

## Puerarin Inhibits Proliferation and Induces Apoptosis by Upregulation of miR-16 in Bladder Cancer Cell Line T24

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Bladder cancer (BC) is a common disease of the urinary system. Puerarin is a flavonoid extracted from *Pueraria lobata*. However, the role of puerarin in BC remains unclear. Hence, this study aimed to investigate the effect of puerarin on BC cells. Cell viability, proliferation, and apoptosis were measured by CCK-8, BrdU assay, and flow cytometry analysis, respectively. The expressions of miR-16, apoptosis-related factors, and the main factors of the NF- $\kappa$ B pathway were analyzed by qRT-PCR and Western blot. In this study, we found that cell viability and proliferation were significantly reduced, cell apoptosis was enhanced, and the mRNA level of miR-16 was upregulated in puerarin-treated T24 cells. Further, silencing of miR-16 inhibited the decrease in cell viability and the increase in apoptosis. The expression of main factors involved in the NF- $\kappa$ B signaling pathway was downregulated in the puerarin group, while miR-16 silencing alleviated these downregulations. More importantly, puerarin deactivated the NF- $\kappa$ B signaling pathway via upregulation of miR-16. Also, miR-16 downregulated COX-2 expression via deactivation of the NF- $\kappa$ B signaling pathway. This study demonstrated that puerarin could inhibit cell proliferation, promote cell apoptosis, and deactivate NF- $\kappa$ B signaling pathway via upregulation of miR-16 in T24 cells.

**Key words: Puerarin; MicroRNA-16 (miR-16); Bladder cancer; Cyclooxygenase 2 (COX-2); Nuclear factor  $\kappa$  light chain enhancer of activated B cells (NF- $\kappa$ B) signaling pathway**

### INTRODUCTION

Bladder cancer (BC) is a malignant tumor that occurs in the bladder mucosa. According to the pathological distinction, BC is mainly divided into urothelial cell carcinoma (UCC), squamous cell carcinoma (SCC), and adenocarcinoma, of which UCC accounts for more than 90% of BC cases<sup>1</sup>. In China, BC has the highest incidence in genitourinary tumors. Whereas the incidence in Western countries is less than in China, it is also the fifth most common cancer<sup>2,3</sup>. The main manifestation of BC is painless hematuria. This disease is commonly treated with transurethral tumor resection, radiation, and chemotherapy<sup>4</sup>, and the drugs include cisplatin, mitomycin C, Bacillus Calmette–Guerin (BCG), and interferon- $\alpha$  (IFN- $\alpha$ )<sup>5,6</sup>. Although these methods have some certain effects, they usually bring severe systemic toxicity and local irritation to the bladder<sup>7,8</sup>. Therefore, research for effective and nontoxic side effects of BC treatment is very urgent. Recently, treatment of BC with Chinese medicines has achieved some good results in China<sup>9</sup>.

Puerarin is a flavonoid extracted from the dried roots of *Pueraria lobata*<sup>10</sup>. It has antitumor, antioxidation,

anti-inflammatory, lipid-lowering, antiapoptosis, and other biological activities<sup>10–12</sup>. Considerable evidence has suggested that puerarin could treat many diseases, like endothelial dysfunction, liver fibrosis, neurotoxicity, and bone damage<sup>13–15</sup>. The molecular mechanisms of the anti-tumor effect of puerarin have been focused on different types of cancers, such as liver cancer, colon cancer, and breast cancer<sup>15–17</sup>. Some studies have also found that high concentrations of puerarin could inhibit the growth of breast cancer cells and induce apoptosis of HT-29 colon cancer cells<sup>16,17</sup>. However, the molecular mechanism of puerarin in BC is still unclear.

MicroRNAs (miRNAs) are a class of noncoding small RNA molecules with a length of about 22 nucleotides that regulate gene expression by directly participating in the interpretation of mRNA or inhibiting translation processes<sup>18</sup>. According to Lewis et al., more than 30% of genes are regulated by miRNAs in the human genome<sup>19</sup>. miRNAs play a key role in most cancers, modulating the initiation, progression, and transmission of tumor microenvironment<sup>20</sup>. In addition, miRNAs are associated with cell proliferation, apoptosis, differentiation, and

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metastasis during tumorigenesis<sup>21</sup>. For example, miR-451a and miR-223 could induce apoptosis in renal cell carcinoma and prostate cancer, respectively<sup>21,22</sup>.

Therefore, this study aimed to investigate the effect of puerarin on cell viability, proliferation, and apoptosis of BC cells to provide an effective approach for BC treatment.

## MATERIALS AND METHODS

### *Cell Culture and Treatment*

The human BC T24 cell line was obtained from the Shanghai Institutes for Biological Sciences of the Chinese Academy of Sciences (CAS; Shanghai, P.R. China). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin–streptomycin mixture (both from Thermo Fisher Scientific) in a humid atmosphere containing 5% CO<sub>2</sub> at 37°C. Different concentrations (0, 10, 50, and 100 µg/ml) of puerarin (Abcam, Cambridge, MA, USA) were used to treat the T24 cell line for 24 h. Based on the results, 50 µg/ml of puerarin was used predominantly in the subsequent experiments. The nuclear factor κ light chain enhancer of activated B cells (NF-κB) inhibitor BAY 11-7082 (Beyotime Biotechnology, Shanghai, P.R. China) was added at a concentration of 1 µM, and the cells were cultured at 37°C for 2 h.

### *Cell Transfection*

miR-16 mimic, miR-16 inhibitor, and their negative controls (NCs) were synthesized by GenePharma Co. (Shanghai, P.R. China). Cell transfections were conducted using Lipofectamine 3000 (Thermo Fisher Scientific) following the manufacturer's instructions. After 48 h of transfection, the stably transfected cells were produced and cultured for about 4 weeks in DMEM containing 0.5 mg/ml antibiotic G418 sulfate (Thermo Fisher Scientific). The G418-resistant cells were then collected for subsequent experiments.

### *Cell Viability Assay*

T24 cells (5 × 10<sup>3</sup> cells/well) were seeded in 96-well plates and detected by a cell counting kit-8 (CCK-8; Dojindo, Gaithersburg, MD, USA) over time. In brief, 10 µl/well CCK-8 solution was added after puerarin treatment of the cells, and the cultures were incubated for 1 h in humidified 95% air and 5% CO<sub>2</sub> at 37°C. The absorbance was measured at 450 nm using a microplate reader (Bio-Rad, Hercules, CA, USA). The experiment was repeated three times.

### *Cell Proliferation Assay*

T24 cells (1 × 10<sup>4</sup> cells/well) were seeded in 24-well plates, with the addition of 1 mg/ml bromodeoxyuridine (BrdU; Sigma-Aldrich, St. Louis, MO, USA) before cell

proliferation was detected, and the cells were cultured for 3 h. Cells were washed with cold phosphate-buffered saline (PBS; Thermo Fisher Scientific) three times, and 300 µl BrdU was added at a dilution of 1:50 overnight. At least 1,000 cells were counted in each condition under a microscope (Zeiss, Heidenheim, Germany). Each condition contained at least five replicates.

### *Cell Apoptosis Assay*

Cells (1 × 10<sup>4</sup> cells/well) were performed using propidium iodide (PI) and fluorescein isothiocyanate (FITC)-conjugated annexin V staining (both from Sigma-Aldrich). Briefly, cells were washed in cold PBS and fixed in 70% cold ethanol (Sigma-Aldrich) overnight. Fixed cells were then washed twice in PBS, stained in PI/FITC-annexin V in the presence of 50 µg/ml RNase A (Sigma-Aldrich), and incubated for 1 h at room temperature in the dark. Flow cytometry analysis was done using a FACScan (Beckman Coulter, Fullerton, CA, USA). The data were analyzed using the FlowJo V10 software (Tree Star, Ashland, OR, USA).

### *Quantitative Real-Time Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR)*

The total RNA was extracted from cells using TRIzol Reagent (Life Technologies Corporation, Carlsbad, CA, USA) according to the manufacturer's instructions. For testing the expression levels of miR-16, the TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific) was used to synthesize cDNA, and TaqMan Universal Master Mix II with the TaqMan MicroRNA Assay (both from Thermo Fisher Scientific) was used for qRT-PCR analysis. Data were normalized to U6 in this study, and fold changes were calculated using the 2<sup>-ΔΔCt</sup> method<sup>23</sup>.

### *Western Blot Assay*

The expression levels of proteins were detected using Western blot analysis. Radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime Biotechnology) supplemented with protease inhibitors (Roche, Basel, Switzerland) was used for extracting the proteins, which were quantified using the BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA). The Western blot system was established using a NuPAGE Bis-Tris Gels System (Bio-Rad) on the basis of the manufacturer's descriptions. Primary antibodies, such as cyclin D1 (ab134175), β-actin (ab8227), B cell lymphoma-2 (Bcl-2; ab59348), Bcl-2 X-associated protein (Bax; ab182733), procaspase 3 (ab32499), cleaved caspase 3 (ab2302), procaspase 9 (ab135544), cleaved caspase 9 (ab2324), NF-κB subunit 1 (p65; ab19870), phosphorylated (p)-p65 (ab86299), NF-κB inhibitor α (IκBα; ab109300), p-IκBα (ab64813), and cyclooxygenase-2 (COX-2; ab52237) (all 1:1,000; Abcam), were prepared

in 5% blocking buffer, incubated with the membrane at 4°C overnight. Then they were washed with PBS and cultivated with goat anti-rabbit antibody (ab190492; 1 mg/ml; Abcam) marked by horseradish peroxidase for 1 h at room temperature. After rinsing, the polyvinylidene difluoride (PVDF) membrane carried blots, and antibodies were transferred into the ChemiDoc™ XRS System (Bio-Rad). Further, 200 µl of Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA) was added to cover the membrane surface. The signals were captured, and the band intensity was quantified using the Image Lab™ Software (Bio-Rad).

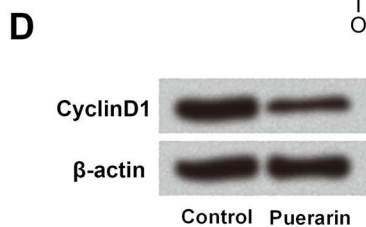
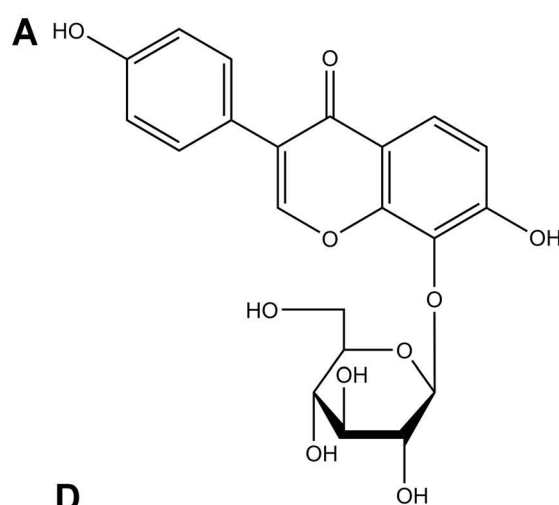
#### Statistical Analysis

The results of the experiments are expressed as the mean ± standard deviation (SD). GraphPad Prism 6.0 statistical software (GraphPad Software Inc., La Jolla, CA, USA) was used for statistical analysis of data. The *p* values were calculated using a one-way analysis of variance (ANOVA) for multigroup comparisons. A value of *p* < 0.05 was considered statistically significant.

## RESULTS

### Puerarin Inhibited Cell Viability and Proliferation, and Promoted Cell Apoptosis in T24 Cells

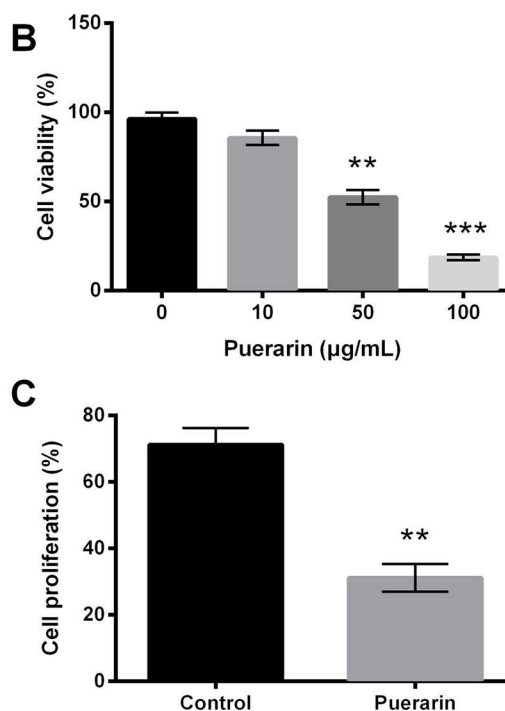
The chemical structure of puerarin is shown in Figure 1A, which is also known as 8-C-glucoside of daidzein.



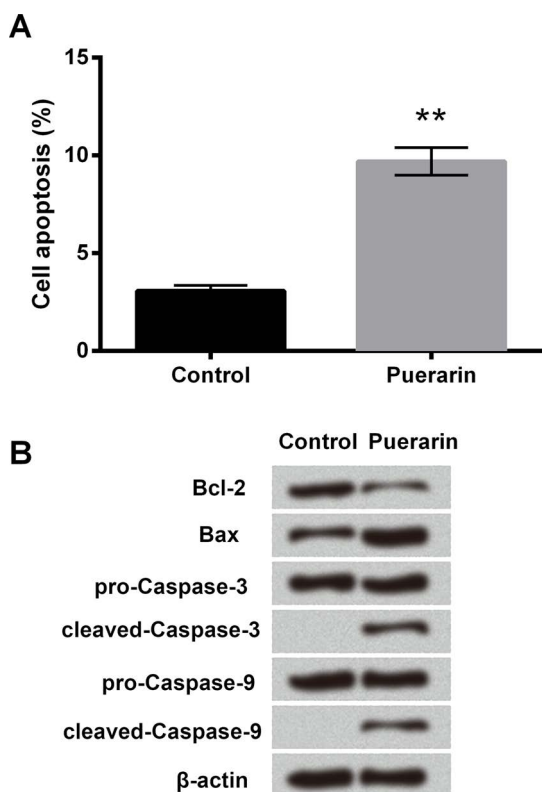
Cell viability was assessed using different concentrations (0, 10, 50, and 100 µg/ml) of puerarin in order to study the effect of puerarin on T24 cells. Although the decrease in cell viability was not obvious in 10 µg/ml of puerarin, it was significantly reduced in the 50 and 100 µg/ml groups (*p* < 0.01 or *p* < 0.001) (Fig. 1B). Therefore, 50 µg/ml of puerarin was selected for the following experiments. Cell proliferation was measured using the BrdU assay, and the results showed it was obviously decreased after puerarin treatment (*p* < 0.01) (Fig. 1C). We next examined the protein level of cyclin D1 associated with cell cycle using Western blot. The expression of cyclin D1 was downregulated in the puerarin group compared with the control group (Fig. 1D). In addition, cell apoptosis was greatly increased, the expression of antiapoptotic factor Bcl-2 was downregulated, and the protein levels of proapoptotic factor Bax, cleaved caspase 3, and cleaved caspase 9 were remarkably upregulated with puerarin exposure (*p* < 0.01) (Fig. 2A and B). These data illustrated that puerarin could inhibit cell viability and proliferation, and promote cell apoptosis in T24 cells.

### Puerarin Inhibited Cell Viability and Proliferation, and Promoted Cell Apoptosis via Upregulation of miR-16 in T24 Cells

The expression and effect of miR-16 were detected in puerarin-treated T24 cells (Fig. 3). qRT-PCR results



**Figure 1.** Puerarin decreased cell viability and proliferation in T24 cells. (A) The chemical structure of puerarin. (B) Cell viability was assessed by cell counting kit-8 (CCK-8) assay. (C) Cell proliferation was measured by bromodeoxyuridine (BrdU) assay. (D) The expression of cyclin D1 was detected by Western blot analysis. \*\**p* < 0.01, \*\*\**p* < 0.001 compared to control.



**Figure 2.** Puerarin increased cell apoptosis in T24 cells. (A) Cell apoptosis was assessed by flow cytometry analysis. (B) The expressions of apoptosis-related proteins were detected by Western blot analysis. \*\* $p < 0.01$ .

showed that the mRNA level of miR-16 was upregulated in the puerarin group ( $p < 0.01$ ) (Fig. 3A). miR-16 expression was significantly downregulated in the miR-16 inhibitor group ( $p < 0.001$ ) (Fig. 3B), illustrating that the miR-16 inhibitor was successfully transfected into cells. Cell viability was enhanced (Fig. 3C), cell apoptosis was reduced ( $p < 0.05$ ) (Fig. 3E), cyclin D1 and Bcl-2 expressions were upregulated (Fig. 3D and F), and the protein levels of Bax and cleaved caspase 3/9 were downregulated (Fig. 3F) in the puerarin+miR-16 inhibitor group compared with the puerarin+NC group. The above results showed that puerarin could inhibit cell viability and promote cell apoptosis via upregulation of miR-16 in T24 cells.

#### *Puerarin Deactivated NF- $\kappa$ B Signaling Pathway via Upregulation of miR-16 in T24 Cells*

A previous report suggested that puerarin inhibited the expression of COX-2 via suppressing NF- $\kappa$ B activation in lipopolysaccharide (LPS)-induced macrophage cells<sup>24</sup>. Hence, this study also focused on the relationship between puerarin and the NF- $\kappa$ B signaling pathway

by Western blot. The expressions of p-p65 and p-I $\kappa$ B $\alpha$  were significantly downregulated by puerarin treatment (Fig. 4A and B). Meanwhile, p-p65 and p-I $\kappa$ B $\alpha$  expressions were upregulated in the puerarin+miR-16 inhibitor group compared with the puerarin+NC group (Fig. 4B). These results indicated that puerarin could inhibit the activation of the NF- $\kappa$ B signaling pathway via upregulation of miR-16 in T24 cells.

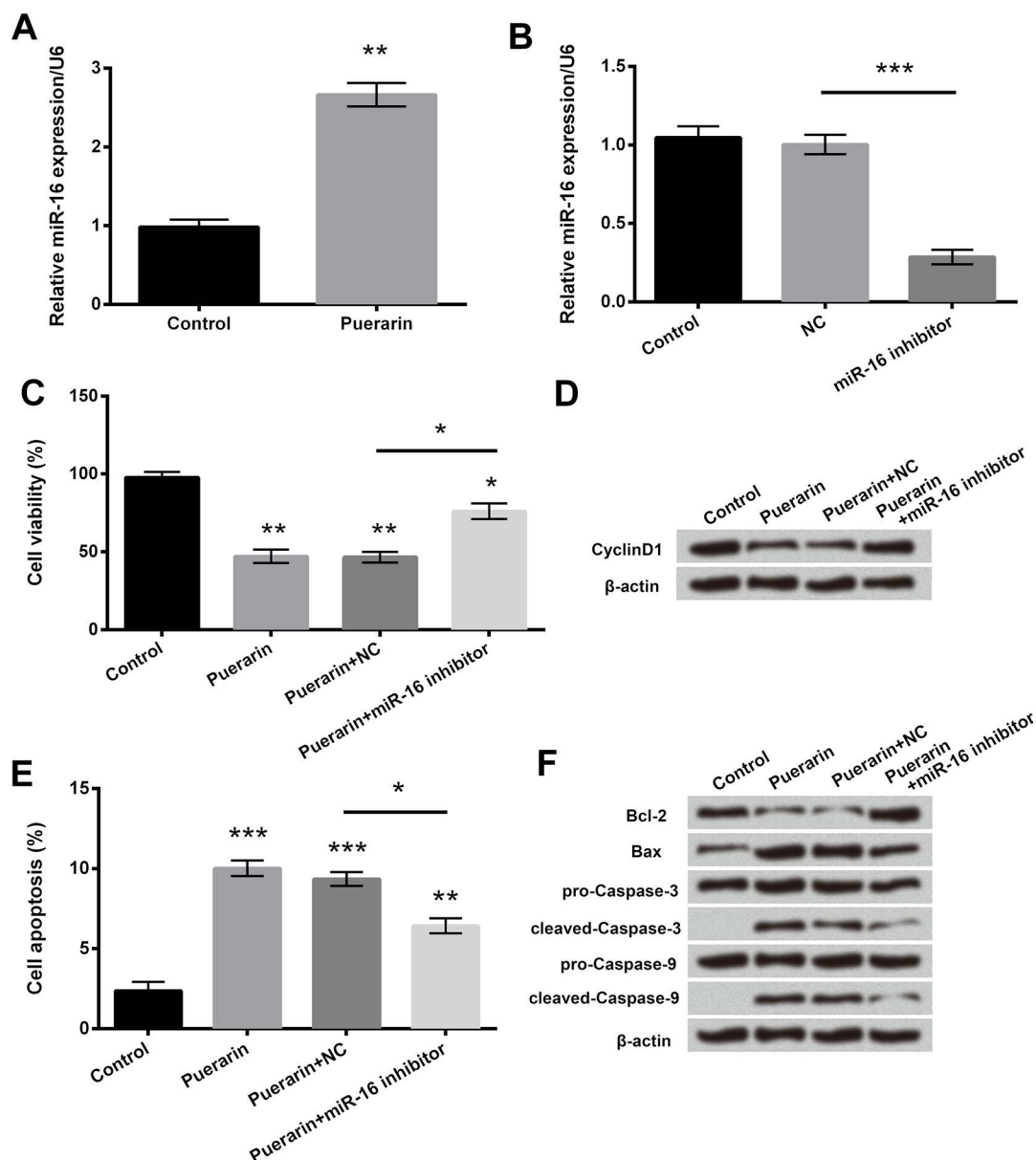
#### *miR-16 Downregulated COX-2 Expression via Deactivation of NF- $\kappa$ B Signaling Pathway*

The regulation mechanisms of miR-16 in T24 cells were detected using qRT-PCR and Western blot. The mRNA level of miR-16 was clearly upregulated when cells were transfected with the miR-16 mimic ( $p < 0.001$ ) (Fig. 5A). Subsequently, p-p65, p-I $\kappa$ B $\alpha$ , and COX-2 expressions were greatly downregulated in the miR-16 mimic group, while the opposite results were observed in the miR-16 inhibitor group (Fig. 5B). Further, the expressions of p-p65, p-I $\kappa$ B $\alpha$ , and COX-2 were significantly downregulated after addition of the NF- $\kappa$ B inhibitor, BAY 11-7082, to the miR-16 inhibitor group (Fig. 5B). Overall, the results suggested that miR-16 downregulated COX-2 expression via suppressing the activation of NF- $\kappa$ B signaling pathway.

## DISCUSSION

BC is the most common malignant tumor in the urinary system and has a serious impact on patients' lives<sup>1</sup>. Puerarin is one of the main effective components extracted from *Pueraria lobata*, which could affect different types of cancer cell proliferation and apoptosis<sup>15-17</sup>. However, the role of puerarin in BC cells is still unclear. Hence, this study aimed to investigate the effect and molecular mechanism of puerarin on BC cells. The results showed that puerarin inhibited cell viability and proliferation and promoted apoptosis via upregulation of miR-16 in T24 cells. Further, puerarin deactivated the NF- $\kappa$ B signaling pathway via upregulation of miR-16 in T24 cells, and miR-16 downregulated COX-2 expression via deactivation of NF- $\kappa$ B signaling pathway.

In recent years, many studies have focused on the antitumor properties of puerarin<sup>17,25</sup>. Lin et al. reported that puerarin has similar estrogen-like effects to other isoflavones<sup>26</sup>. Moreover, high concentrations of puerarin could inhibit breast cancer cell growth and promote apoptosis<sup>26</sup>. The study also found that puerarin treatment led to a dose-dependent inhibition of cell growth in breast cancer<sup>26</sup>. Consequently, we used different concentrations (0, 10, 50, and 100  $\mu$ g/ml) of puerarin to assess cell viability. The present study confirmed that puerarin at high concentrations significantly decreased T24 cell viability



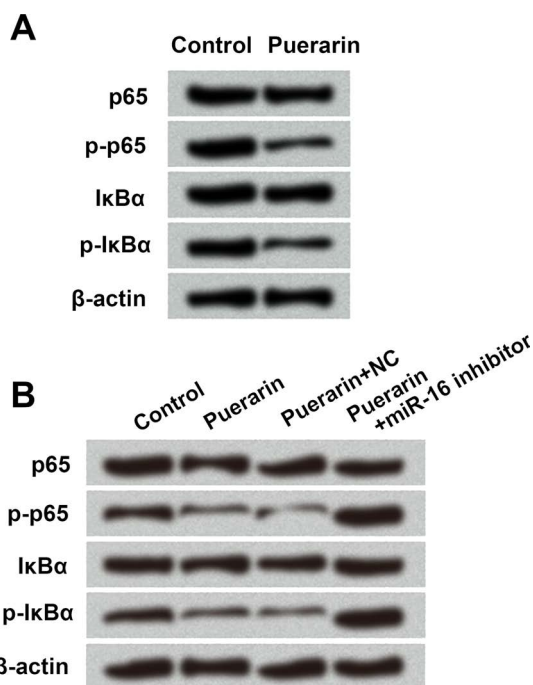
**Figure 3.** Puerarin inhibited cell viability and promoted cell apoptosis via upregulation of miR-16 in T24 cells. The mRNA levels of miR-16 in (A) puerarin-treated cells or (B) miR-16 inhibitor-transfected cells were detected by quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR), respectively. (C) Cell viability was assessed by CCK-8 assay. (D) The expression of cyclin D1 was examined by Western blot analysis. (E) Cell apoptosis was measured by flow cytometry analysis. (F) The expressions of apoptosis-related proteins were determined by Western blot analysis. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

in a dose-dependent manner. Further, 50  $\mu\text{g/ml}$  of puerarin was selected in our following study. Cyclin D1 is a critical cell cycle regulator that controls the progression of  $G_0/G_1$ , promotes cell proliferation, and is associated with different types of cancer<sup>27</sup>. According to Kopparapu et al., the expression of cyclin D1 protein was higher in BC tissues than in benign bladder tissues, which is linked to the degree of invasiveness and cancer recurrence<sup>28</sup>.

Moreover, puerarin induced cell apoptosis through a caspase 3-dependent pathway and mediated cell cycle arrest in the  $G_2/M$  phase<sup>26,29</sup>. Thus, in this research, cell proliferation, apoptosis, and the expression of cyclin D1 were detected. Our results showed that puerarin could inhibit cell proliferation and promote apoptosis in BC cells.

With the development of molecular biology technology, researchers have begun to study the molecular

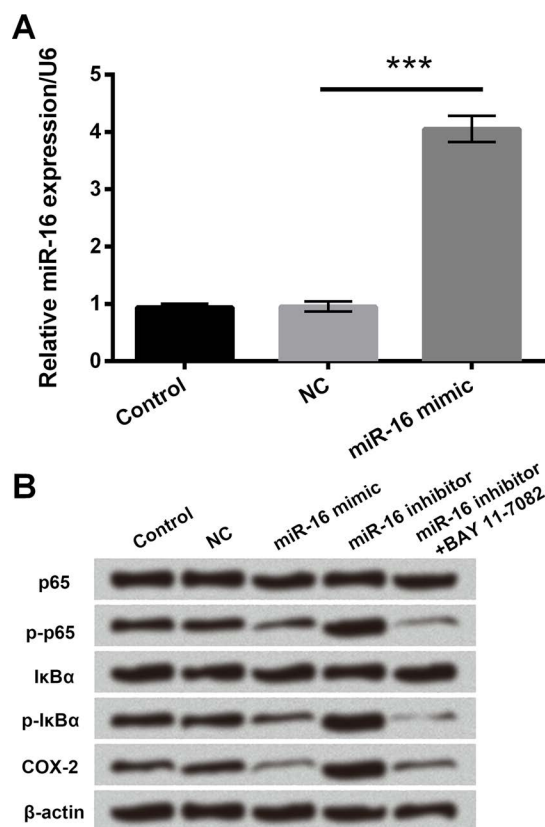




**Figure 4.** Puerarin deactivated NF- $\kappa$ B signaling pathway via upregulation of miR-16 in T24 cells. The expression of the main factors in the NF- $\kappa$ B signaling pathway was detected in (A) puerarin-treated cells and (B) miR-16 inhibitor-transfected cells by Western blot analysis, respectively. NC, negative control; NF- $\kappa$ B, nuclear factor  $\kappa$  light chain enhancer of activated B cells; p65, NF- $\kappa$ B subunit 1; p, phosphorylated; I $\kappa$ B $\alpha$ , NF- $\kappa$ B inhibitor.

mechanism of BC genesis, development, and prognosis, and to search for BC-related genes<sup>30</sup>. Some studies have demonstrated that different miRNAs could participate in cell proliferation and apoptosis of BC, such as miR-145, miR-133a, and miR-16<sup>31,32</sup>. Moreover, the study of Jiang et al. revealed that expression of miR-16 was downregulated in BC, and cyclin D1 expression was negatively regulated by miR-16<sup>31</sup>. Another study reported that urothelial carcinoma-associated 1 (UCA1) regulated the glutaminase 2 (GLS2) expression by interfering with miR-16 in BC cells<sup>33</sup>. Therefore, we hypothesized that puerarin might have an impact on BC cells via regulation of miR-16. Increased cell viability and proliferation, and reduced apoptosis were detected when cells were treated with puerarin plus miR-16 inhibition. Finally, our study revealed that puerarin could decrease cell viability and proliferation and increase cell apoptosis via upregulation of miR-16 in T24 cells.

NF- $\kappa$ B is a widespread cell protective factor whose activation induces cell transformation, promotes cancer cell proliferation, and inhibits cell apoptosis and angiogenesis<sup>34</sup>. A previous report suggested that puerarin inhibited the expression of COX-2 via suppressing NF- $\kappa$ B activation in LPS-induced macrophage cells<sup>24</sup>. In



**Figure 5.** miR-16 downregulated COX-2 expression via deactivation of NF- $\kappa$ B signaling pathway. (A) The mRNA levels of miR-16 in miR-16 mimic or NC-transfected cells were detected by qRT-PCR. (B) The expressions of the main factors in NF- $\kappa$ B signaling pathway were measured by Western blot analysis. COX-2, cyclooxygenase-2. \*\*\* $p < 0.001$ .

addition, Zuo et al. reported that artesunate induced cytotoxicity and apoptosis by increasing miR-16 expression, which resulted in the decrease in COX-2 expression<sup>30</sup>. Therefore, we predicted that the regulatory mechanism of puerarin in BC cells was similar to the previous studies<sup>24,30</sup>. The phosphorylation levels of factors involved in NF- $\kappa$ B signaling pathway and COX-2 expression were measured by Western blot in this study. In the end, experiments have confirmed our conjecture that puerarin could deactivate NF- $\kappa$ B signaling pathway via upregulation of miR-16, and miR-16 could downregulate COX-2 expression via deactivation of NF- $\kappa$ B signaling pathway.

In summary, puerarin inhibited cell proliferation, promoted cell apoptosis, and deactivated NF- $\kappa$ B signaling pathway in T24 cells via upregulation of miR-16. These findings may provide a novel, safe, and effective theoretical method for the treatment of BC with drugs in the future.

**ACKNOWLEDGMENTS:** The authors declare no conflicts of interest.

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