

# YAP knockdown inhibits proliferation and induces apoptosis of human prostate cancer DU145 cells

XIN JIN\*, WENCHAO ZHAO\*, PENG ZHOU\* and TIANLI NIU

Department of Urology, The Affiliated Taizhou People's Hospital of Nantong University, Taizhou, Jiangsu 225300, P.R. China

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**Abstract.** The role of yes-associated protein (YAP) in human prostate cancer DU145 cells and its underlying molecular mechanisms were explored in the present study. Initially, the expression levels of YAP were detected in DU145 cells, which revealed that YAP was highly expressed in these cells. To investigate the role of YAP in DU145 cells, a stable YAP-silenced DU145 cell line was generated using YAP-small interfering RNA. Reverse transcription-quantitative polymerase chain reaction and western blotting were performed for mRNA and protein detection, respectively. An MTT assay and flow cytometry were performed to investigate the proliferation and apoptosis of DU145 cells. The results demonstrated that YAP knockdown significantly decreased the proliferative ability of DU145 cells, whereas the percentage of apoptotic cells was markedly increased, compared with the control. In addition, the mRNA and protein expression levels of connective tissue growth factor and cysteine-rich angiogenic factor 61 were notably decreased, the ratio of B-cell lymphoma 2 (Bcl-2)/Bcl-2-associated X protein (Bax) was significantly reduced, and the expression levels of caspase 3 were significantly decreased within YAP-silenced DU145 cells. In conclusion, YAP knockdown reduced the proliferation and induced apoptosis of DU145 cells. Therefore, the gene transcription and protein expression of YAP may be involved in the development of prostate cancer and may be considered a potential target for the treatment of such cancers.

## Introduction

Prostate cancer (PCa) is the most common malignancy that is frequently diagnosed in males, and is a major cause of

cancer-associated mortality worldwide (1). Androgen deprivation therapy is the most effective treatment for advanced PCa; however, most patients with PCa develop castration-resistant prostate cancer (CRPC) due to therapy-associated resistance (2,3). Unfortunately, CRPC remains incurable (2) and the underlying mechanisms of CRPC development are yet to be revealed.

The Hippo signaling pathway, which was originally identified in fruit flies, is involved in tumor development via the regulation of cell proliferation and apoptosis (3-7). The core components of the Hippo signaling pathway within mammals comprise protein kinases, including mammalian sterile 20-like 1 and 2, large tumor suppressor 1 and 2, and the adaptor proteins WW domain-containing protein and Mps one binder 1. The core functions of the Hippo signaling pathway serve tumor-suppressing roles via yes-associated protein (YAP), and TAZ phosphorylation and inactivation. YAP, which is a 65-kDa protein, is an effector protein of the Hippo signaling pathway and is a transcriptional co-activator of numerous transcription factors. Previous studies have confirmed that YAP functions as an oncogenic protein in mammalian cells via promoting cell growth (8-10). Additionally, YAP overexpression has been associated with various human cancers, including PCa, and breast, ovarian, lung, liver and gastric cancer (11-17).

Increasing evidence has suggested the important roles of YAP in regulating PCa cell behavior (18,19). To the best of our knowledge, no previous study has focused on the role of YAP within human PCa DU145 cells. Therefore, the role of YAP in human PCa DU145 cells and the underlying molecular mechanisms were explored in the present study.

## Materials and methods

**Materials.** The human PCa DU145 cells and RWPE-1 cells were obtained from the American Type Culture Collection (Manassas, VA, USA); serum-free keratinocyte medium (K-SFM) was obtained from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA); the RPMI-1640 medium, fetal bovine serum (FBS) and polyvinylidene fluoride (PVDF) membrane were obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Primary antibodies against connective tissue growth factor (CTGF, cat. no. sc-14939) were purchased from Santa Cruz Biotechnology, Inc., Dallas, TX, USA, while, cysteine-rich angiogenic inducer 61 antibodies (CYR61, cat. no. 14476, 1:1,000), B-cell lymphoma 2 antibodies

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*Correspondence to:* Dr Tianli Niu, Department of Urology, The Affiliated Taizhou People's Hospital of Nantong University, 210 Yingchun Road, Taizhou, Jiangsu 225300, P.R. China  
E-mail: niutl0905@163.com

\*Contributed equally

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(Bcl-2, cat. no. 2872, 1:1,000), Bcl-2-associated X protein antibodies (Bax, cat. no. 2774, 1:1,000), caspase 3 antibodies (cat. no. 9662, 1:1,000), GAPDH antibodies (cat. no. 5174, 1:1,000) and secondary antibodies (cat. no. 7076, 1:1,000) were obtained from Cell Signaling Technology Inc., (Danvers, MA, USA); MTT and enhanced chemiluminescence (ECL) Plus reagent were obtained from Eli Lilly & Co., (Indianapolis, IN, USA); Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit was obtained from Vazyme Biotech Co., (Nanjing, China). YAP-small interfering RNA (YAP-siRNA, cat. no. sc-38637), control (Con)-siRNA (cat. no. sc-37007) and the siRNA Transfection Reagent (cat. no. sc-29528) were obtained from Santa Cruz Biotechnology, Inc.

**Cell culture.** Human PCa DU145 cells were cultured in RPMI-1640 supplemented with 10% FBS and 1% penicillin-streptomycin. RWPE-1 cells were cultured in 10 ml complete SFM containing 5 ng/ml recombinant epidermal growth factor (CYT-217, ProSpec-Tany TechnoGene Ltd., East Brunswick, NJ, USA), 50 µg/ml bovine pituitary extract (CC023; [macgene.bion.com.cn/](http://macgene.bion.com.cn/)), 100 IU/ml penicillin and 100 IU/ml streptomycin. Cells were incubated in standard cell culture conditions (5% CO<sub>2</sub>, 95% humidity) at 37°C. Human PCa DU145 cells were passaged every 2-3 days, and RWPE-1 cells were passaged every ~5 days.

**Cell transfection.** Human PCa DU145 cells were plated in a 6-well plate 1 day prior to transfection, the transfection assay was performed once the cells reached 60-70% confluence. DU145 cells (2x10<sup>5</sup> cells/well) were transfected with YAP-siRNA or Con-siRNA (1 µg) using 30 µl transfection reagent plus and incubated at 37°C for 6 h in 5% CO<sub>2</sub> incubator, according to the manufacturer's protocol. A total of 24 h following transfection, the transfected DU145 cells were used for subsequent experiments, and were collected for protein analysis after 72 h incubation.

**RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was extracted from human PCa DU145 and RWPE-1 cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. To determine the mRNA expression levels within the cells, RT was performed using PrimeScript™ RT reagent kit (Takara Bio Inc., Otsu, Japan) according to the manufacturer's protocol. For qPCR, SYBR® Premix Ex Taq™ II (Takara Bio Inc.) was used and all reactions were performed in triplicate with the following conditions: 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 72°C for 30 sec, 78°C for 1.5 min for 35 cycles, after which samples were stored at 4°C. GAPDH was used as an internal control. The 2<sup>-ΔΔC<sub>q</sub></sup> method was performed to analyze the relative amounts of each transcript (20). The qPCR primers are presented in Table I.

**Western blot analysis.** Total cellular protein from human PCa DU145 and RWPE-1 cells was extracted using radioimmuno-precipitation assay buffer (P0013B, Beyotime Biotechnology, Nanjing, China) and SDS-PAGE analysis was performed to separate the total protein. Protein concentration was determined by bicinchoninic protein assay kit. Proteins (25 µg)

were resolved by 10% sodium dodecyl sulfate polyacrylamide gels and were then transferred to a polyvinylidene membrane. The membrane was blocked with 5% skim milk for 1 h at room temperature. Subsequently, the membrane was blotted overnight at 4°C with the following primary antibodies: CTGF (1:1,000 dilution), CYR61 (1:1,000 dilution), Bcl-2 (1:1,000 dilution), Bax (1:1,000 dilution), caspase 3 (1:1,000 dilution) and GAPDH (1:2,000 dilution). The membrane was then incubated with horseradish peroxidase-conjugated anti-mouse and anti-rabbit immunoglobulin G secondary antibodies (1:5,000 dilution) at room temperature for 2 h. Protein bands were observed using enhanced chemiluminescence Plus reagent prior to imaging and analysis. **MTT assay.** Cell proliferation was detected using an MTT assay. YAP-siRNA and Con-siRNA were transfected into DU145 cells. Subsequently, log-phase human PCa DU145 cells were harvested with 0.25% trypsin and seeded in 96-well plates (5x10<sup>3</sup> cells/well). After 24 h incubation at 37°C with 5% CO<sub>2</sub>, MTT was added to the cell culture medium and incubated for 4 h at 37°C. Then DMSO was employed for formazan crystals dissolving. Optical density was detected at 490 nm using a spectrophotometer. Each experiment was repeated in triplicate and data were displayed as the mean ± standard deviation.

**Apoptosis analysis.** To detect alterations in cell apoptosis, an Annexin V-FITC apoptosis detection kit was utilized. DU145 cells were transfected with YAP-siRNA or Con-siRNA for 24 h. Following transfection, cells were rinsed with cold PBS. Cells (5x10<sup>5</sup> cells/well) were then labeled with Annexin V-FITC and propidium iodide, according to the manufacturer's protocol. Flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA) was used for cell apoptosis analysis. Version 2.5 WinMDI (Purdue University Cytometry Laboratories; [www.cyto.purdue.edu/flowcyt/software/Catalog.htm](http://www.cyto.purdue.edu/flowcyt/software/Catalog.htm)) was applied for data analysis. Each test was repeated in triplicate.

**Statistical analysis.** All tests were performed in triplicate. Data are displayed as the mean ± standard deviation. SPSS 17.0 statistical software (SPSS, Inc., Chicago, IL, USA) was applied for all statistical analyses. A Student's t-test was used to analyze the difference between the two groups. P<0.05 was considered to indicate a statistically significant difference.

## Results

**YAP expression in human PCa DU145 cells.** Firstly, the mRNA and protein expression levels of YAP were detected in human PCa DU145 cells via RT-qPCR and western blotting, respectively. As demonstrated in Fig. 1, the mRNA and protein expression levels of YAP were significantly higher in human PCa DU145 cells compared with in normal prostate epithelial RWPE-1 cells. Based on these results, the expression levels of YAP were significantly increased in human PCa DU145 cells.

**mRNA and protein expression levels of YAP in DU145 cells following cell transfection.** To investigate the role of YAP in human PCa DU145 cells, a stable YAP-silenced DU145 cell line was generated using YAP-siRNA. The results of RT-qPCR, compared with the blank group, indicated that the expression levels of YAP within YAP-siRNA-transfected cells

Table I. Primer sequences for polymerase chain reaction.

Gene	Primer sequence (5'-3')
YAP-Forward	ACGTTCATCTGGGACAGCAT
YAP-Reverse	GTTGGGAGATGGCAAAGACA
CTGF-Forward	TTGGCAGGCTGATTTCTAGG
CTGF-Reverse	GGTGCAAACATGTAACCTTTTG
Cyr61-Forward	CCCGTTTTGGTAGATTCTGG
Cyr61-Reverse	GCTGGAATGCAACTTCGG
Bcl-2-Forward	ATGTGTGTGGAGAGCGTCAA
Bcl-2-Reverse	ACAGTTCCACAAAGGCATCC
Bax-Forward	GGCCACCAGCTCTGAGCAGA
Bax-Reverse	GCCACGTGGGCGTCCCAAAGT
Caspase 3-Forward	CTGGTTGGCGTCGCCTTG
Caspase 3-Reverse	GAATCCACTGAGTTTTCAG
GAPDH-Forward	CTTTGGTATCGTGGAAGGACTC
GAPDH-Reverse	GTAGAGGCAGGGATGATGTTCT

Bax, B-cell lymphoma 2-associated X protein; Bcl-2, B-cell lymphoma 2; CTGF, connective tissue growth factor; CYR61, cysteine-rich angiogenic inducer 61; YAP, yes-associated protein.

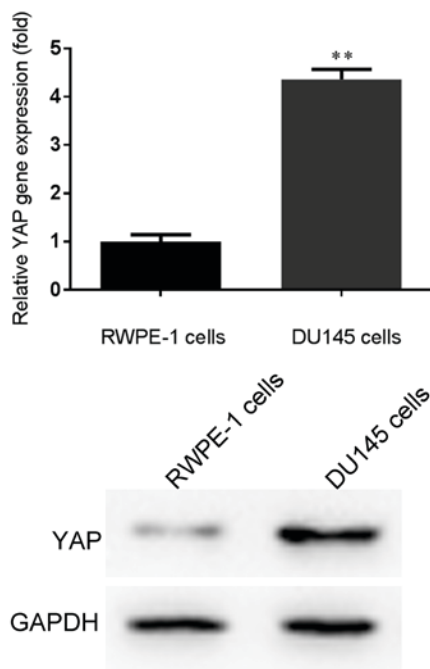


Figure 1. Expression levels of YAP in DU145 and RWPE-1 cells. mRNA and protein expression levels of YAP were determined in DU145 and RWPE-1 cells by reverse transcription-quantitative polymerase chain reaction and western blot analysis, respectively. All data are presented as the mean  $\pm$  standard deviation of three independent experiments. \*\* $P < 0.01$ . YAP, yes-associated protein.

were markedly decreased, whereas Con-siRNA did not affect YAP expression. Reduced YAP protein expression was also revealed by western blotting in the YAP-siRNA group (Fig. 2). These data indicated that YAP-siRNA may effectively inhibit YAP expression.

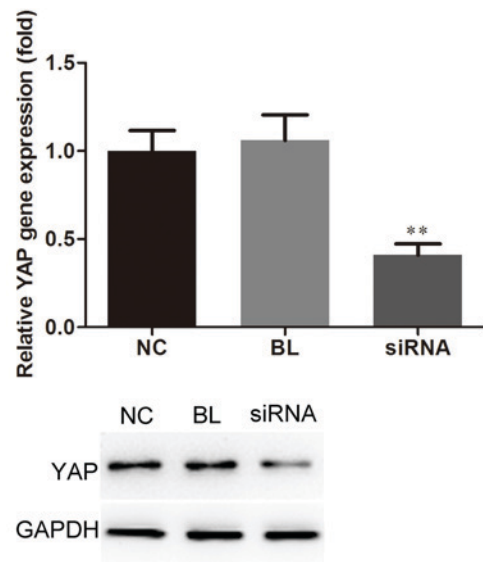


Figure 2. YAP was effectively inhibited by YAP-siRNA. DU145 cells were transfected with YAP-siRNA or Con-siRNA. A total of 24 h post-transfection, the mRNA and protein expression levels of YAP in DU145 cells were determined by reverse transcription-quantitative polymerase chain reaction and western blot analysis, respectively. NC, cells transfected with Con-siRNA; BL, cells without treatment; siRNA, cells transfected with YAP-siRNA. All data are presented as the mean  $\pm$  standard deviation of three independent experiments. \*\* $P < 0.01$  vs. the negative control. BL, blank; NC, negative control; siRNA, small interfering RNA; YAP, yes-associated protein.

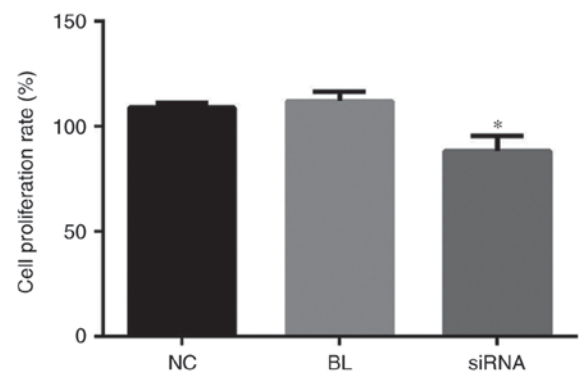


Figure 3. YAP inhibition inhibits the proliferation of DU145 cells. Cell proliferation was analyzed using an MTT assay 24 h after DU145 cells were transfected with YAP-siRNA or Con-siRNA. NC, cells transfected with Con-siRNA; BL, cells without treatment; siRNA, cells transfected with YAP-siRNA. \* $P < 0.05$ . All data are presented as the mean  $\pm$  standard deviation of three independent experiments. BL, blank; NC, negative control; siRNA, small interfering RNA; YAP, yes-associated protein.

*Downregulation of YAP reduces the proliferative ability of DU145 cells.* To investigate the effects of YAP expression on DU145 cell proliferation, YAP-siRNA and Con-siRNA were transfected into DU145 cells, and an MTT assay was performed. The results of the present study demonstrated that compared with the blank and control group, cell proliferation was markedly inhibited in YAP-siRNA-transfected DU145 cells. Furthermore, colony formation ability of cells was measured by MTT assay. The results indicated that YAP knockdown significantly suppressed the DU145 cell colony-forming ability (Fig. 3). These results indicated

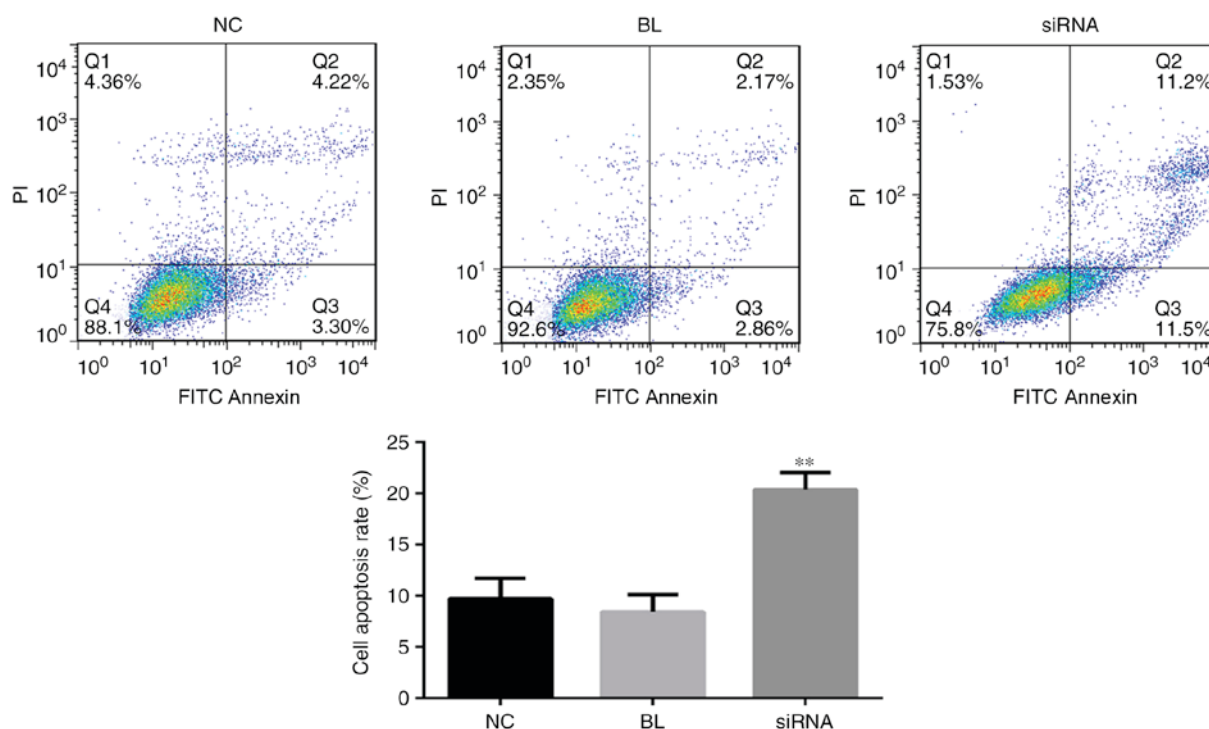


Figure 4. YAP inhibition induces the apoptosis of DU145 cells. A total of 24 h after DU145 cells were transfected with YAP-siRNA or Con-siRNA, flow cytometry was performed to detect cell apoptosis measuring the sum of cell percentage in quadrants Q2 and Q3. NC, cells transfected with Con-siRNA; BL, cells without any treatment; siRNA, cells transfected with YAP-siRNA. All data are presented as the mean  $\pm$  standard deviation of three independent experiments. \*\* $P < 0.01$ . BL, blank; FITC, fluorescein isothiocyanate; NC, negative control; PI, propidium iodide; siRNA, small interfering RNA; YAP, yes-associated protein.

that YAP downregulation reduced the proliferation and colony-forming ability of DU145 cells.

**Downregulation of YAP increases apoptosis of DU145 cells.** The observed growth inhibition in YAP-siRNA-transfected DU145 cells was investigated using an Annexin V-FITC apoptosis detection kit for cell apoptosis via flow cytometry. The results revealed that YAP knockdown significantly induced DU145 cell apoptosis compared with in the control group (Fig. 4).

**Downregulation of YAP alters the expression of CTGF, CYR61, Bcl-2, Bax and caspase 3.** CTGF and CYR61 are downstream genes of YAP in the Hippo signaling pathway, and are regulated by YAP proteins. It has been reported that CTGF and CYR61 serve important roles in promoting cell proliferation, migration and invasion in cancer (21). Following transfection of DU145 cells with YAP-siRNA, the mRNA and protein expression levels of CTGF and CYR61 genes were significantly reduced compared with in Con-siRNA-transfected cells (Fig. 5).

Western blotting and RT-qPCR were performed to further explore the mechanism of YAP knockdown-induced cell apoptosis through the expression levels of the apoptosis-associated proteins Bax, Bcl-2 and caspase 3. The results of the present study suggested that the Bcl-2/Bax ratio was markedly lower in DU145 cells transfected with YAP-siRNA compared with in cells transfected with Con-siRNA. As expected, the expression levels of caspase 3 were notably decreased following YAP-siRNA transfection (Fig. 5).

## Discussion

The major findings of the present study included the high expression of YAP in human PCa DU145 cells compared with in normal prostate epithelial RWPE-1 cells. Silencing of the YAP gene in DU145 cells was associated with reduced proliferative ability and a significant increase in apoptosis. The expression levels of tumor-associated genes, including CTGF, CYR61, Bcl-2, BAX and caspase 3, were also altered in DU145 cells when YAP expression was knocked down. These findings suggested that YAP may function as an oncogene in human PCa.

An imbalance between cell proliferation and apoptosis may result in cancer (22). The Hippo signaling pathway serves important roles in the regulation of tumor cell and tissue growth. YAP, which is a core component of the Hippo-YAP signaling pathway, has been confirmed to be associated with tumor metastasis, grade and stage (23,24). It has also been reported that the function of YAP may be dysregulated in numerous cancers, serving oncogenic and tumor suppressive roles; previous studies have also indicated that YAP depletion may inhibit the growth and metastasis of tumor cells (25-30).

In the present study, YAP expression was detected in human PCa DU145 cells, and the effects of YAP knockdown on the proliferative ability and apoptosis of DU145 cells were determined *in vitro*. The results suggested that YAP was highly expressed in DU145 cells. However, the proliferative ability of cells was reduced and cell apoptosis was increased following YAP downregulation. The underlying mechanism of the regulation of cell behavior via YAP knockdown was



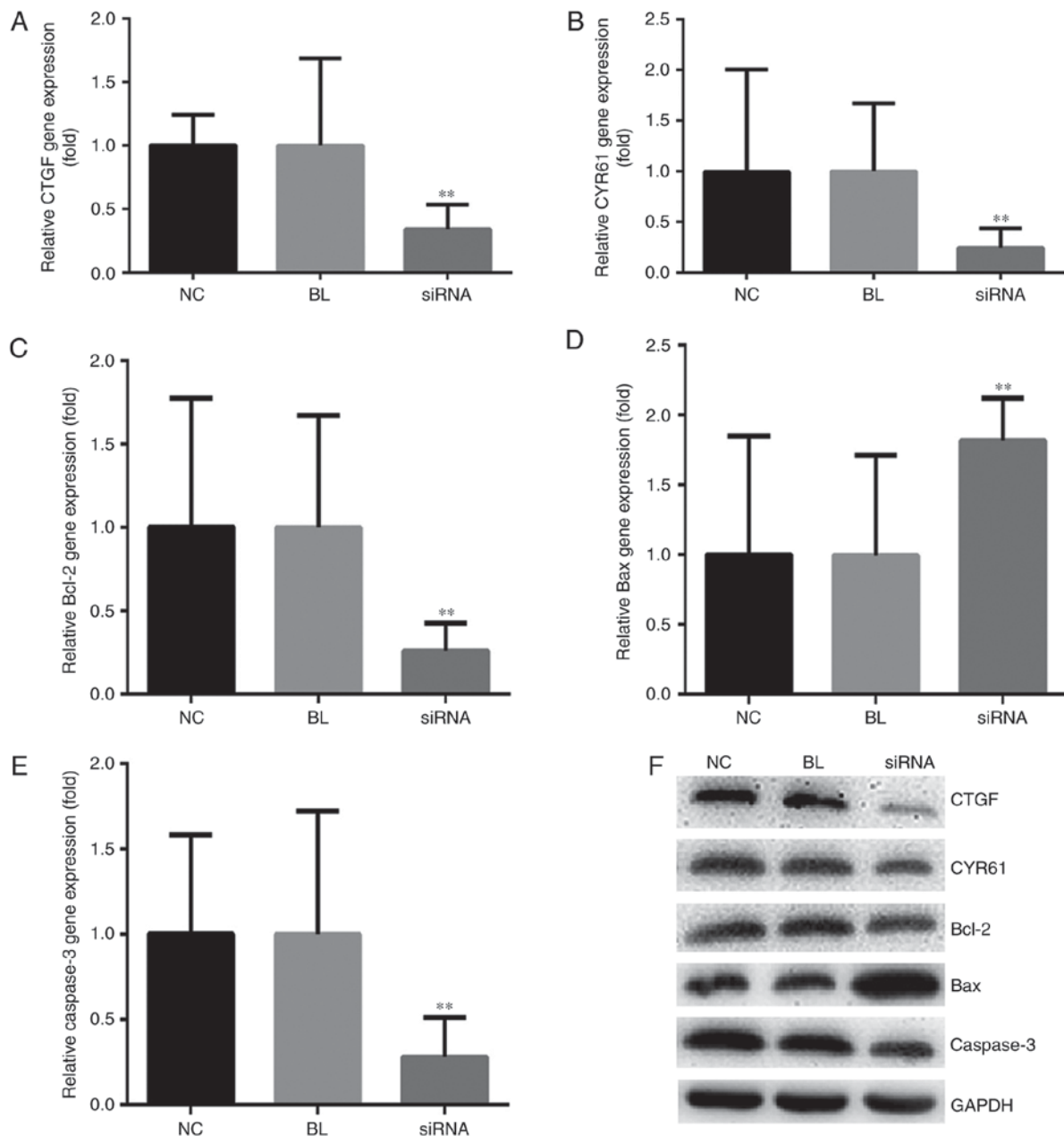


Figure 5. YAP inhibition alters the expression of related genes in DU145 cells. DU145 cells were transfected with YAP-siRNA or Con-siRNA. A total of 24 h after transfection, CTGF, CYR61, Bcl-2, Bax and caspase 3 expression levels were measured using (A-E) reverse transcription-quantitative polymerase chain reaction and (F) western blotting, respectively. NC, cells transfected with Con-siRNA; BL, cells without any treatment; siRNA, cells transfected with YAP-siRNA. \*\* $P < 0.01$ . Bax, B-cell lymphoma 2-associated X protein; Bcl-2, B-cell lymphoma 2; CTGF, connective tissue growth factor; CYR61, cysteine-rich angiogenic inducer 61; siRNA, small interfering RNA; YAP, yes-associated protein.

investigated through the detection of tumor-associated genes. CYR61 and CTGF are matricellular proteins involved in numerous physiological and pathological processes, including carcinogenesis, which serve critical roles in regulating tumor cell growth (31). Following YAP knockdown in DU145 cells, decreased expression levels of CTGF and CYR61 were detected. Furthermore, the expression levels of apoptosis-associated proteins (Bax, Bcl-2 and caspase 3) were analyzed. The data revealed that the Bcl-2/Bax ratio was markedly reduced in YAP-silenced DU145 cells and the expression levels of caspase 3 were significantly decreased compared with in the control group.

In conclusion, these results indicated that YAP knockdown suppressed the proliferation, and induced apoptosis of human PCa DU145 cells. Therefore, the YAP gene may be associated with tumorigenesis and the development of PCa which may serve as a potential future treatment target.

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