

# Oridonin suppresses the growth of glioblastoma cells via inhibiting Hippo/YAP axis

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## Research Article

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# Abstract

Glioma is a brain tumor that originated from brain or spine glial cells. Despite utilizing alternative treatments, the overall survival remains poor. Oridonin (ORI) is purified from the Chinese herb *Rabdosia rubescens* which exhibited anti-cancer effects on human tumorigenesis. The aim of this study was to investigate the effect of ORI on U87MG glioblastoma cells and whether Hippo/YAP-related signaling pathway was involved in. Here, we found that ORI inhibited cell proliferation and promoted cell apoptosis in a dose-dependent manner in U87MG cells. Moreover, ORI inhibited Bcl-2, YAP, c-Myc protein expression but increased Bax, caspase-3, p-YAP protein expression. Furthermore, these anti-cancer effects of ORI were also confirmed in a mouse model bearing glioma. Further study suggested that the YAP inhibitor Verteporfin (VP) showed the similar effect of ORI, but ORI reversed the effect of over-expression of YAP. Collectively, Oridonin suppressed glioblastoma oncogenesis via the Hippo/YAP signaling pathway and could be a potential therapeutic target in the treatment of glioblastoma.

## 1. Introduction

Glioblastoma is a devastating disease in which morbidity and mortality rates are high [1]. Despite utilizing alternative treatments, including surgery, chemotherapy and radiotherapy, the overall survival remains poor [2]. Thus, searching for new therapeutic strategy of glioblastoma was imperative. Oridonin (ORI) is a major component derived from the traditional Chinese medicine, *Rabdosia rubescens*, which exhibits anti-inflammatory, anti-infectious and anti-neoplastic properties [3, 4]. The effect of ORI on tumor suppression has been documented in solid cancers, including colon cancer [5], ovarian cancer [6] and nasopharyngeal carcinoma [7]. Lin *et al.* reported that ORI induces glioma cell apoptosis via downregulation of RanGAP1 protein [8]. However, the underlying mechanism remains elusive.

Hippo signaling pathway plays an important role in tumor pathogenesis [9]. Yes-associated protein (YAP), one of the key components in Hippo pathway, works interactively with cellular-myelocytomatosis oncogene (c-Myc) to promote tumor progression. Previous investigations performed by others have found that YAP is closely related to the malignant progression of gliomas [10]. In this study, we reported that Oridonin blunted glioblastoma growth both *in vivo* and *in vitro*. In addition, Oridonin suppressed glioblastoma oncogenesis via the Hippo/YAP signaling pathway. Therefore, Oridonin is a therapeutic target in the treatment of glioblastoma.

## 2. Materials And Methods

### 2.1. Chemicals and reagents

Oridonin (ORI, White powder, purity 99.5%, molecular weight 364.44) and Verteporfin (VP, Green power, purity 99.5%, molecular weight 718.79) were purchased from MCE (NJ, USA). 3-(4,5-dimethyl-2-thiazolyl)-2,5-dimethyl-2H-tetrazolium bromide (MTT) and were purchased from Saimike (Chongqing, China). Annexin V-FITC apoptosis detection kit, annexin V-mCherry apoptosis detection kit and cell cycle

analysis kit were purchased from Beyotime (Shanghai, China). Primary antibodies against  $\alpha$ -tubulin, caspase-3, Bax, Bcl-2, YAP, c-Myc, p-YAP and the corresponding secondary antibodies (goat anti-rabbit, goat anti-mouse, and Cy3-conjugated affinipure goat anti-rabbit IgG) were all purchased from Proteintech (Hubei, China). Lentiviral vectors harboring YAP1 (YAP) overexpression vectors were obtained from Genechem (Shanghai, China).

## ***2.2. Cell culture***

The cell lines U87MG were procured from the ATCC and maintained in DMEM medium supplemented with 5% fetal bovine serum. The cells were cultured in incubator under a 5% CO<sub>2</sub> and 95% air atmosphere at 37°C.

## ***2.3. Lentivirus transduction***

U87MG cells were infected with prepared Lentiviral vectors harboring YAP as per provided directions, the transfected cells showed green fluorescence after which Western blotting were used to assess changes in target gene expression. All viruses were tagged with GFP tracking. The control vector contained empty Lentivirus expressing GFP only (AdGFP). The Lentiviral vectors harboring YAP expressed GFP (AdGFP) and YAP (adYAP).

## ***2.4. Cell viability assay***

U87MG cells were seeded into a 96-well plate at a density of  $5 \times 10^3$  cells/well and cultured in the medium containing different concentrations of Oridonin (4, 6, 8, 10, and 12  $\mu$ M/L). After treatment for 24 or 48 h, a total of 10  $\mu$ l MTT solution (5 mg/mL) was added to each well and incubated for 4 h under 37°C in dark. The Optical Density (OD) was measured through a microplate reader (Bio-Rad, USA) at 490nm.

## ***2.5. Colony formation assay***

A colony formation assay was performed to detect the clonogenicity capability. According to the manufacturer's instruction, the cells were seeded in a 6-well plate (200 cells per well) and cultured in cell incubator for 2-3 weeks. After rinsed twice with ice-cold phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 15 min, the colonies were stained with 1% crystal violet and counted.

## ***2.6. Flow cytometry***

For cell cycle analyses, cells ( $50 \times 10^4$ ) were collected and fixed with 70% ethanol overnight at 4°C. Cells were treated with staining buffer with PI (50  $\mu$ g/mL) and RNase (10 mg/mL) at 37°C in dark for 30 min, followed by analysis on CytoFLEX flow cytometry (Beckman Coulter, USA). For cell apoptosis analyses, cells ( $50 \times 10^4$ ) were stained with annexin V/PI or V-mCherry at 4°C in dark for 15 min, analysed by CytoFLEX flow cytometry.

## ***2.7. In vivo tumor xenograft study***

Male athymic nude mice (4–6 weeks old with an initial body weight of  $20 \pm 2$  g) were obtained from Beijing HFK Bioscience Co., Ltd. (Beijing, China). The animals were acclimatized at a temperature of  $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$  and a relative humidity of  $70\% \pm 5\%$  under natural light/dark conditions for 1 week with ad libitum access food and water. All animal treatments were performed in strict accordance with international ethical guidelines and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Cells in log-phase growth were collected in serum-free culture medium (at a density of  $1 \times 10^6$  cells in 0.1 mL),  $100\mu\text{M}$  cell suspension was injected into the right flank of nude mice, and groups were randomly divided into three groups: blank-control group (10% DMSO with 90% PBS), ORI (15 mg/kg)-administrated group, and VP (15 mg/kg)-administrated group. The frequency of administration was once every two days. The tumor volume was estimated using the following formula: tumor volume ( $\text{mm}^3$ ) =  $(L \times W^2)/2$ , where L and W represent the length and width of the tumor. On day 21, animals were sacrificed, and tumor tissue was removed and weighed.

## ***2.8. Western blotting***

The sample proteins were lysates by RIPA from cultured cells or tissues. Concentration of protein samples were determined by BCA kit (Beyotime, Shanghai, China). The sample proteins were separated by 10% SDS-PAGE systems and transferred to polyvinylidene difluoride membranes (Beyotime, Shanghai, China). After blocked with 5% bovine serum albumin (BCA, Beyotime, Shanghai, China) for 2h at room temperature, the membranes were incubated with primary antibodies overnight at  $4^{\circ}\text{C}$ . The antibodies and dilution factors were as follows: Bax (1: 2000); Bcl-2 (1:1000); caspase-3 (1:1000); YAP (1:5000); p-YAP (1:800); c-Myc (1:2000);  $\alpha$ -tubulin (1:8000). Membranes were incubated with secondary anti-rabbit or anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5000 dilution) for 1 h at room temperature. Antibody binding was detected by ECL detection kit (Beyotime, Shanghai, China), and the densitometric analysis was conducted using a quantitative imaging system (Bio-Rad, USA).

## ***2.9. Immunohistochemistry and immunofluorescence***

For Immunohistochemistry (IHC) analysis, Tumor tissues were fixed in formaldehyde at room temperature, embedded in paraffin and cut into sections of 4-mm thickness. Following antigen retrieval, stained with Ki67 primary antibodies at 1:200 dilutions. Images were captured by microscope (Nikon, Japan). For Immunofluorescence (IF) analysis, Cells were attached to  $20 \times 20$  mm glass slides, permeabilized and fixed with paraformaldehyde at room temperature, then stained with Ki67 primary antibodies at  $4^{\circ}\text{C}$  overnight. The nuclear staining were performed with DAPI. Images were captured by fluorescence microscopy (Nikon, Japan).

## ***2.10. TUNEL staining, Haematoxylin and Eosin staining***

For TUNEL staining, tissue sections were permeabilized and blocked, then using TUNEL reaction mixtures (The proportion of TdT with dUTP at 1:9) to stain tissues for 2 h at 37 °C, followed by nuclear staining with DAPI. The result were measured by fluorescent microscope (Nikon, Japan). For HE staining, tissue sections were deparaffinized and rehydrated, the hematoxylin solution were used to stain for 5 min, then washed twice before incubating with eosin for 3 min. The result was observed by light microscope (Nikon, Japan).

### ***2.11. Statistical analysis***

All experimental data were expressed as means  $\pm$  standard deviation (SD). The statistical analysis were conducted using SPSS 25.0 and GraphPad Prism 8.0. One-way ANOVA and T-tests were used to assess the statistical significance of the differences between groups. Analyses were repeated in triplicate, and the statistical significance was defined as  $P < 0.05$ .

## **3. Result**

### ***3.1 ORI inhibits cells viability, clone formation and proliferation.***

ORI inhibited the viability of U87MG cells in a time- and dose-dependent manner (Fig. 1A). The cell viability of U87MG infected with empty vector expressing YAP virus (NC) was no different. For the U87MG cells infected with YAP virus expressing (overYAP), the cell viability was significantly increased, but it could be significantly reduced by ORI (Fig. 1B). For clone formation assay, the increasing of ORI concentration significantly reduced the number of cell colony formation (Fig. 1C). over-expression of YAP significantly increased the cell colony formation, but ORI (8  $\mu$ M) reversed the effect of over-expression of YAP (Fig. 1D). Moreover, the proliferation ability was detected by Ki67 expression. The results showed that ORI decreased Ki67 expression in U87MG, over-expression of YAP increased the Ki67 expression and ORI significantly decreased the Ki67 expression in overYAP cells (Fig. 1E). In addition, Verteporfin (VP, a YAP inhibitor) also reduced the Ki67 expression in U87MG (Fig. 1F).

### ***3.2 ORI alters the cell cycle and promotes apoptosis in U87MG.***

For cell cycle, ORI increased the number of G2/M phase cells in U87MG cells in a concentration-dependent manner (Fig. 2A). As shown in Fig. 2B, compared to U87MG, over-expression of YAP increased the S-phase cells and decreased the G2/M phase cells. However, ORI (8  $\mu$ M) reversed the cell cycle in overYAP cells. In parallel with ORI, VP (30  $\mu$ M) increased the G2/M phase cells and decreased the S-phase cells in U87MG (Fig. 2C). Moreover, ORI promoted cell apoptosis in U87MG with the increase of concentration (Fig. 2D). NC and overYAP cell were no significant difference compared with the Ctrl, but ORI (8  $\mu$ M) promoted the cell apoptosis both in U87MG and overYAP cells (Fig. 2E). Interestingly, VP (30  $\mu$ M) significantly increased the cell apoptosis of U87MG (Fig. 2F).

### ***3.3 ORI represses the tumor growth in a mouse xenograft***

As shown in Fig. 3, compared with Ctrl group, ORI (15 mg/kg) and VP (15 mg/kg) significantly inhibited the tumor weight and tumor volume. over-expression of YAP increased the tumor weight and tumor volume, but ORI (15 mg/kg) abolished the effect of over-expression of YAP in overYAP group.

### ***3.4 ORI inhibits tumor cells proliferation and promotes tumor cells apoptosis in vivo***

The results of HE staining (Fig. 4A) showed that the nuclei of U87MG cells were full and spherical. Compared with U87MG group, ORI (15 mg/kg) or VP (15 mg/kg) caused wrinkling of cytoplasm and nuclei, while the morphology of overYAP cells was not greatly affected. compared with overYAP group, ORI (15 mg/kg) caused increasing nucleoplasmic contraction of tumor cells. IHC study (Fig. 4B) showed that Ki67 expression was reduced after treatment of ORI (15 mg/kg) or VP (15 mg/kg) in *vivo*, and Ki67 expression was increased in overYAP group. Compared with overYAP group, ORI (15 mg/kg) significantly reduced the expression of Ki67. We further analyzed tumor cells apoptosis in *vivo* using TUNEL staining. The results (Fig. 4C) showed that ORI (15 mg/kg) or VP (15 mg/kg) increased tumor cells apoptosis in *vivo*. Over-expression of YAP decreased the tumor cell apoptosis. However, ORI (15 mg/kg) significantly increased the cell apoptosis in overYAP group.

### ***3.5 ORI regulates apoptosis-related proteins.***

Caspase-3, Bcl-2, Bax are apoptosis-related proteins and play vital roles in the initiation and execution of apoptosis. Therefore, we assessed these apoptosis-related proteins expressions. In *vitro*, with the concentration increasing, ORI reduced the protein expression of Bcl-2 while increased the protein expression of Bax and caspase-3 (Fig. 5A). In addition, VP (30  $\mu$ M) appeared the similar variation trend with ORI in protein expression of Bcl-2, Bax and caspase-3 (Fig. 5B). Over-expression of YAP in U87MG cells increased the protein expression of Bcl-2, decreased the protein expression of Bax and caspase-3 (Fig. 5C). However, ORI (8  $\mu$ M) reversed the effect of over-expression of YAP on apoptosis-related proteins. In *vivo*, ORI (15 mg/kg) and VP (15 mg/kg) down-regulated the protein expression of Bcl-2, up-regulated the protein expression of Bax and caspase-3. Meanwhile, compared with overYAP group, ORI (15 mg/kg) increased the protein expression of Bax and caspase-3 while decreased the protein expression of Bcl-2 in *vivo* (Fig. 5D).

### ***3.6 ORI regulates protein of YAP, P-YAP and c-Myc.***

In *vitro*, ORI down-regulated the protein expression of YAP and c-Myc, up-regulated the protein expression of p-YAP in a dose-depended manner (Fig. 6A). VP (30  $\mu$ M) decreased the protein expression of YAP, p-YAP and c-Myc (Fig. 6B). Over-expression of YAP increased the protein expression of YAP and P-YAP, but decreased the protein expression of c-Myc (Fig. 6C). In *vivo*, ORI (15 mg/kg) and VP (15 mg/kg) down-regulated the protein expression of YAP and c-Myc, but up-regulated the protein expression of p-YAP. Over-expression of YAP increased the protein expression of YAP, p-YAP and c-Myc. However, ORI (15 mg/kg) reversed the protein expression of YAP and c-Myc (Fig. 6D).

## **4. Discussion**

Glioma is the most common primary intracranial tumor, which develops rapidly and cause high fatality rate [11]. Therefore, finding effective drug for the treatment of glioma is extraordinarily important. As a active diterpenoid and the main active factor of *Rabdosia rubescens*, ORI could inhibit multiple cancers include small cell lung cancer and gastric cancer [12, 13]. However, the understanding about anti-glioma effect of ORI is limited.

The process of tumorigenesis involves cell proliferation, cell migration and cell apoptosis. Cell proliferation and migration are negative factors that promote tumor development, while cell apoptosis cause reverse result. Ki67 is a commonly used marker for cancer cell proliferation. It is expressed in S, G2, and M cell phase [14]. The expression level of Ki67 indicate the proliferation activity of cancer cells. Bcl-2 (B-cell lymphoma-2), as a tumor-promoting gene, can significantly inhibit cell apoptosis [15]. After cell apoptosis begin, the expression of Bcl-2-associated X protein Bax (Bcl-2-Associated X) increases and the expression of Bcl-2 decreases, which in turn releases cytochrome C and activates the caspase-3 (cysteine-dependent aspartate-specific protease 3), induce cell apoptosis eventually [16]. In this study, ORI reduced the viability of U87MG cells in a dose-time-dependent manner, reduced the colony formation of U87MG cells, and blocked the U87MG cell cycle in G2/M phase. Meanwhile, ORI also reduced the expression of tumor proliferation marker Ki67, increased the protein expression of Bax and caspase-3, and reduced the protein expression of Bcl-2. These results indicated that ORI could inhibit the proliferation of U87MG cells and promote its apoptosis. In *in vivo* tumor xenograft, oridonin (15 mg/kg) inhibited the growth and weight of tumor significantly, increased the protein expression of caspase-3 and Bax, reduced the protein expression of Bcl-2, and increased tumor cell apoptosis. Both the results *in vivo* and *in vitro* showed that oridonin could inhibit glioma and promote the apoptosis of glioma cells. However, the underlying mechanism between ORI and its anti-glioma effect is still largely unknown.

Previous studies have found that Hippo signaling pathway was activated in many types of cancers which contain lung cancer, colorectal cancer, ovarian cancer and liver cancer [17]. Hippo signaling pathway is involved in regulating cell proliferation and organ function. YAP is the core transcriptional co-activator in the Hippo signaling pathway [18]. YAP is overexpressed and highly activated in various types of malignant tumors, promoted tumor cell proliferation, migration, and reduces apoptosis [19]. After entering the nucleus, YAP can directly regulate the transcription of c-Myc (cellular-myelocytomatosis oncogene) [20]. c-Myc participated in the regulation of cell cycle. c-Myc is also related to cell growth, differentiation, metabolism and apoptosis. The overexpression of c-Myc is closely related to the occurrence and metastasis of cancer [21]. Otherwise, when the Hippo signaling pathway is inhibited, the YAP residue S127 is phosphorylated. Therefore, the p-YAP (Ser127) cannot enter the nucleus and degrade in the cytoplasm, reducing the transcriptional effect of YAP [22, 23]. The decrease of YAP could cause down-regulation of c-Myc expression, which in turn inhibits cell proliferation and promotes apoptosis. In this experiment, oridonin not only inhibited the proliferation and growth of U87MG cells and tumor tissue *in vivo*, but also decreased the expression of YAP, c-Myc, Bcl-2, and increased the expression of p-YAP, Bax, and caspase-3. Overexpression of YAP increased the expression of YAP, c-Myc, Bcl-2 and decreased the expression of Bax and caspase-3. Interestingly, the effect of overexpressing YAP could be reversed by

ORI. These results suggest that oridonin may exert its anti-glioma effect by inhibiting the YAP pathway at least partly.

In conclusion, ORI can inhibit the proliferation of glioblastoma and promote its apoptosis, and its anti-glioblastoma effect may be achieved by mediating Hippo/YAP. This study provides a theoretical basis for the anti-glioblastoma effect of ORI, and may provide a potential therapeutic approach for the clinical treatment of glioblastoma. However, the occurrence of glioblastoma involves variety of factors, and further experiments such as more glioblastoma cell lines are needed to verify the anti-glioblastoma effect of ORI.

## **Declarations**

### **Acknowledgments**

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### **Author Contributions**

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Yonghong Zhang, Qingsong Jiang and Shuang Chen. The first draft of the manuscript was written by Chen Wang. The manuscript were revised by Liang Zhang and Hongmei Qiu. All authors read and approved the final manuscript.

### **Ethics declarations:**

#### **Ethics approval**

All animal treatments were performed in strict accordance with international ethical guidelines and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Approval was granted by the Ethics Committee of Chongqing Medical University.

#### **Consent to Participate**

Not applicable.

#### **Consent for Publication**

All authors read and approved the final manuscript.

## Competing Interests

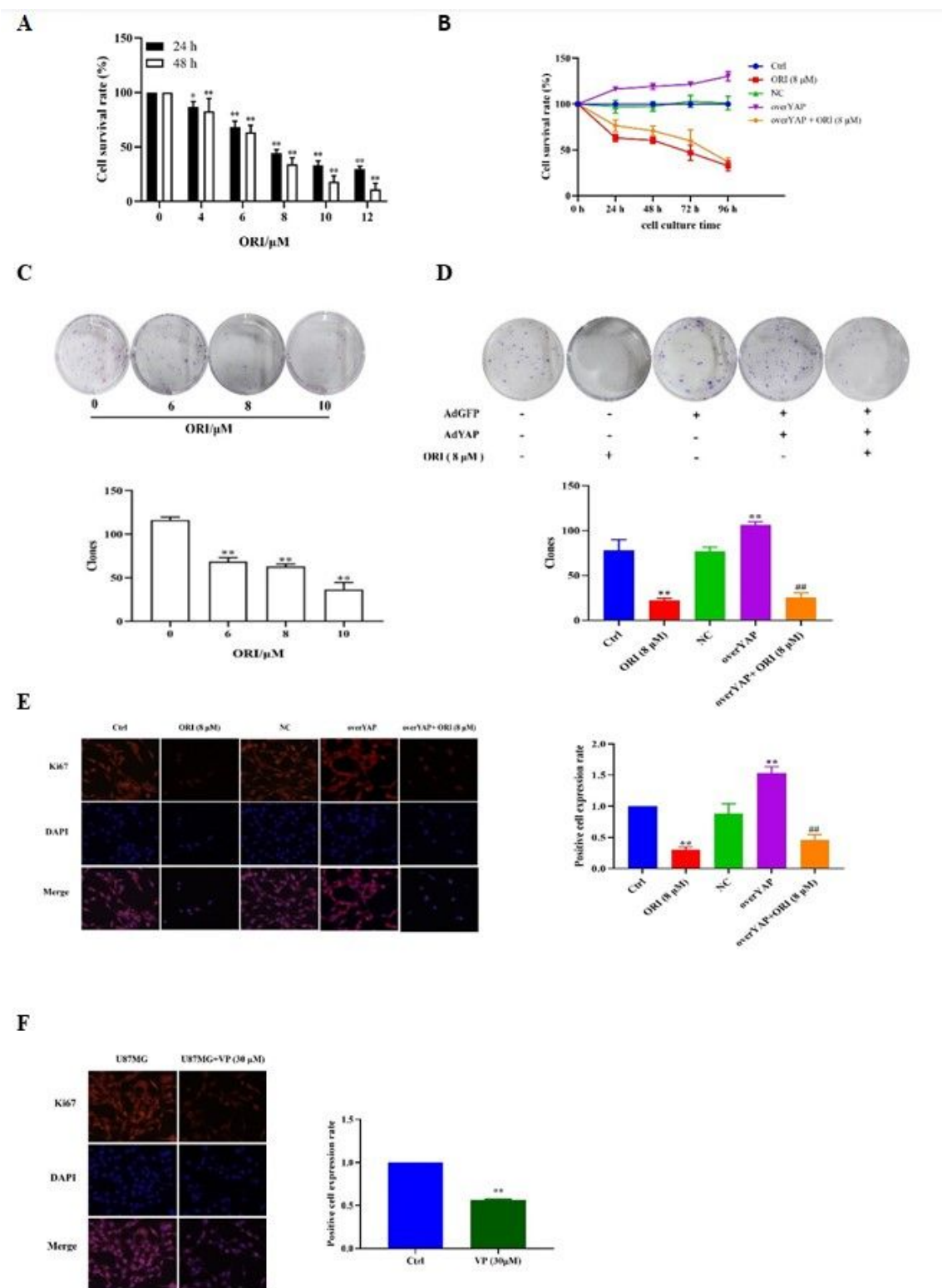
The authors declared no potential conflicts of interest.

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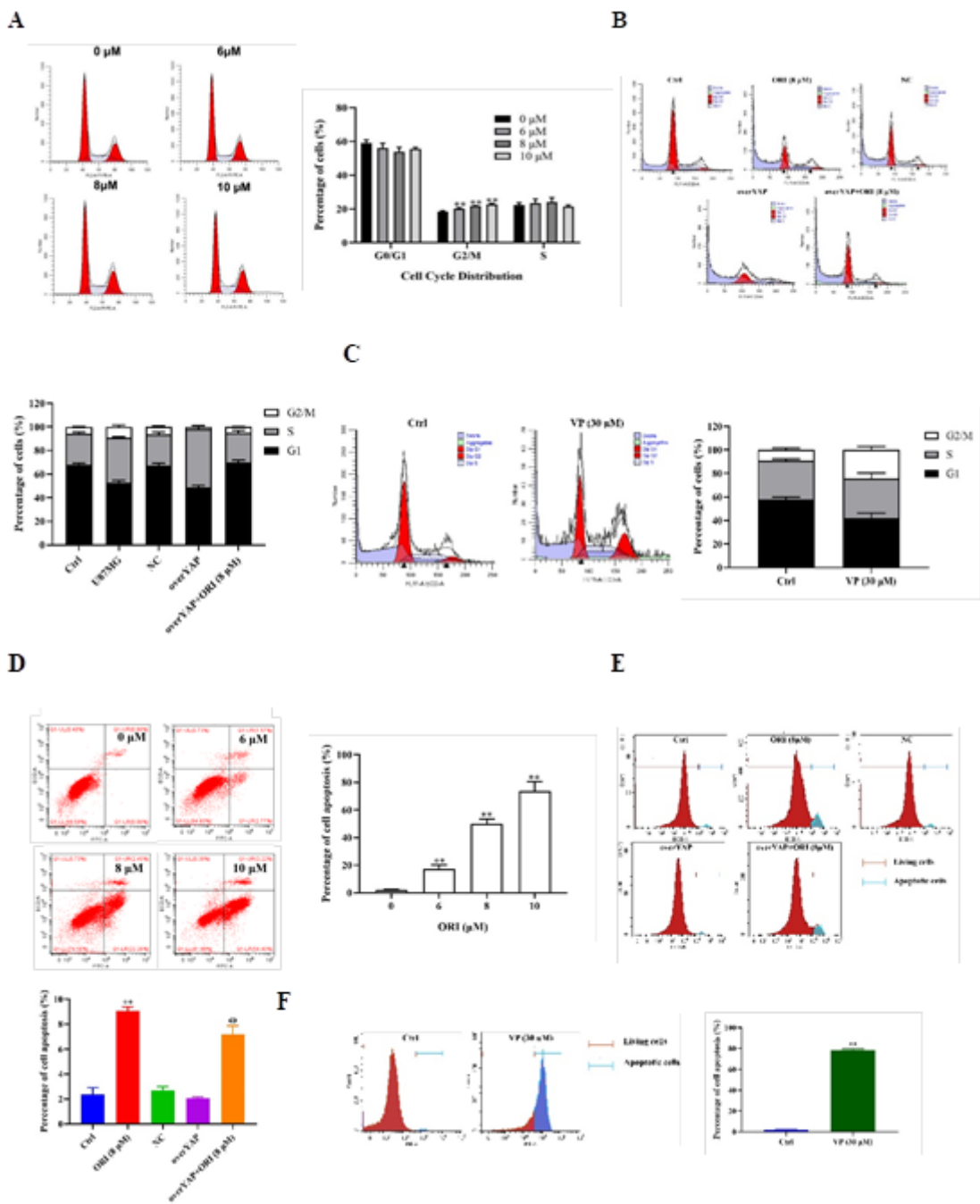
## Figures



**Figure 1**

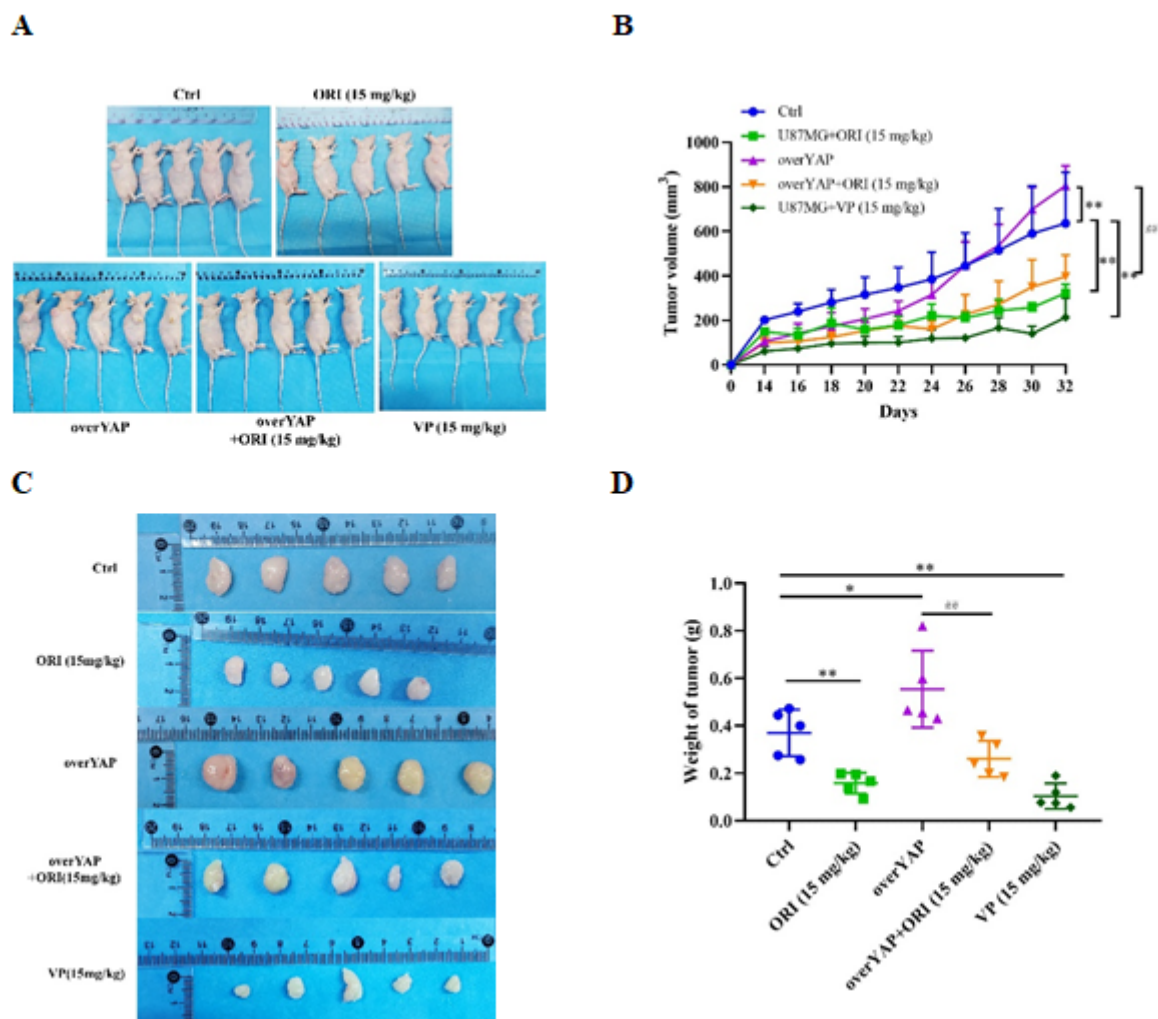
Effect of ORI on cell viability, colony formation and cell proliferation under different treatment factors. **(A)** Effect of ORI on U87MG cell viability under different concentrations. **(B)** Effects of overexpression of YAP and ORI on cell viability. **(C)** Effect of ORI on U87MG cell colony formation under different concentrations. **(D)** Effects of overexpression of YAP and ORI on colony formation. **(E)** Effects of overexpression of YAP and ORI on cell proliferation. **(F)** Effect of VP on U87MG cell proliferation. The data are expressed as the

mean  $\pm$  SD (n = 3). \* $P$  < 0.05, \*\* $P$  < 0.01 vs the control (U87MG) group. # $P$  < 0.05, ## $P$  < 0.01 vs the overYAP-U87MG group.



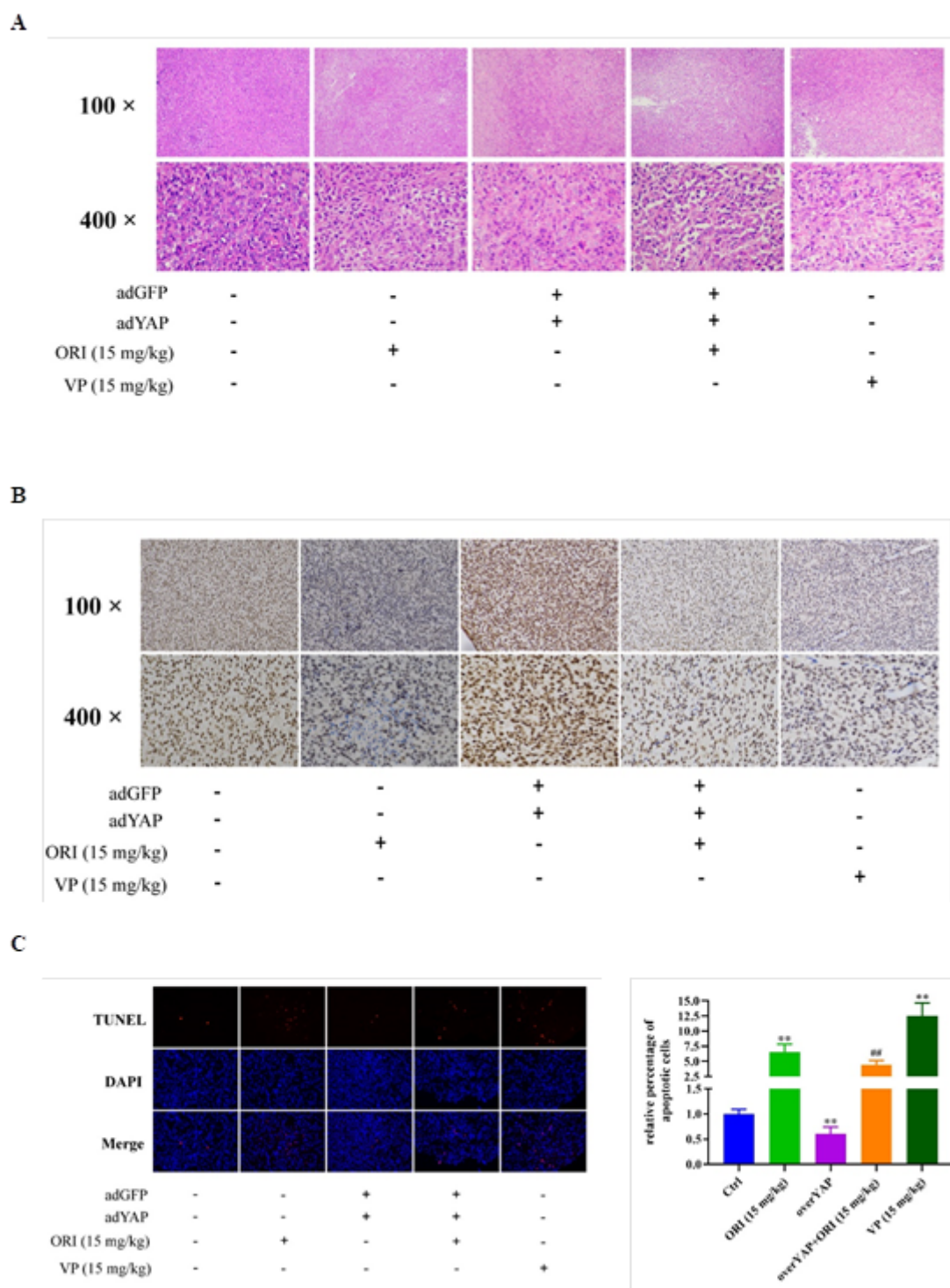
**Figure 2**

Cell cycle and apoptosis of U87MG after treatment with different factors. **(A)** Changes in cell cycle with different concentrations of ORI. **(B)** Changes in cell cycle with overexpression of YAP and ORI. **(C)** Effects of overexpression of YAP and ORI on cell cycle. **(D)** Changes in apoptosis with different concentrations of ORI. **(E)** Changes in apoptosis with overexpression of YAP and ORI. **(F)** Effects of overexpression of YAP and ORI on apoptosis. The data are expressed as the mean  $\pm$  SD (n = 3). \* $P$  < 0.05, \*\* $P$  < 0.01 vs the Ctrl group. # $P$  < 0.05, ## $P$  < 0.01 vs the overYAP group.



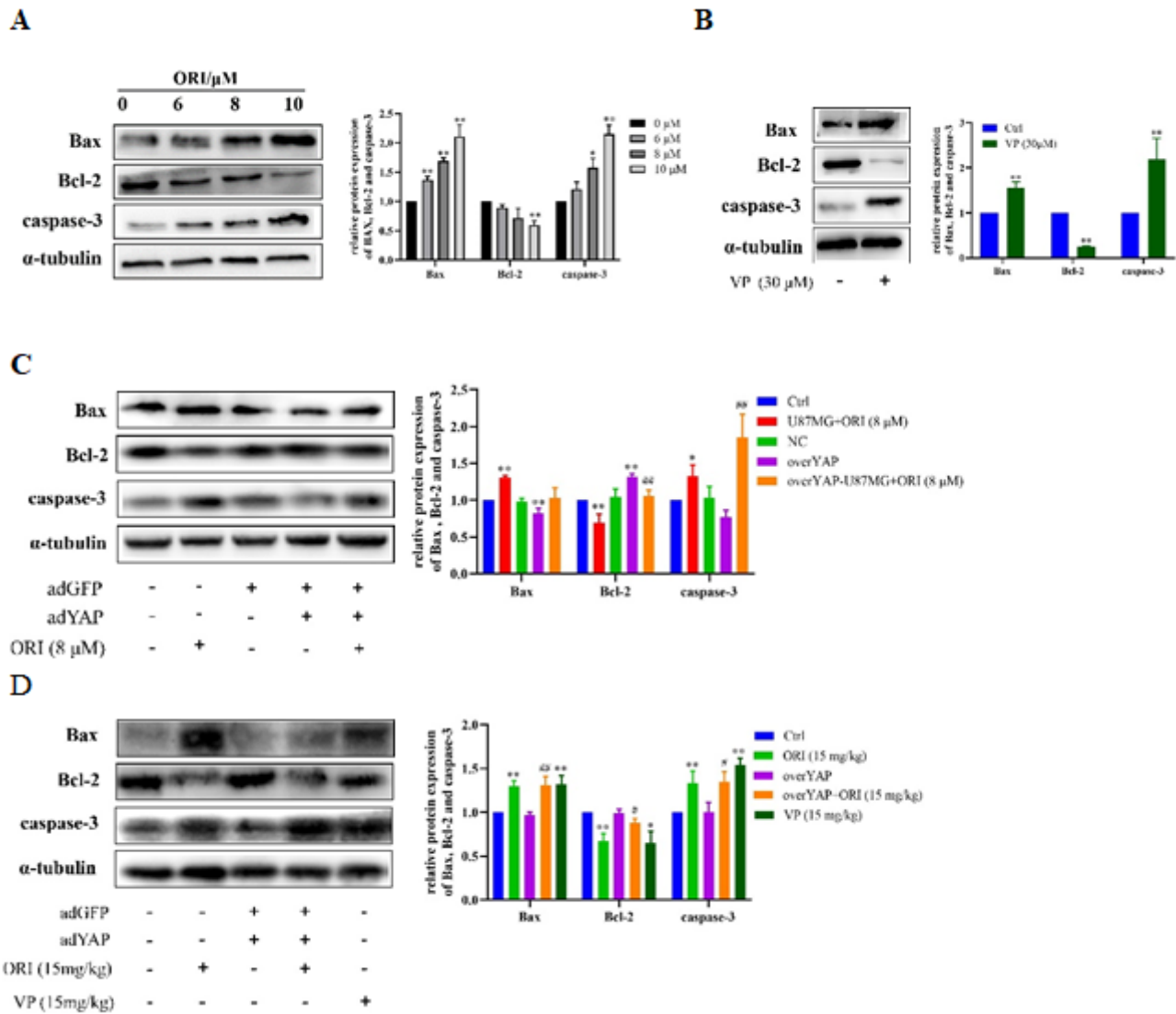
**Figure 3**

ORI represses the tumor growth in *vivo*. **(A)** Photographs of different groups of thymus-free nude mice after 32 days of subcutaneous tumor formation. **(B)** The periodic monitor of tumor dimensions. **(C)** and **(D)** Tumors were removed from the animals and weighed. The data are expressed as the mean  $\pm$  SD ( $n = 5$ ). \* $P < 0.05$ , \*\* $P < 0.01$  vs the Ctrl group. # $P < 0.05$ , ## $P < 0.01$  vs the overYAP group.



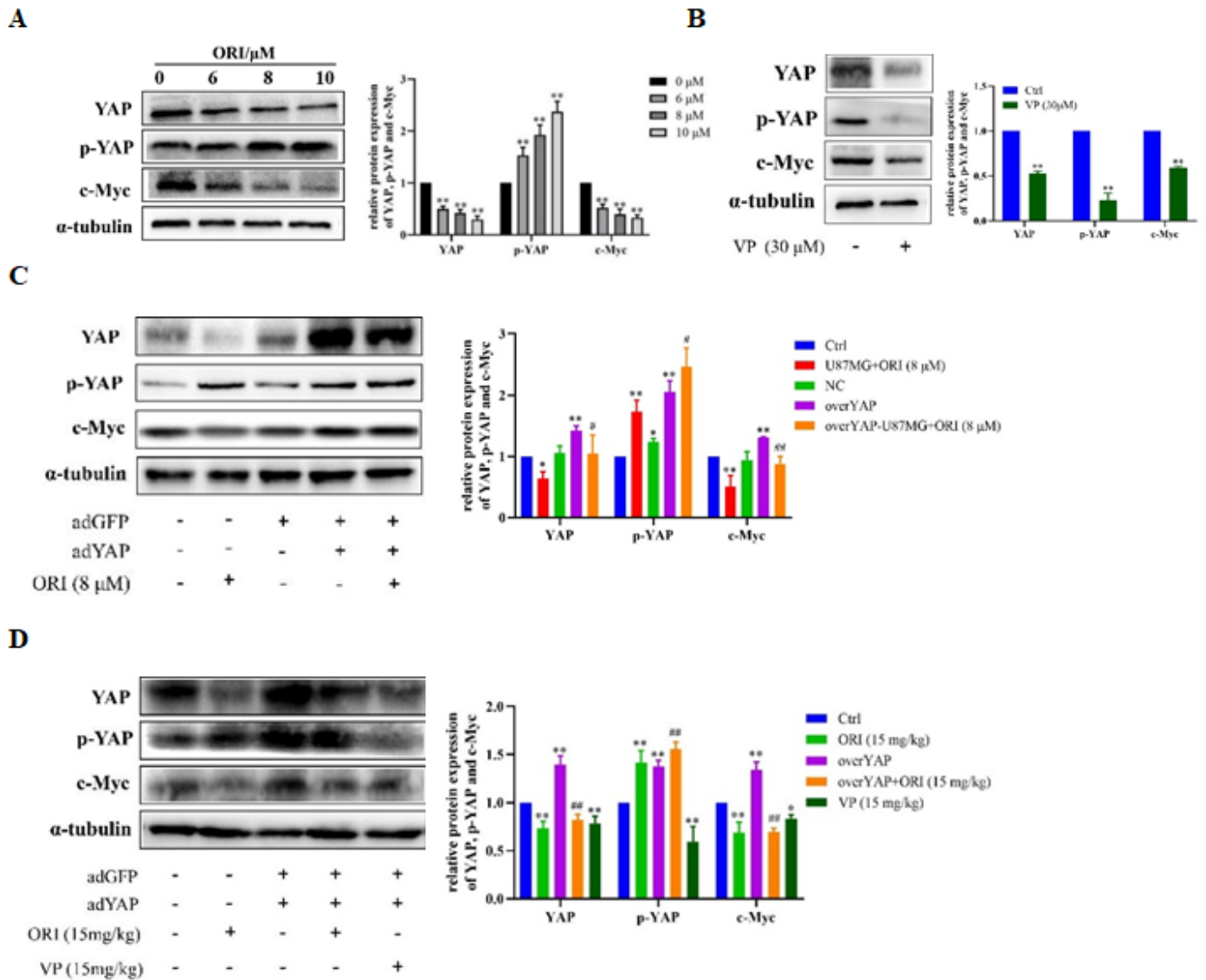
**Figure 4**

ORI inhibits tumor cells proliferation and promotes tumor cells apoptosis *in vivo*. **(A)** HE staining results of tumor tissues. **(B)** IHC staining results of tumor tissues. **(C)** TUNEL staining results and statistics of tumor tissues. The data are expressed as the mean  $\pm$  SD ( $n = 3$ ). \* $P < 0.05$ , \*\* $P < 0.01$  vs the Ctrl group. # $P < 0.05$ , ## $P < 0.01$  vs the overYAP group.



**Figure 5**

ORI regulates Bax, Bcl-2, caspase-3. **(A)** Effect of ORI-activated U87MG on Bax, Bcl-2 and caspase-3 under different concentrations. **(B)** Effect of VP-activated U87MG on Bax, Bcl-2 and caspase-3. **(C)** Effect of ORI and overexpression of YAP on Bax, Bcl-2 and caspase-3 in *vitro*. **(D)** Effect of ORI and overexpression of YAP on Bax, Bcl-2 and caspase-3 in *vivo*. The data are expressed as the mean  $\pm$  SD ( $n = 3$ ). \* $P < 0.05$ , \*\* $P < 0.01$  vs the Ctrl group. # $P < 0.05$ , ## $P < 0.01$  vs the overYAP group.



**Figure 6**

ORI regulates YAP, p-YAP and c-Myc. **(A)** Effect of ORI-activated U87MG on YAP, p-YAP and c-Myc under different concentrations. **(B)** Effect of VP-activated U87MG on YAP, p-YAP and c-Myc. **(C)** Effect of ORI and overexpression of YAP on YAP, p-YAP and c-Myc in *vitro*. **(D)** Effect of ORI and overexpression of YAP on YAP, p-YAP and c-Myc in *in vivo*. The data are expressed as the mean  $\pm$  SD ( $n = 3$ ). \* $P < 0.05$ , \*\* $P < 0.01$  vs the Ctrl group. # $P < 0.05$ , ## $P < 0.01$  vs the overYAP group.