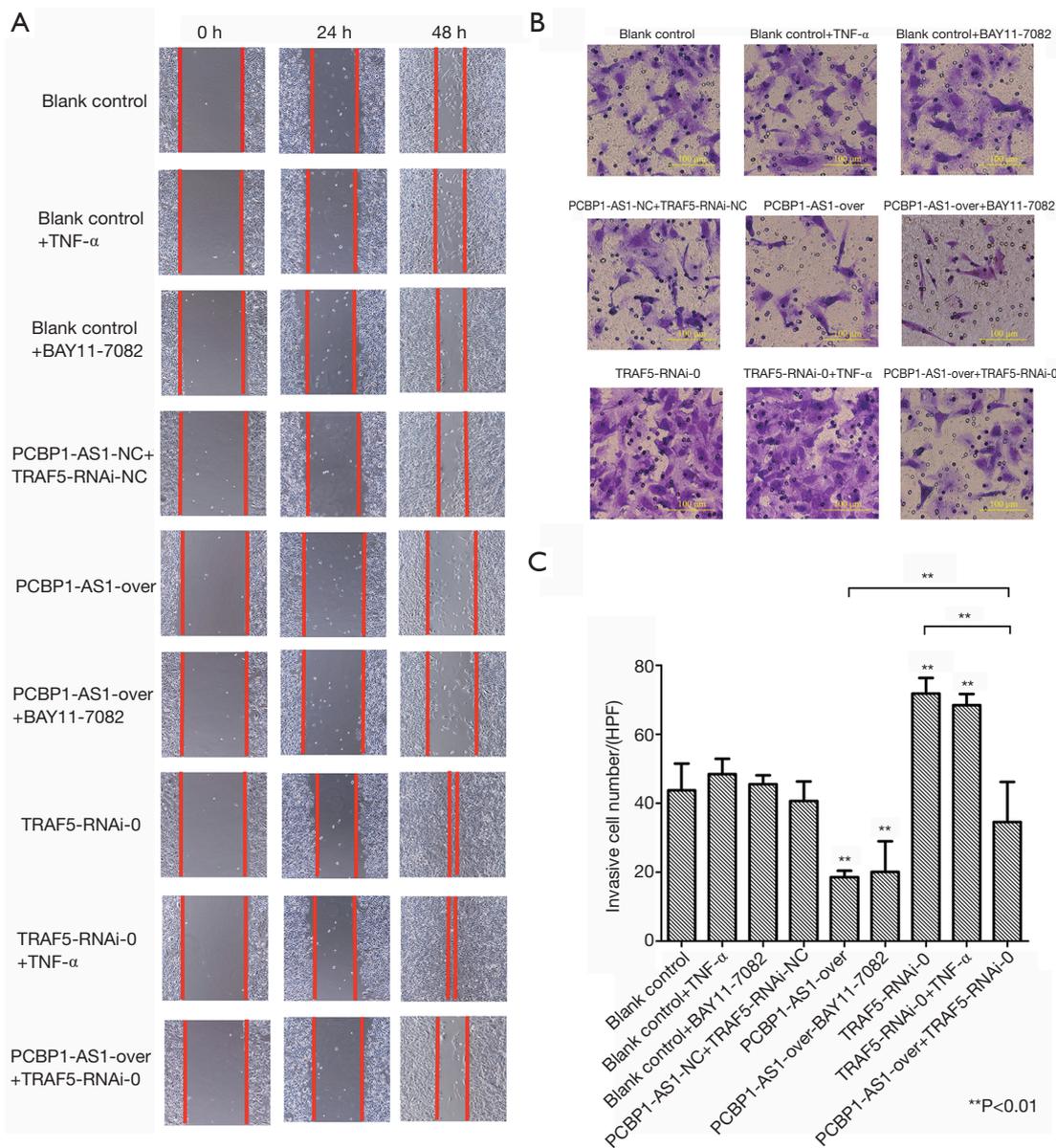
The average number of invasive cells in each experimental group is shown in *Figure 4B,C*. The Transwell assay was conducted, and the number of invasive cells was counted at the 24-h time point. No significant difference was found in the number of invasive cells in the PCBP1-AS1-NC + TRAF5-RNAi-NC group compared with the blank control group. However, the average number of invasive cells in the PCBP1-AS1-over group was significantly decreased ( $P=0.006$ ) compared with the blank control group, and the number of invasive cells in the TRAF5-RNAi-0 group was greatly increased ( $P=0.009$ ) compared with the blank control group. Compared with cells in the PCBP1-AS1-over group, the average number of invasive cells in the PCBP1-AS1-over + TRAF5-RNAi-0 group was increased ( $P=0.009$ ), but was decreased compared with the number of cells in the TRAF5-RNAi-0 group ( $P=0.005$ ). Furthermore, compared with the blank control group, no apparent change was noticed in the average number of invasive cells in the blank control + TNF- $\alpha$  group and blank control + BAY11-7082 group ( $P>0.05$ ). No significant difference was found in the number of invasive cells between the TRAF5-RNAi-0 group and TRAF5-

RNAi-0 + TNF- $\alpha$  group ( $P>0.05$ ). Similarly, no obvious difference was found in the average number of invasive cells in the PCBP1-AS1-over group compared with that in the PCBP1-AS1-over + BAY11-7082 group ( $P>0.05$ ).

### **Discussion**

Abnormally expressed lncRNA has been identified in various diverse tumor types (5-8). Moreover, abnormal expression of lncRNA is closely related to the proliferative, migratory, invasive, and metastatic abilities of tumor cells and regulation of apoptosis (9-11). However, the expression lncRNAs in VSCC remains to be fully elucidated. In our study, we provide evidence that PCBP1-AS1 is aberrantly downregulated and plays a tumor suppressor role in the progression of VSCC. We demonstrated that PCBP1-AS1 positively regulated the expression of TRAF5. Moreover, PCBP1-AS1 exerts the biological effects on SW954 cells via TRAF5-mediated regulation of the NF- $\kappa$ B pathway. Therefore, our study provides a theoretical basis to utilize PCBP1-AS1 in the diagnosis and treatment of VSCC.

Our group has validated the differential expression of HOAIR, MALAT1, MEG3, NEAT1, MIR31HG, and LINC00478 in VSCC and corresponding adjacent normal vulvar tissues (3). Based on the microarray results, we determined that the expression of PCBP1-AS1 was significantly downregulated in VSCC. Presently, only two published reports regarding PCBP1-AS1 are available worldwide. In May 2017, Feng *et al.* performed transcriptome analysis of oral squamous cell carcinoma compared with healthy oral mucosa; the authors found that PCBP1-AS1 was significantly differentially expressed (12). In the same year, in July, Mo *et al.* identified a functional lncRNA-mRNA co-expression module between PCBP1-AS1 and target gene mRNA in human peripheral blood mononuclear cells (13). In this study, we measured the relative expression level of PCBP1-AS1 and TRAF5 mRNA in VSCC tissues using qRT-PCR and analyzed the correlation between the expression level of PCBP1-AS1 and TRAF5 mRNA and clinicopathological characteristics of patients with VSCC. Our results indicated that the expression level of PCBP1-AS1 and TRAF5 mRNA in VSCC tissues was significantly downregulated compared with that in corresponding adjacent normal vulvar tissues. PCBP1-AS1 expression was closely related to the tumor size, tumor differentiation, FIGO stage, lymph node metastasis, distant metastasis, and SCC-Ag, while TRAF5 mRNA expression was significantly correlated with the



**Figure 4** PCBP1-AS1 influences SW954 cell migration and invasion by regulating TRAF5 in an NF- $\kappa$ B-independent manner. (A) Wound healing assay to detect the migration ability in SW954 cells in the blank control, blank control + TNF- $\alpha$ , blank control + BAY11-7082, PCBP1-AS1-over, PCBP1-AS1-over + BAY11-7082, TRAF5-RNAi-0, and TRAF5-RNAi-0 + TNF- $\alpha$  groups. (B,C) Transwell invasion assay (B) and average number of invasive cells (C) to detect the invasion ability in each experimental group. PCBP1-AS1, poly C binding protein-1 antisense RNA1; TRAF5, tumor necrosis factor receptor associated factor 5; NF- $\kappa$ B, nuclear factor kappa B.

FIGO stage and lymph node metastasis. These results suggest that PCBP1-AS1 and TRAF5 mRNA might be used as indicators for the diagnosis and prognosis of VSCC.

NF- $\kappa$ B, a family of transcription factor proteins, consists of five subunits: c-Rel (Rel), p65 (RelA, NF- $\kappa$ B3), RelB, p50 (NF- $\kappa$ B1), and p52. The five subunits have Rel

homology domains and, thus, can form homo- or heterodimers, which bind to I $\kappa$ B at rest to form complexes that are present in the cytoplasm in an inactive state. In the classical pathway of activation, after a specific stimulus, I $\kappa$ B $\alpha$  is phosphorylated and ubiquitinated and is degraded by the proteasome; thus, it is dissociated from NF- $\kappa$ B. After

being phosphorylated, NF- $\kappa$ B enters the nucleus and binds to a fixed nucleotide sequence in the promoter region of the target gene to regulate the transcription of the target gene (14,15). The NF- $\kappa$ B signaling pathway is thought to play a dual role in the progression of cancer (15-18). Studies have shown that activation of the NF- $\kappa$ B/p65 pathway promotes the progression of breast cancer, colorectal cancer, and head and neck carcinoma (19-21), while the NF- $\kappa$ B/c-Rel pathway is activated in breast and pancreatic cancers (22,23). However, other studies have shown that the NF- $\kappa$ B/p65 pathway is involved in the apoptosis of tumor cells and that the inhibition of the NF- $\kappa$ B/p65 pathway leads to the rapid development of skin cancer (24,25). The NF- $\kappa$ B/c-Rel pathway also plays a tumor suppressor role in lymphomas and gastric carcinomas (26,27). The role of TRAF5-mediated regulation of the NF- $\kappa$ B signaling pathway in inflammation has been widely recognized, and its role in the progression of tumors has also been described (4,28). In our study, these results indicated that the expression of TRAF5, p65, p-p65, c-Rel, and I $\kappa$ B $\alpha$  protein were all significantly downregulated in VSCC tissue. Furthermore, activation of the TRAF5-mediated NF- $\kappa$ B/p65 pathway and NF- $\kappa$ B/c-Rel pathway in normal vulvar tissue and a corresponding decrease in TRAF5 protein expression in VSCC tissue, leading to inhibition of the NF- $\kappa$ B/p65 pathway, with decreased protein expression of p65, I $\kappa$ B $\alpha$ , and p-p65. Additionally, the NF- $\kappa$ B/c-Rel pathway is inhibited in VSCC tissue, resulting in decreased expression of c-Rel protein. Our results demonstrated that TRAF5 mRNA was increased when PCBP1-AS1 expression was upregulated, while PCBP1-AS1 expression was unchanged after TRAF5 expression was downregulated. Upregulation of PCBP1-AS1 significantly increased NF- $\kappa$ B activity, while downregulation of TRAF5 markedly decreased NF- $\kappa$ B activity (Figure S1). Thus, aberrant expression of PCBP1-AS1 can regulate TRAF5 expression both at the level of transcription and translation, and mediate changes in NF- $\kappa$ B pathway activity.

Upregulation of PCBP1-AS1 significantly increased apoptosis and inhibited cell proliferation, migration and invasion; meanwhile, knockdown of TRAF5 significantly inhibited apoptosis and enhanced cell proliferation, migration, and invasion. Moreover, activation or inhibition of the NF- $\kappa$ B signaling pathway suppressed or enhanced proliferation and increased or reduced apoptosis. However, activation and inhibition of the NF- $\kappa$ B signaling pathway had no significant effect on the migration and

invasion abilities of the cells. These results suggest that PCBP1-AS1 may regulate the proliferation and apoptosis of VSCC SW954 cells via TRAF5-mediated regulation of the NF- $\kappa$ B signaling pathway, influencing the progression of VSCC, and may play a role in the migration and invasion ability of SW954 cells via an NF- $\kappa$ B signaling pathway-independent mechanism.

Moreover, some limitations exist in our study. First, our sample size was limited, and more VSCC tissues are needed for further study. Second, the detailed molecular mechanism by which PCBP1-AS1 regulated TRAF5 also remains to be fully elucidated. Finally, although PCBP1-AS1 and TRAF5 can regulate the migration and invasion of VSCC, which signaling pathways involved remains unclear in our study.

## Conclusions

This study demonstrates that, as a tumor suppressor gene, PCBP1-AS1 plays an important role in the progression of VSCC. PCBP1-AS1 can positively regulate the TRAF5-mediated NF- $\kappa$ B signaling pathway to affect the proliferation and apoptosis of VSCC SW954 cells. Additionally, PCBP1-AS1 can influence the migration and invasion of SW954 cells by regulating TRAF5, but not through the NF- $\kappa$ B signaling pathway.

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## Footnote

*Conflicts of Interest:* All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/tcr.2019.08.11>). The authors have no conflicts of interest to declare.

*Ethical Statement:* The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as

revised in 2013). Patients in this study all provided written informed consent. This study was approved by the Ethics Committee of Shengjing Hospital affiliated with China Medical University.

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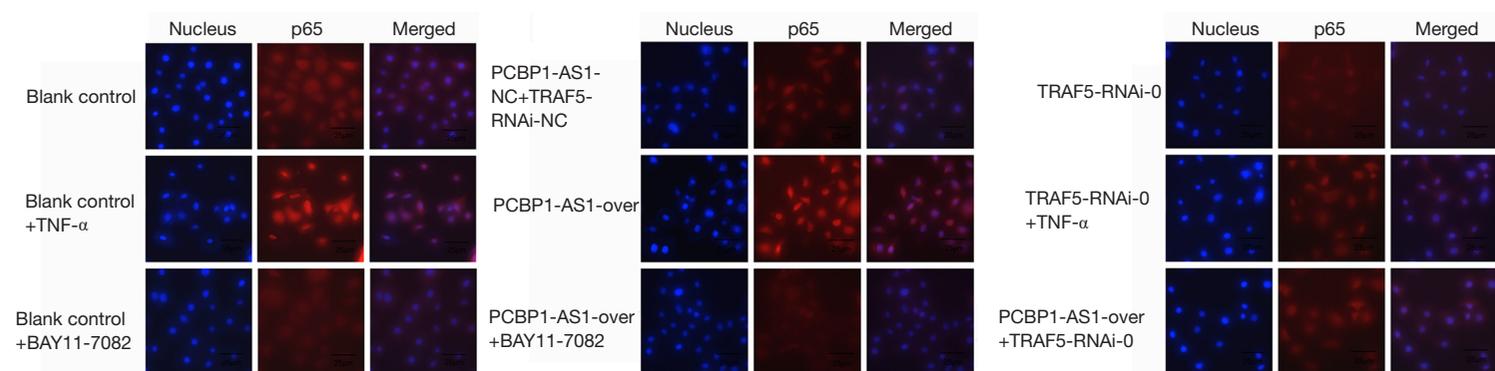
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## Supplementary



**Figure S1** NF- $\kappa$ B activity in SW954 cells in the blank control, blank control + TNF- $\alpha$ , blank control + BAY11-7082, PCBP1-AS1-over, PCBP1-AS1-over + BAY11-7082, TRAF5-RNAi-0, and TRAF5-RNAi-0 + TNF- $\alpha$  groups. Compared with the blank control group, no significant change was found in the amount of nuclear NF- $\kappa$ B subunit p65 (red fluorescence) activated and transported into the nucleus (blue fluorescence) in the PCBP1-AS1-NC + TRAF5-RNAi-NC group. However, in the PCBP1-AS1-over group, compared with the blank control group, the amount of nuclear NF- $\kappa$ B subunit p65 was markedly increased and nuclear NF- $\kappa$ B subunit p65 was significantly decreased in the TRAF5-RNAi-0 group. In the PCBP1-AS1-over + TRAF5-RNAi group, the amount of nuclear NF- $\kappa$ B subunit p65 was less than that in the PCBP1-AS1-over group but more than that in the TRAF5-RNAi-0 group. Compared with the corresponding control groups (blank control group, PCBP1-AS1-over group, TRAF5-RNAi-0 group), the amount of nuclear NF- $\kappa$ B subunit p65 was increased in the activation groups (blank control + TNF- $\alpha$ , TRAF5-RNAi-0 + TNF- $\alpha$ ) but was decreased in the inhibition groups (blank control + BAY11-7082, PCBP1-AS1-over + BAY11-7082). PCBP1-AS1, poly C binding protein-1 antisense RNA1; TRAF5, tumor necrosis factor receptor associated factor 5; NF- $\kappa$ B, nuclear factor kappa B.