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Original Article

Beta-catenin knockdown impairs the viability of ovarian cancer cells by modulating YAP-dependent glycolysis

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Abstract: Objectives: Ovarian cancer (OC) ranks fifth among the main causes of cancer-related deaths in women worldwide. PCLAF/KIAA0101 and Yes-associated protein (YAP) have been linked to several human malignant cancers, including OC. However, the roles of KIAA0101 and YAP in glycolysis-dependent OC cell proliferation remain unknown. Methods: qRT-PCR and western blot were performed to analyze the KIAA0101 expression. Short hairpin RNA transfection was performed to silence KIAA0101 expression in cells. Cell viability and apoptosis were assayed by colony formation and flow cytometry, respectively. Glucose uptake, lactate production, and glycolytic enzyme expression were assessed to determine the level of cellular glycolysis. Phosphorylation and the nuclear localization of YAP were assessed to determine YAP activation. Results: OC tissue and cell lines exhibited higher KIAA0101 expression than the non-cancerous tissues and cells. KIAA0101 silencing reduced the proliferation and increased the apoptosis of both A2780 and ES-2 OC cell lines. Furthermore, KIAA0101 depletion suppressed glycolysis and YAP activation, as evidenced by increased YAP phosphorylation and decreased nuclear localization. Reactivation of YAP was performed by administration of mitochonic acid 5 in both OC cell lines with KIAA0101 knockdown. Glucose uptake, lactate production, phosphofructokinase, pyruvate dehydrogenase beta, pyruvate kinase M2, triosephosphate isomerase 1, glucose-6-phosphate dehydrogenase, enolase 1, and lactate dehydrogenase expression levels in cells recovered after the reactivation of YAP. Additionally, YAP reactivation increased cell proliferation and inhibited apoptosis. Conclusions: This study showed that KIAA0101 could promote glycolysis during nasopharyngeal carcinoma development through YAP signaling activation, suggesting that KIAA0101 could serve as a target for OC treatment.

Keywords: Ovarian cancer, KIAA0101, Yes-associated protein, glycolysis, MA5

Introduction

Epithelial ovarian cancer (OC) is a main gynecologic malignancy, with 3-12/100,000 women being diagnosed with OC annually [1]. Traditional therapies for OC include surgery, chemotherapy based on Taxol and platinum, and radiation therapy [1]. Early OC symptoms are difficult to detect, and most patients with OC are diagnosed at the terminal stage. Therefore, there is an urgent need to enhance early diagnosis of OC and establish novel treatment methods for effective intervention.

PCNA clamp associated factor (PCLAF), also called KIAA0101, is a PCNA-associated protein [2] that possesses a conserved PCNA-binding motif and competes with the CDK inhibitor p21 for PCNA binding. KIAA0101 ubiquitination is able to facilitate its binding with PCNA at the late S-phase [3]. KIAA0101 is involved in modulating diverse biologic processes, such as DNA repair [4], cell proliferation and cell cycle progression [5]. KIAA0101 acts as an oncogene in various human malignant cancers, such as OC, renal cell carcinoma, hepatocellular carcinoma, gastric, esophageal cancer and breast cancer [6-12]. However, the biologic mechanisms of KIAA0101 in OC remain unclear.

The hippo pathway is a highly conserved tumor inhibitor pathway, that mainly consists of large tumor suppressor 1/2 (LATS1/2), mammalian Ste20-like kinases 1/2 (MST1/2), Yes-associated protein (YAP), and/or its paralog TAZ (also called WWTR1). LATS1/2 and MST1/2 are onco-suppressive kinases. Once this pathway is activated, MST1/2 will phosphorylate and

activate LATS1/2, which in turn causes YAP phosphorylation and inhibition [13]. Hippo pathway inactivation is tightly associated with the occurrence and progression of various tumors [14]. Abnormal overexpression of YAP has been observed in various tumors. *YAP* can promote tumorigenesis and is considered to be an oncogene in many solid cancers [15, 16], including OC [17-19].

Tumor cells, unlike healthy cells, are largely dependent on glycolysis to generate energy and metabolites that are essential to support cellular proliferation, even under normoxic conditions, which is known as the Warburg effect [20]. Aerobic glycolysis is widely regarded as a downstream consequence of tumor progression and may be triggered by oncogenes. These observations indicate that glycolysis is capable of facilitating tumor malignancy through modulating certain oncogenic signals, such as those elicited by YAP activation [21, 22]. These studies suggest that YAP-dependent glycolysis plays a role in OC development.

The aim of this study was to explore the role of KIAA0101 in YAP-dependent glycolysis in OC cells. Expression of KIAA0101 was detected in non-cancerous tissue as well as in OC tissue samples. KIAA0101 was then knocked down in two OC cell lines to determine its roles in cell proliferation, apoptosis, YAP activation, and glycolysis. Moreover, reactivation of YAP was utilized to reveal its involvement in the KIAA0101-regulated cell proliferation, apoptosis and glycolysis in OC cells.

Materials and methods

Patients and tissue samples

Totally, 22 OC and 8 healthy fallopian tube tissue samples were harvested at the Obstetrics and Gynecology Department (Changning Maternity and Infant Health Hospital, East China Normal University) between 2015 and 2019. All patients were followed up for no less than two years. Histological grade and tumor stage were defined according to the recently revised staging system from *International Federation of Gynecology and Obstetrics 2013* [23]. Informed consent was obtained from patients or their guardians. The study was approved by the Ethics Committee of Changning Maternity and Infant Health Hospital, East China Normal University.

Cell culture and transfection

Human OC cells (CAOV3, OVCAR3, SKOV-3, A2780, HO-8910 and ES-2) and immortalized human FTE187 cells were obtained from the American Type Culture Collection (ATCC). OC cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS, Invitrogen), penicillin (100 mg/mL, Sigma-Aldrich) and streptomycin (100 mg/mL, Sigma-Aldrich). FTE187 cells were cultured in MCDB105 medium (Sigma-Aldrich) and Medium 199 (Thermo Fisher Scientific) at a ratio of 1:1, supplemented with epidermal growth factor (10 ng/mL) and 10% FBS. All cells were cultured at 37°C in a humidified 5% CO_2 atmosphere.

Transfection

The short hairpin RNA encoding KIAA0101 (shRNA; 5'-AGA GCT ACG AGC TGC CTG AC-3') and the shRNA encoding NC (5'-TCC TCT TGG AGA TCA GCC G-3') were obtained from RiboBio (Guangzhou, China). shRNAs (100 nM) were used for the transfection of each cell line using RNAiMAX (Thermo Fisher).

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

RNA was isolated with TRIzol®, and RNA concentrations were measured using a NanoDrop™ 2000 device (OD260). cDNA was prepared via reverse transcription with the use of Oligo $(dT)_{20}$ primer and an MMLV First-Strand Kit for real-time PCR, SYBR™ Select Master Mix (Invitrogen). qRT-PCR was conducted using a QIAGEN SYBR™ Green Based One-Step qRT-PCR Kit. PCR was carried out as the following procedures: first denaturation (95°C, 10 min), followed by 40 denaturation cycles (95°C, 15 s) and extension (60°C, 40 s). mRNA expression was calculated through 2-ΔΔCT method with *GAPDH* as an internal reference. The primers used for qRT-PCR are shown in **Table 1**. All experiments were performed in triplicate.

Western blotting (WB)

Whole-cell lysates were obtained by incubating the cells with RIPA buffer (pH 8.0, Thermo Fisher Scientific) containing a protease inhibi-

Table 1. Sequences of primers used for qRT-PCR

Primer	Sequences
KIAA0101 Forward	5'-CCA ATA TAA ACT GTG GCG GG-3'
KIAA0101 Reverse	5'-TGG CCT CAA GTG ATC CTC-3'
GAPDH Forward	5'-GGT GAA GGT CGG AGT CAA CGG A-3'
GAPDH Reverse	5'-GAG GGA TCT CGC TCC TGG AAG A-3'

tor cocktail (Abcam). Protein concentration was measured using BCA kit (Thermo Fisher Scientific). The proteins extracted were subjected to SDS-PAGE and electrophoretic transfer onto PVDF membranes (Bio-rad). Next, the unoccupied sites on the membranes were blocked with skimmed milk, and the membranes were incubated with primary antibodies overnight at 4°C, followed by rinsing with TBST. The immunoblots were then incubated with secondary antibodies (Beyotime) at room temperature for 1 h. The antibodies used for the WB included the following: anti-KIAA0101 (1:1000, PA5-87838, Thermo Fisher Scientific), anti-b-actin (1:5000, ab8227, Abcam), anti-p-YAP (1:2000, 4911S, Cell Signaling Technology), anti-YAP (1:2000, 4912, Cell Signaling Technology), anti-lamin B1 (1:500, ab65986, Abcam), anti-PFK (1:1000, sc-377346, Santa Cruz Biotechnology), anti-TPI (1:1000, ab96-696, Abcam), anti-G6PD (1:500, sc-3738-86, Santa Cruz Biotechnology), anti-PKM2 (1:1000, 4053, Cell Signaling Technology), anti-ENO1 (1:1000, ab155102, Abcam), anti-LDHA (1:2000, 2012, Cell Signaling Technology) and anti-PDHB (1:1000, ab110331, Abcam). After rinsing several times with TBST, the antigen bands were visualized using a maximum sensitivity substrate kit (Thermo Fisher Scientific).

Colony formation assay

Cells were inoculated in 6-well plates (5×10^3 cells/well) and cultivated overnight until colonies were observed. Cells were next subjected to crystal violet staining. ImageJ software was used for measuring the colony number. Each experiment was conducted in triplicate and repeated three times.

Flow cytometry (FC)

Cell apoptosis was evaluated by FC, using an annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) - based apoptosis detection kit (Thermo Fisher Scientific). After suspension in binding buffer (20 μL), cells were subjected to Annexin V-FITC (10 μL) and PI (5 μL). A flow cytometer (Agilent) was used to test the cell apoptosis rate.

Determination of lactate and glucose levels

DT cell lactate and glucose levels were measured using Lactate Assay Kit II and High Sensitivity Glucose Assay Kit (Sigma, Shanghai, China), respectively.

Ethics statement

The study on human patient samples was approved by the Ethics Committee of Changning Maternity and Infant Health Hospital, East China Normal University.

Data analysis

All data were analyzed using SPSS software and GraphPad Prism 7 software. All data were shown as mean ± SD. Student's *t*-test was used to assess the differences between two experimental groups. ANOVA and Tukey's post hoc test were used for comparison among multiple groups. $P < 0.05$ indicated statistical significance.

Results

Elevated KIAA0101 mRNA expression in OC cells and tissue samples

qRT-PCR analysis revealed higher *KIAA0101* mRNA level in the 22 OC tissue samples than in the 8 healthy control samples (**Figure 1A**). Furthermore, compared to non-cancerous FTE187 cells, the two OC cell lines exhibited elevated KIAA0101 expressions at both mRNA and protein levels (**Figure 1B**, **1C**).

KIAA0101 silencing suppressed proliferation and YAP-dependent glycolysis and elicited the apoptosis of A2780 and ES-2 cells

A decrease in KIAA0101 expression at both the mRNA and protein levels, relative to its levels in the shNC transfection group, was observed after the cells were subjected to shKIAA0101 transfection (**Figure 2A-D**).

The proliferative capacity of A2780 and ES-2 cells was assayed by colony formation. The

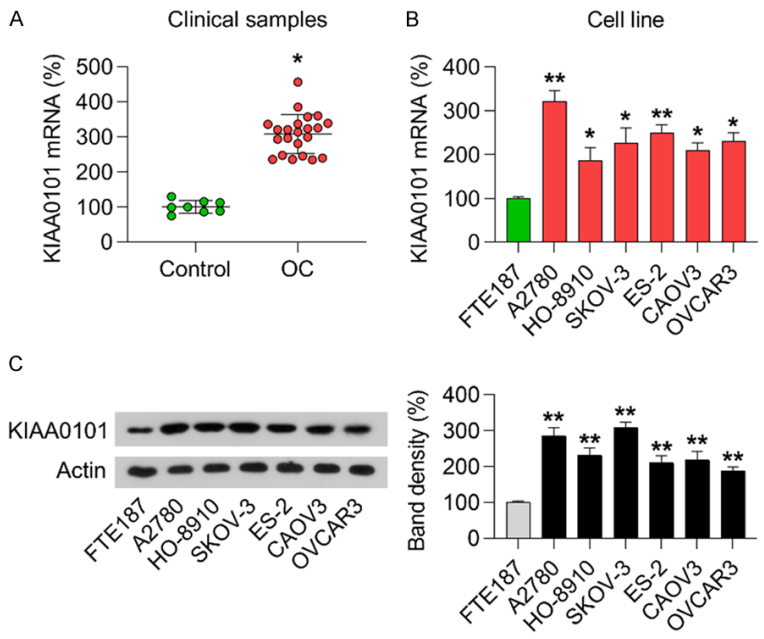

Figure 1. Expression of PCNA clamp associated factor (PCLAF/KIAA0101) in ovarian cancer (OC) clinical cells and samples. A. Quantitative reverse transcription-PCR (qRT-PCR) showing different expression levels of KIAA0101 in OC tissue samples (n = 22) and samples from healthy volunteers (n = 8). B, C. qRT-PCR and western blot analyses of KIAA0101 mRNA and protein expression levels, respectively, in OC cells (A2780, HO-8910, SKOV-3, ES-2, CAOV3, and OVCAR3) and FTE187 cells. *P < 0.05, **P < 0.01 vs. Control/FTE187 group.

data from this experiment showed that the reduction in KIAA0101 expression resulted in a decline in the number of colonies of A2780 and ES-2 cells (**Figure 3A**, **3B**).

To determine the effect of KIAA0101 depletion on the apoptosis of OC cells, FC with annexin V-FITC and PI staining was carried out. The cells transfected with shNC exhibited a low proportion of apoptotic cells, while silencing of KIAA0101 increased the proportion of apoptotic cells (approximately 25%-30%) (**Figure 3C**, **3D**). These findings demonstrate that KIAA0101 silencing can induce apoptosis in OC cell lines.

As mentioned above, YAP can activate glycolysis, which is required for tumor cell proliferation [13]. Thus, activation of YAP in OC cells was determined by examining its phosphorylation and nuclear localization. WB results revealed an increase in the phosphorylation of YAP, as well as a decrease in its nuclear localization in A2780 and ES-2 cells in response to KIAA0101 knockdown (**Figure 4A**, **4B**). This suggests that KIAA0101 depletion can lead to the deactivation of YAP in OC cell lines.

To determine whether KIAA0101 is involved in aerobic glycolysis in DT cells, glucose consumption and lactate generation were evaluated. Depletion of KIAA0101 reduced glucose consumption and lactate generation in OC cells relative to those in the shNC transfection group (**Figure 4C-F**). WB was performed to determine the expression levels of key glycolytic enzymes in DT-06 and DT-09 cells after β-catenin silencing. The results showed that the levels of phosphofructokinase (PFK), pyruvate dehydrogenase beta (PDHB), pyruvate kinase M2 (PKM2), triosephosphate isomerase 1 (TPI), glucose-6-phosphate dehydrogenase (G6PD), enolase 1 (ENO1) and lactate dehydrogenase (LDHA) decreased after KIAA0101 expression was downregulated in OC cells (**Figure 4G**, **4H**). These findings suggest that KIAA0101 silencing can attenuate YAP-dependent glycolysis in OC cells.

YAP reactivation counteracted the effect of KIAA0101 knockdown in A2780 and ES-2 cells

To elucidate the interaction between KIAA0101 and YAP, cells were transfected with shKIAA0101 and then exposed to 5 μM of mitochonic acid 5 (MA5). WB revealed the phosphorylation of YAP to be noticeably reduced, while there was an increase in the nuclear localization of YAP in OC cells with KIAA0101 knockdown in the MA5 treatment group, relative to that in the control group (**Figure 5A**, **5B**). These findings indicate that MA5 treatment can reactivate YAP in OC cells with KIAA0101 silencing.

Glucose consumption and lactate generation were examined in KIAA0101-silenced OC cells with or without MA5 treatment. MA5 treatment restored glucose consumption and lactate gen-

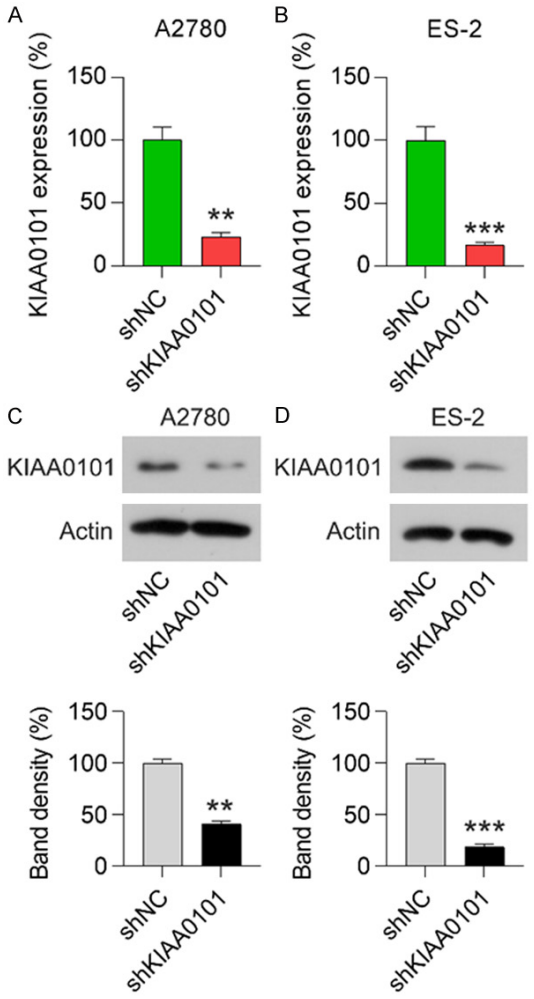

Figure 2. Silencing of KIAA0101 in A2780 and ES-2 cells. Cells were treated with shRNA-NC (shNC) or shRNA-KIAA0101 (shKIAA0101) for 1 d. (A, B) qRT-PCR and (C, D) western blot analyses of KIAA0101 mRNA and protein levels, respectively, in both cell lines. **P < 0.01, ***P < 0.001 vs. shNC group.

eration in OC cells with KIAA0101 silencing (**Figure 6A-D**). WB showed a recovery in PFK, PDHB, PKM2, TPI, G6PD, ENO1, and LDHA expression levels in the KIAA0101-silenced OC cells, in response to MA5 administration (**Figure 6E**, **6F**). These results demonstrate that YAP reactivation can offset the impact of KIAA0101 silencing on OC cell glycolysis.

Colony formation assays showed that the administration of MA5 restored the proliferative capacity of KIAA0101-silenced OC cells compared to that of the untreated cells (**Figure 7A**, **7B**). Next, the role of YAP reactivation in KIAA0101 depletion-triggered apoptosis of A2780 and ES-2 cells was determined. MA5 treatment reduced the rate of apoptosis, as demonstrated via FC (**Figure 7C**, **7D**). These data indicate that YAP-dependent glycolysis can modulate the regulatory effects of KIAA0101 on cell viability and OC cell death.

Discussion

Several studies have indicated a role of KIAA0101 in modulating the proliferation, migration, and chemoresistance of OC cancer cells [11, 12], but the relevant mechanisms that underlie its role in the glycolysis of OC cells are yet unclear. In the present study, KIAA0101 expression was shown to be upregulated in OC cells and tissue samples and to be positively associated with the activation of YAP signaling. Additionally, we found that KIAA0101 served as a positive modulator of glycolysis by increasing nuclear localization, as well as downregulating the phosphorylation of YAP. Furthermore, use of the YAP activator MA5 effectively counteracted the effects of glycolysis on OC cell proliferation and apoptosis in vitro. Collectively, these results indicate KIAA0101 to be a possible target for OC therapy.

Although the functional roles of KIAA0101 in tumor cell proliferation, migration, and chemoresistance have been well established, the underlying mechanisms through which KIAA0101 promotes OC cell proliferation and inhibits its apoptosis remain unclear. Chen et al. found KIAA0101 expression to be upregulated in metastatic epithelial OC tissue compared to that in primary epithelial OC tissue. KIAA0101 was noted to be involved in epithelial cell migration and chemoresistance through the upregulation of the Wnt/β-catenin signaling pathway [11]. From a therapeutic perspective, targeting KIAA0101 is of particular interest, as we observed an upregulation of KIAA0101 expression in OC, which was found to be associated with OC cell proliferation. Our findings are concordant with those of Chen et al. [11].

A key finding of this study is the role of KIAA0101 in glycolysis. Even in the presence of sufficient oxygen, tumor cells are able to generate energy by glycolysis. Activation of the glycolysis pathway has been reported to be closely associated with the occurrence and progression of tumors [24, 25] and to play a critical role in tumor

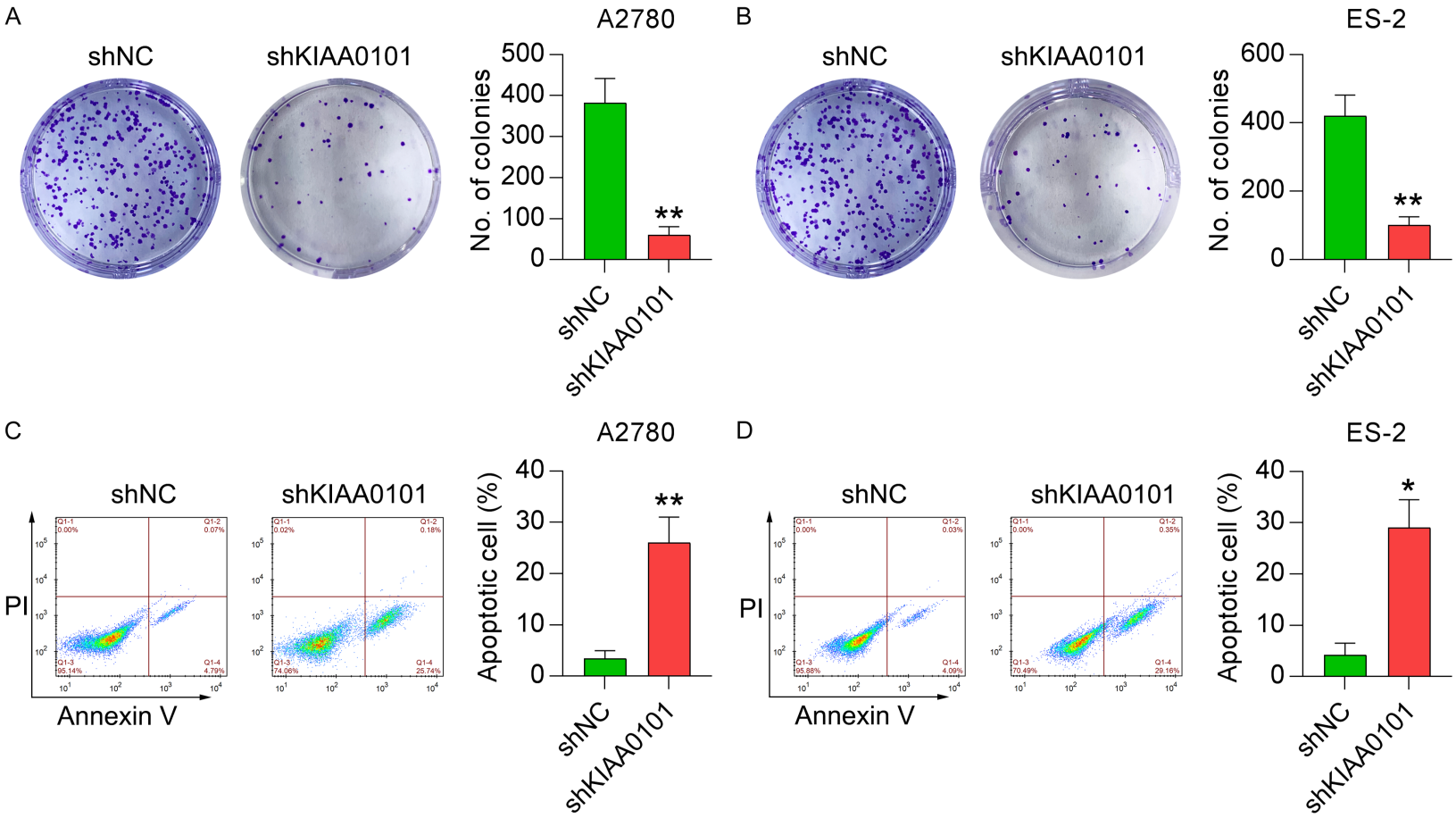

Figure 3. Effect of KIAA0101 silencing on A2780 and ES-2 cell growth and apoptosis. Cells were treated with shRNA-NC (shNC) or shRNA-KIAA0101 (shKIAA0101) for 1 d. A, B. Cell growth measured by colony formation assay. C, D. Flow cytometry analysis of apoptosis. *P < 0.05, **P < 0.01 vs. shNC group.

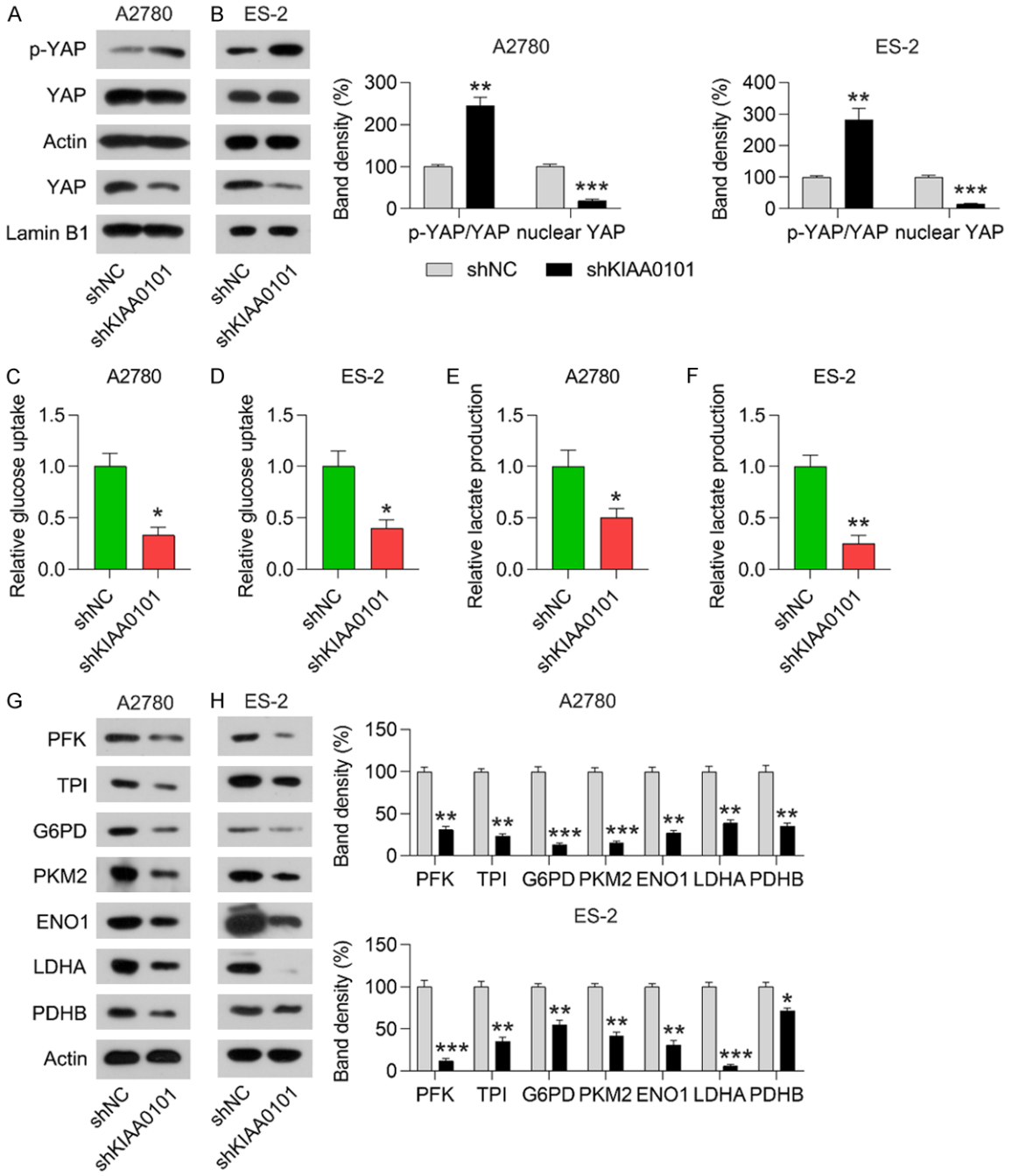

Figure 4. Effect of KIAA0101 silencing on Yes-associated protein (YAP)-dependent glycolysis in A2780 and ES-2 cells. Cells were treated with shRNA-NC (shNC) or shRNA-KIAA0101 (shKIAA0101) for 1 d. (A, B) Western blot analysis of the expression, phosphorylation and nuclear localization of YAP in both cell lines. Quantification of (C, D) glucose consumption and (E, F) lactate production. (G, H) Western blot analysis of phosphofructokinase (PFK), pyruvate dehydrogenase beta (PDHB), pyruvate kinase M2 (PKM2), triosephosphate isomerase 1 (TPI), glucose-6-phosphate dehydrogenase (G6PD), enolase 1 (ENO1) and lactate dehydrogenase (LDHA) expressions in both cell lines. *P < 0.05, **P < 0.01, ***P < 0.001 vs. shNC group.

growth and resistance to therapy [26]. A number of enzymes in the glycolytic pathway have been considered as possible chemotherapeutic targets [27-30]. To the best of our knowledge, this is the first study to establish a link between KIAA0101 and cancer cell glycolysis.

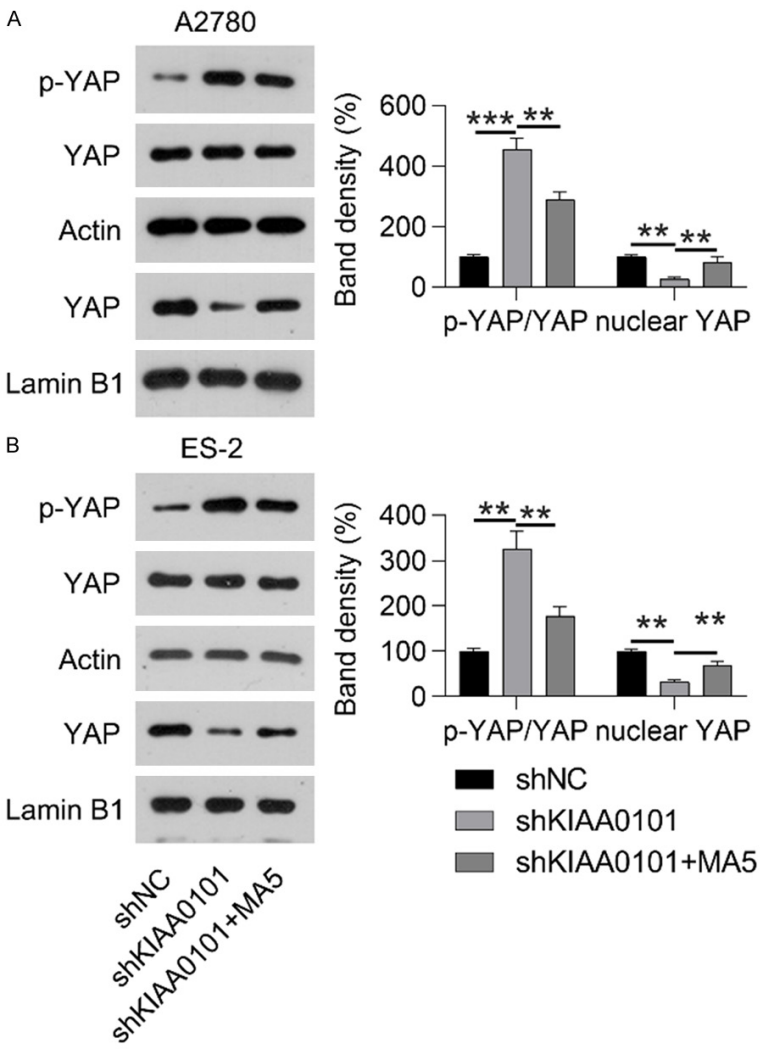

Figure 5. Reactivation of Yes-associated protein (YAP) in ovarian cancer cells with KIAA0101 silencing. Cells were treated with shRNA-NC (shNC) or shRNA-KIAA0101 (shKIAA0101) for 1 d and then exposed to 5 μM MA-5 for 1 d. A, B. Western blot analysis of the expression, phosphorylation and nuclear localization of YAP in both cell lines. **P < 0.01, ***P < 0.001, vs. shNC/shKIAA0101 group.

During metabolic processes, the expression of key enzymes, such as PKM2, is upregulated [13]. Dysregulation of some pathways is also associated with metabolic reprogramming in cancer [31, 32]. YAP is a major effector molecule of the Hippo pathway and can become sequestered in the cytoplasm, leading to protein degradation and the inhibition of cell proliferation [33-35]. YAP can activate transcription through promoters with specific recognition sites and plays a critical role in gastric cancer [36]. Moreover, it is considered to be a multiple-input downstream effector, similar to the Hippo, WNT/β-catenin, and JNK pathways [37-39]. YAP activation also promotes glycolysis in tumor cells. YAP mutation can result in a stable active form of YAP, which causes an elevation in glucose uptake and lactate generation [40, 41]. Therefore, we examined whether YAP is involved in KIAA0101-mediated glycolysis, although an association between YAP and KIAA0101 has not been reported to date. Here, YAP was found to be deactivated in OC cell lines following KIAA0101 depletion, accompanied by attenuated glucose uptake, lactate production and low expression of glycolytic enzymes. MA5 treatment counteracted the effects of KIAA0101 knockdown on these glycolysis-associated phenotypic changes. These observations suggest that YAP plays a role in KIAA0101-regulated glycolysis during OC development.

The present study has several limitations. First, the role of KIAA0101 in glycolysis during OC tumorigenesis and development was not assessed in animals. Second, the mechanism by which KIAA0101 cause dephosphorylation of YAP was not investigated, and this underlying mechanism of action needs to be investigated in the future. Third, the mechanism, how does the transcriptional modulator YAP regulate glycolytic enzymes, such as PKM2, was not investigated in the current study. In our future studies, these three key points will be further explored.

In summary, this study demonstrated that KIAA0101 could promote glycolysis through activation of YAP signaling, suggesting that KIAA0101 is a OC treatment target. Despite

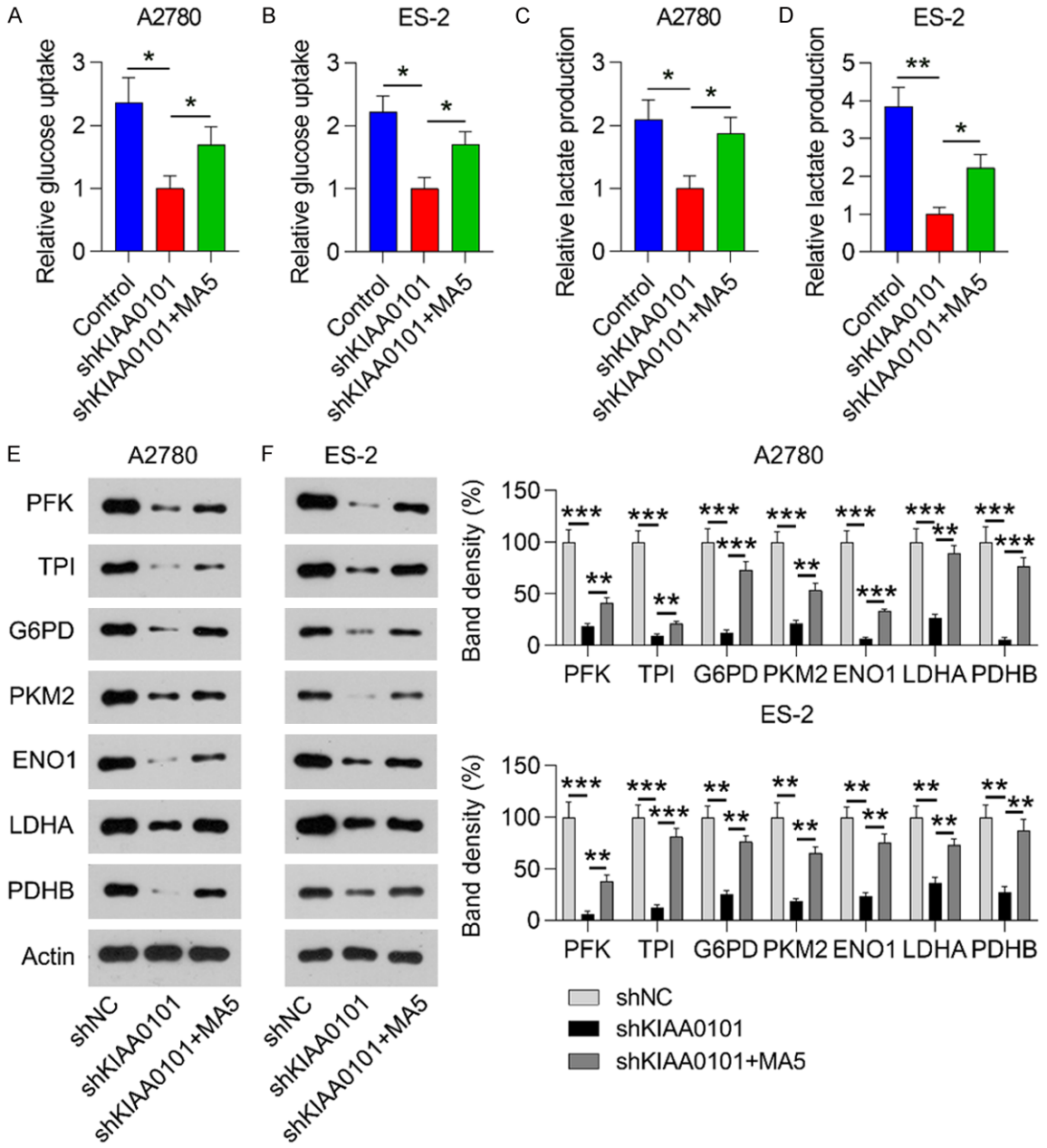

Figure 6. Reactivation of Yes-associated protein restored glycolysis in ovarian cancer cells with KIAA0101 silencing. Cells were treated with shRNA-NC (shNC) or shRNA-KIAA0101 (shKIAA0101) for 1 d and then exposed to 5 μM mitochonic acid 5 (MA5) for 1 d. A, B. Measurement of glucose consumption. C, D. Measurement of lactate levels. E, F. Western blot analysis of phosphofructokinase (PFK), pyruvate dehydrogenase beta (PDHB), pyruvate kinase M2 (PKM2), triosephosphate isomerase 1 (TPI), glucose-6-phosphate dehydrogenase (G6PD), enolase 1 (ENO1) and lactate dehydrogenase (LDHA) expressions in both cell lines. *P < 0.05, **P < 0.01, ***P < 0.001, vs. shNC/shKIAA0101 group.

these advances in the understanding of the role of KIAA0101 in OC, whether KIAA0101 can serve as a diagnostic marker or can help in the development of clinical candidates for OC treatment still needs to be confirmed.

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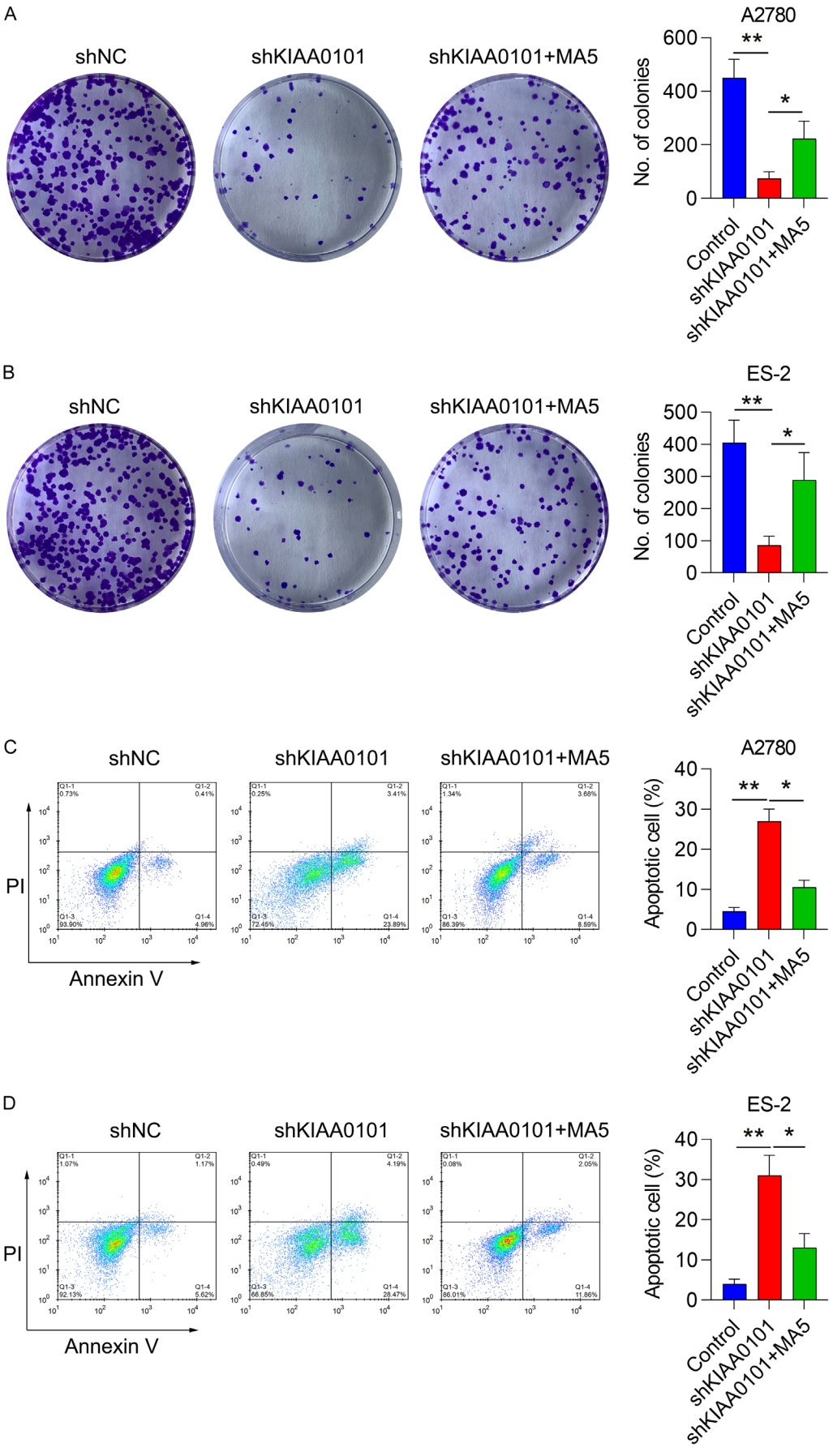
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shNC
shKIAA0101
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No. of colonies
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Control
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B
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Control
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PI
Annexin V
Q1-1 0.73%
Q1-2 0.41%
Q1-3 93.90%
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Q1-4 23.89%
Q1-1 1.34%
Q1-2 3.68%
Q1-3 86.39%
Q1-4 8.59%
A2780
Apoptotic cell (%)
40
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shKIAA0101+MA5
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Annexin V
Q1-1 1.07%
Q1-2 1.17%
Q1-3 92.13%
Q1-4 5.62%
Q1-1 0.49%
Q1-2 4.19%
Q1-3 66.85%
Q1-4 28.47%
Q1-1 0.08%
Q1-2 2.05%
Q1-3 86.01%
Q1-4 11.86%
ES-2
Apoptotic cell (%)
40
30
20
10
0
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*
Control
shKIAA0101
shKIAA0101+MA5

Figure 7. Reactivation of Yes-associated protein recovered proliferation and suppressed apoptosis of ovarian cancer cells with KIAA0101 silencing. Cells were treated with shRNA-NC (shNC) or shRNA-KIAA0101 (shKIAA0101) for 1 d and then exposed to 5 μM MA-5 for 1 d. A, B. Cell growth measured by colony formation assay. C, D. Flow cytometry analysis of apoptosis. *P < 0.05, **P < 0.01 vs. shNC/shKIAA0101 group.

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Disclosure of conflict of interest

None.

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