CIP2A interacts with AKT1 to promote the malignant biological behaviors of oral squamous cell carcinoma by upregulating the GSK-3β/β-catenin pathway

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Abstract. Oral squamous cell carcinoma (OSCC) is one of the most common malignancies worldwide, which is associated with a poor prognosis. The present study aimed to investigate the role of cancerous inhibitor of protein phosphatase 2A (CIP2A) in OSCC and its regulatory effect on AKT1. Firstly, CIP2A and AKT1 expression in OSCC cells was detected by western blotting. After silencing CIP2A, cell viability and cell proliferation were assessed using the Cell Counting Kit-8 assay and 5-ethynyl-2'-deoxyuridine staining. Cell apoptosis was evaluated by TUNEL staining and the expression of apoptosis-related proteins was assessed using western blotting. Wound healing, Transwell and tube formation assays were performed to evaluate CAL-27 cell migration, invasion and human umbilical vein endothelial cell (HUVEC) tube formation. The interaction between CIP2A and AKT1 was identified by co-immunoprecipitation (co-IP). In addition, AKT1 was overexpressed in CIP2A-silenced CAL-27 cells to perform rescue experiments to analyze the malignant biological functions of CAL-27 cells. Finally, the expression of proteins in the glycogen synthase kinase (GSK)-3β/β-catenin pathway was determined by western blot analysis. Markedly elevated CIP2A and AKT1 expression was observed in OSCC cells. CIP2A knockdown inhibited the viability, proliferation, migration and invasion, and promoted the apoptosis of CAL-27 cells. Concurrently, CIP2A loss-of-function attenuated tube formation. Results of Co-IP confirmed there was an interaction between CIP2A and AKT1. Rescue experiments suggested that AKT1 overexpression alleviated the inhibitory effects of CIP2A knockdown on the viability, proliferation, migration and invasion of CAL-27 cells, as well as tube formation in HUVECs . Additionally, CIP2A silencing significantly downregulated phosphorylated-GSK-3β and β-catenin expression, which was reversed by AKT1 overexpression. In conclusion, CIP2A could interact with AKT1 to promote the malignant biological behaviors of OSCC cells by upregulating the GSK-3β/β-catenin pathway. These findings may provide a targeted therapy for OSCC treatment.

Introduction

Oral squamous cell carcinoma (OSCC) represents the most frequent form of head and neck squamous cell carcinoma, accounting for 90% of all oral cancer cases worldwide. In addition, OSCC is associated with a poor prognosis with a 5-year overall survival rate of only ~50% globally (1,2). A previous study reported that >30% of patients with OSCC may develop multiple tumors within 5-10 years, which further aggravates this prognosis (3). Although great progress has been made in surgical techniques, radiotherapy and chemotherapy, the survival rate remains relatively low, due to the local recurrence and metastasis of the disease (4,5). Therefore, an in-depth understanding of the molecular mechanism underlying the occurrence and development of OSCC is of crucial importance for the development of new therapeutic strategies.

Cancerous inhibitor of protein phosphatase 2A (CIP2A), originally named KIAA1524 or p90, has been reported to act as an oncogene by promoting tumor growth in several types of cancer, including bladder cancer, non-small cell lung cancer and colorectal cancer (6-8). Notably, CIP2A is also highly expressed in OSCC tissues, and high CIP2A expression is significantly related to poor prognosis and short survival time (9,10). A previous study indicated that CIP2A participates in the regulation of tumor angiogenesis (11), and downregulation of CIP2A has been shown to suppress the proliferation and vascularization of renal clear cell carcinoma cells (12). In addition, the glycogen synthase kinase (GSK)-3β/β-catenin pathway serves a crucial role in regulating the proliferation, migration and angiogenesis of cancer cells (13,14). Inhibiting GSK-3β/β-catenin signaling has been demonstrated to suppress the progression of OSCC (15). Whether CIP2A can affect the malignant phenotypes and angiogenesis of OSCC by regulating the GSK-3β/β-catenin pathway remains to be investigated. The STRING database

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predicted that there may be an interaction between CIP2A and AKT1. It has also been reported that CIP2A regulates cell proliferation via the AKT signaling pathway in human lung cancer (16), and that CIP2A downregulation may induce apoptosis via inhibition of the AKT signaling pathway in breast cancer (17). AKT1 has been reported to be involved in accelerating the malignant progression of various types of cancer, including pancreatic cancer, epithelial ovarian cancer and colorectal cancer (18-20). In particular, AKT1 expression has been shown to be significantly elevated in OSCC tissues (21). Furthermore, AKT1 has been demonstrated to promote tumor angiogenesis (22) and to regulate GSK-3β/β-catenin signaling to participate in the progression of various types of cancer, including prostate cancer, glioma and breast cancer (23-25). Therefore, the present study aimed to assess the relationship between CIP2A and AKT1, and to determine their roles in the malignant progression and angiogenesis of OSCC.

In the present study, the expression of CIP2A and AKT1 in OSCC cell lines was detected. Subsequently, further experiments analyzed the effects of CIP2A silencing on the malignant progression and angiogenesis of OSCC cells, and explored the relationship between CIP2A and AKT1 to reveal the mechanisms underlying OSCC.

Materials and methods

Cell culture. Three human OSCC cell lines, HN-4, SCC-9 and CAL-27, provided by the American Type Culture Collection were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.). The human normal oral keratinocytes (HOKs; cat. no. 2610) and human umbilical vein endothelial cells (HUVECs; cat. no. 8000) were obtained from ScienCell Research Laboratories, Inc. The HOK cells were grown in oral keratinocyte growth medium (ScienCell Research Laboratories, Inc.) and the HUVECs were cultured in endothelial cell medium (Gibco; Thermo Fisher Scientific, Inc.). All media were supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and the cells were cultured in a humidified atmosphere containing 5% CO_2 at 37˚C.

Transfection. For transfection, the short hairpin RNAs (shRNAs) targeting CIP2A (shRNA-CIP2A-1, sense, 5'-GCAGTGCATTCAACTTCTACA-3', antisense, 5'-TGTAGAAGTTGAATGCACTGC-3'; shRNA-CIP2A-2, sense, 5'-CGCTGGTTAAGCCAACCTTTG-3', antisense, 5'-CAAAGGTTGGCTTAACCAGCG-3'), the empty shRNA plasmid (shRNA-NC, sense, 5'-TTCTCCGAACGTGTCACGT-3', antisense, 5'-ACGTGACACGTTCGGAGAA-3'), pc-DNA3.1 vectors containing the complete sequence of AKT1 (Ov-AKT1) and the empty vector plasmid (Ov-NC) were supplied by Shanghai GenePharma Co., Ltd. CAL-27 cells at the logarithmic phase were seeded in a 6-well plate ($1x10^5$ cells/well) and were incubated at 37˚C for 24 h. A total of 100 nM recombinant was transfected into cells at 70% confluence using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) for 48 h at 37˚C. The transfected cells were collected 48 h post-transfection for subsequent experiments.

Cell viability assay. The transfected CAL-27 cells were inoculated in 96-well plates at a density of 2,000 cells/well. After incubation for 24, 48 and 72 h, 10 µl Cell Counting Kit-8 (CCK-8) solution (Beijing Solarbio Science & Technology Co., Ltd.) was added to each well. The 96-well plate was cultured in the incubator for 1 h and the optical density at 450 nm was detected using an enzyme-labeled instrument.

5-ethynyl-2'-deoxyuridine (EdU) staining. An EdU kit (BeyoClick™ EdU Cell Proliferation Kit with Alexa Fluor 488; Beyotime Institute of Biotechnology) was used to detect cell proliferation. Following incubation for 3 days in 24-well plates, CAL-27 cells were incubated with 50 µM EdU, fixed with 4% paraformaldehyde for 30 min at 37˚C and stained with DAPI in the dark at room temperature for 30 min. Finally, the proliferation of cells was observed and images were captured using an inverted fluorescence microscope (Nikon Corporation).

TUNEL staining. The apoptosis of transfected cells was assessed using the TUNEL assay (Beyotime Institute of Biotechnology) according to the manufacturer's instructions. CAL-27 cells ($2x10^4$ cells/well) were cultured on glass coverslips overnight. After being fixed with 4% paraformaldehyde at 37˚C for 1 h, the cells were blocked with 3% H_2O_2 for 20 min at room temperature. After permeabilization with 0.1% Triton X-100 at 4˚C for 2 min, the slides were incubated with 50 µl TUNEL reaction mixture for 1 h at 37˚C, mounted with Vectashield® mounting medium containing 1 mg/ml DAPI solution for 5 min at 37˚C in the dark, and cell samples in five randomly selected fields were analyzed using an inverted fluorescence microscope (Nikon Corporation).

Wound healing assay. The CAL-27 cells were seeded into six-well plates at a density of $2x10^5$/well and cultured until the cells reached ~100% confluence. The confluent cells were scratched using a sterilized 10-µl pipette tip. The detached cells were removed by washing with PBS. Subsequently, the medium was replaced with serum-free DMEM and the cells were incubated at 37˚C. After 24 h, images of cell migration were captured using an inverted light microscope (Nikon Corporation) and the wound area was measured using ImageJ software version 1.7.0 (National Institutes of Health).

Transwell invasion assay. After transfection, CAL-27 cells were collected and $5x10^4$ cells were added to 200 µl serum-free medium in the upper chambers precoated with Matrigel (BD Biosciences) at 37˚C for 30 min. Normal medium containing 10% FBS was added to the lower chamber. After culturing for 24 h, the invasive cells were fixed with 4% paraformaldehyde at room temperature for 30 min and stained with 0.1% crystal violet at room temperature for 10 min. Observation of invasive cells was performed using an inverted light microscope (Nikon Corporation).

HUVECs tube formation assay. Matrigel (200 µl) was pipetted into each well of 24-well plates and melted overnight at 4˚C. HUVECs ($5x10^4$) were serum-starved in medium overnight at 37˚C and then seeded into plates precoated with Matrigel. After incubation at 37˚C for 30 min, the starved HUVECs

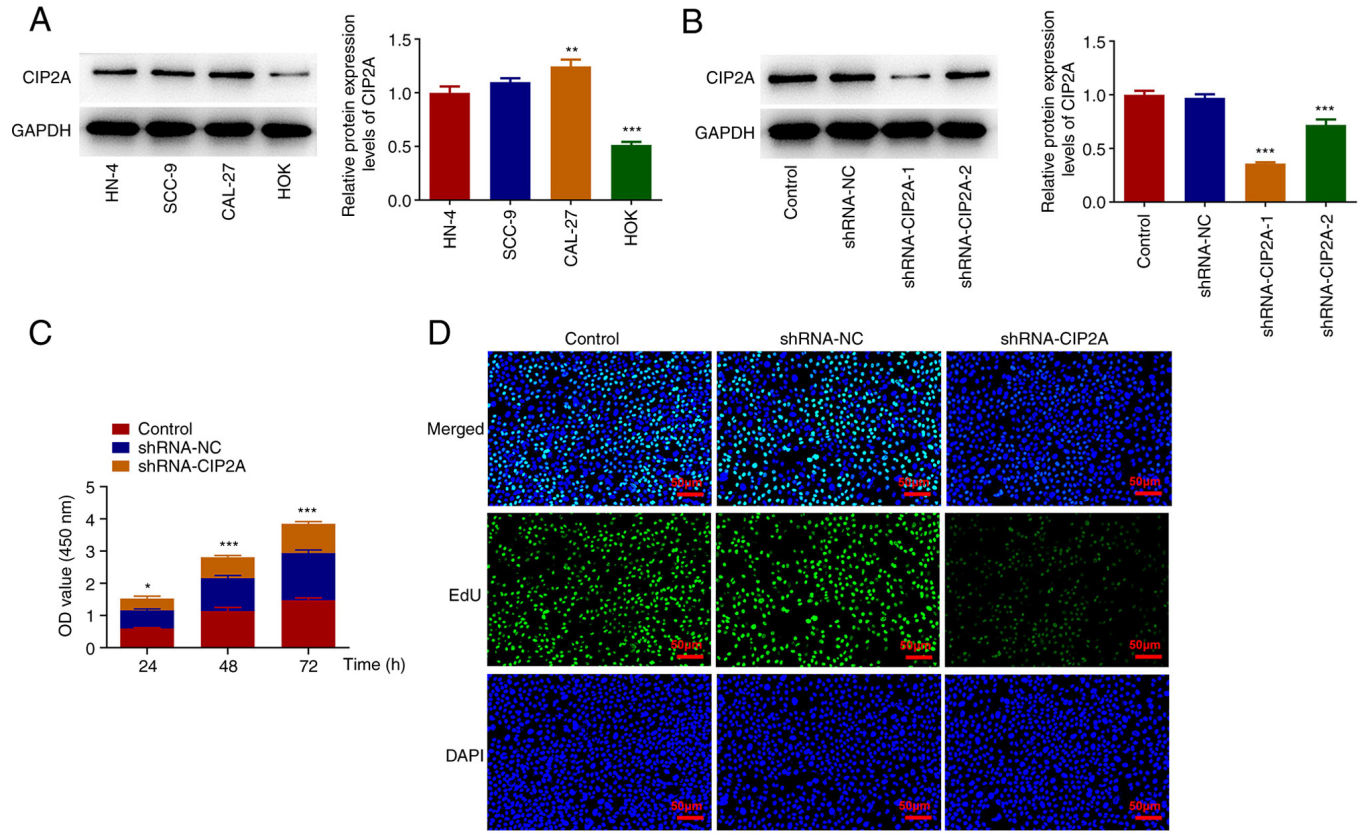

Figure 1. CIP2A is highly expressed in OSCC cells and knockdown of CIP2A inhibits the proliferation of OSCC cells. (A) CIP2A expression in OSCC cells and HOK cells was evaluated by western blotting. ***P<0.001 vs. HOK. (B) CIP2A expression in CAL-27 cells transfected with shRNA-CIP2A-1/2 was detected by western blotting. Proliferation of CAL-27 cells after CIP2A knockdown was measured by (C) Cell Counting Kit-8 assay and (D) EdU staining. *P<0.05, **P<0.01 and ***P<0.001 vs. shRNA-NC. CIP2A, cancerous inhibitor of protein phosphatase 2A; EdU, 5-ethynyl-2'-deoxyuridine; HOK, human oral keratinocyte; NC, negative control; OSCC, oral squamous cell carcinoma; shRNA, short hairpin RNA.

were cultured with 200 μl conditioned medium for 8 h at 37˚C with 5% CO_2. The conditioned medium was collected from the supernatants of transfected CAL-27 cells through centrifugation at 500 x g at room temperature for 10 min. Tube formation was viewed and images were captured under an inverted light microscope (Nikon Corporation).

Co-immunoprecipitation (Co-IP) assay. The transfected cells were lysed on ice for 30 min in RIPA lysis buffer (Beyotime Institute of Biotechnology) containing protease inhibitors. The supernatant was collected after centrifugation at 13,000 x g for 10 min at 4˚C. Then, 0.2 mg protein A agarose beads (Thermo Fisher Scientific, Inc.) were exposed to 500 μg lysis buffer and incubated with 2 μg IgG antibody (cat. no. ab313801; 1:30; Abcam) or CIP2A antibody (cat. no. #14805; 1:100; Cell Signaling Technology) or AKT1 antibody (cat. no. ab182729; 1:40; Abcam) overnight at 4˚C. Following the IP reaction and centrifugation at 1,000 x g at 4˚C for 2 min, the agarose beads were rinsed utilizing lysis buffer and boiled for 5 min at 100˚C. Western blot analysis was performed to analyze the expression of target proteins.

Western blot analysis. The cells were lysed in RIPA lysis buffer (Beyotime Institute of Biotechnology) and the lysates were centrifuged at 12,000 x g for 10 min at 4˚C to obtain the proteins, the concentration of which was determined using the bicinchoninic acid method. Protein samples (40 μg/per lane) were separated by SDS-PAGE on 10% gels and then transferred to PVDF membranes. After blocking in 5% non-fat milk for 1 h at room temperature, these membranes were probed with primary antibodies overnight at 4˚C, followed by incubation with a HRP-conjugated secondary antibody (cat. no. 7074P2; 1:5,000; Cell Signaling Technology, Inc.) at room temperature for 1 h. Chemiluminescence was used to expose the immunoreactive protein bands on the membrane using an enhanced chemiluminescence kit (Thermo Fisher Scientific, Inc.). The band intensities were semi-quantified relative to GAPDH and gray intensity analysis was performed using ImageJ software version 1.8.0 (National Institutes of Health). Anti-CIP2A (cat. no. 14805S; 1:1,000), anti-Bcl2 (cat. no. 4223T; 1:1,000), anti-Bax (cat. no. 41162S; 1:1,000), anti-AKT1 (cat. no. 75692S; 1:1,000), anti-phosphorylated (p)-GSK-3β (cat. no. 5558T; 1:1,000), anti-GSK-3β (cat. no. 12456T; 1:1,000), anti-β-catenin (cat. no. 8480T; 1:1,000) and anti-GAPDH (cat. no. 5174T; 1:1,000) antibodies were obtained from Cell Signaling Technology, Inc.

Bioinformatics tools. STRING database (https://string-db.org/) was used to predict the proteins that can interact with CIP2A.

Statistical analysis. GraphPad Prism 8.0 (Dotmatics) was used for the statistical analysis. All experimental data are presented as the mean ± standard deviation of three experiments.

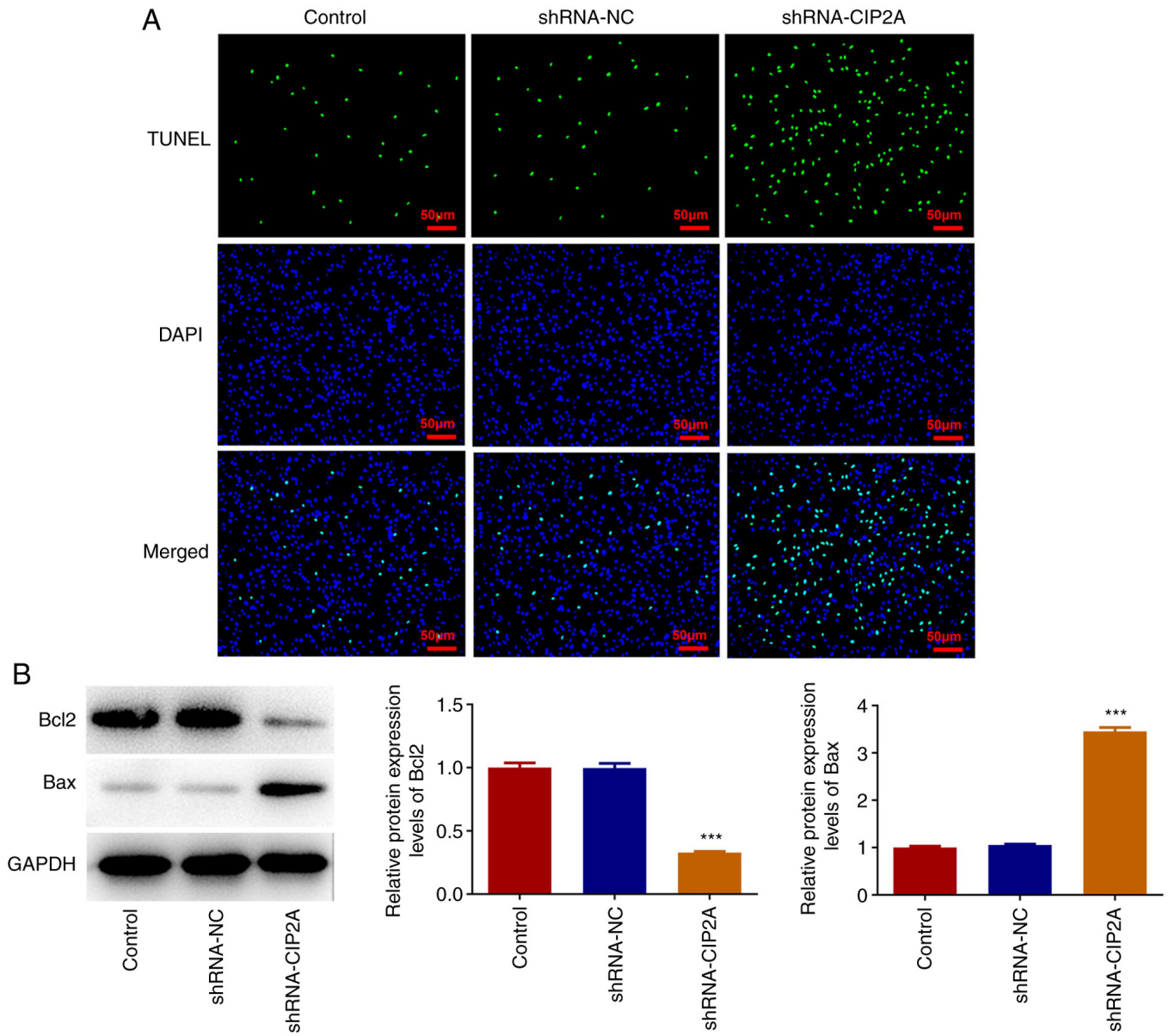

Figure 2. Knockdown of CIP2A accelerates the apoptosis of oral squamous cell carcinoma cells. (A) TUNEL staining was used to evaluate the apoptosis of CAL-27 cells. (B) Expression of Bcl2 and Bax was assessed using western blotting. ***P<0.001 vs. shRNA-NC. CIP2A, cancerous inhibitor of protein phosphatase 2A; NC, negative control; shRNA, short hairpin RNA.

One-way analysis of variance followed by Tukey's post hoc test was performed to compare the data from multiple groups. P<0.05 was considered to indicate a statistically significant difference.

Results

CIP2A is highly expressed in OSCC cells and interference with CIP2A inhibits the proliferation of OSCC cells. Firstly, CIP2A expression in three human OSCC cell lines and HOKs was assessed by western blotting. As shown in Fig. 1A, CIP2A protein expression levels were significantly elevated in HN-4, SCC-9 and CAL-27 cells compared with those in HOKs. CAL-27 cells were chosen to perform the subsequent experiments, as they had the highest expression of CIP2A in all of the aforementioned OSCC cell lines. CIP2A was silenced in CAL-27 cells to observe its effect on cell viability and cell proliferation. CIP2A expression was significantly downregulated post-transfection with shRNA-CIP2A-1/2 compared with that in the shRNA-NC group. The lowest CIP2A expression was observed in the shRNA-CIP2A-1 group; therefore, shRNA-CIP2A-1 was selected for the subsequent experiments. As shown in Fig. 1C, knockdown of CIP2A reduced the viability of CAL-27 cells relative to the shRNA-NC group. Consistently, the marked decrease in EdU fluorescence intensity and increase in TUNEL fluorescence intensity of the shRNA-CIP2A group suggested the inhibitory effect of CIP2A silencing on proliferation and the promoting effect of CIP2A silencing on the apoptosis of CAL-27 cells (Figs. 1D and 2A). In addition, CIP2A knockdown significantly downregulated Bcl2 expression and upregulated Bax expression when compared with the shRNA-NC group (Fig. 2B). These results indicated that knockdown of CIP2A may inhibit the proliferation of OSCC cells.

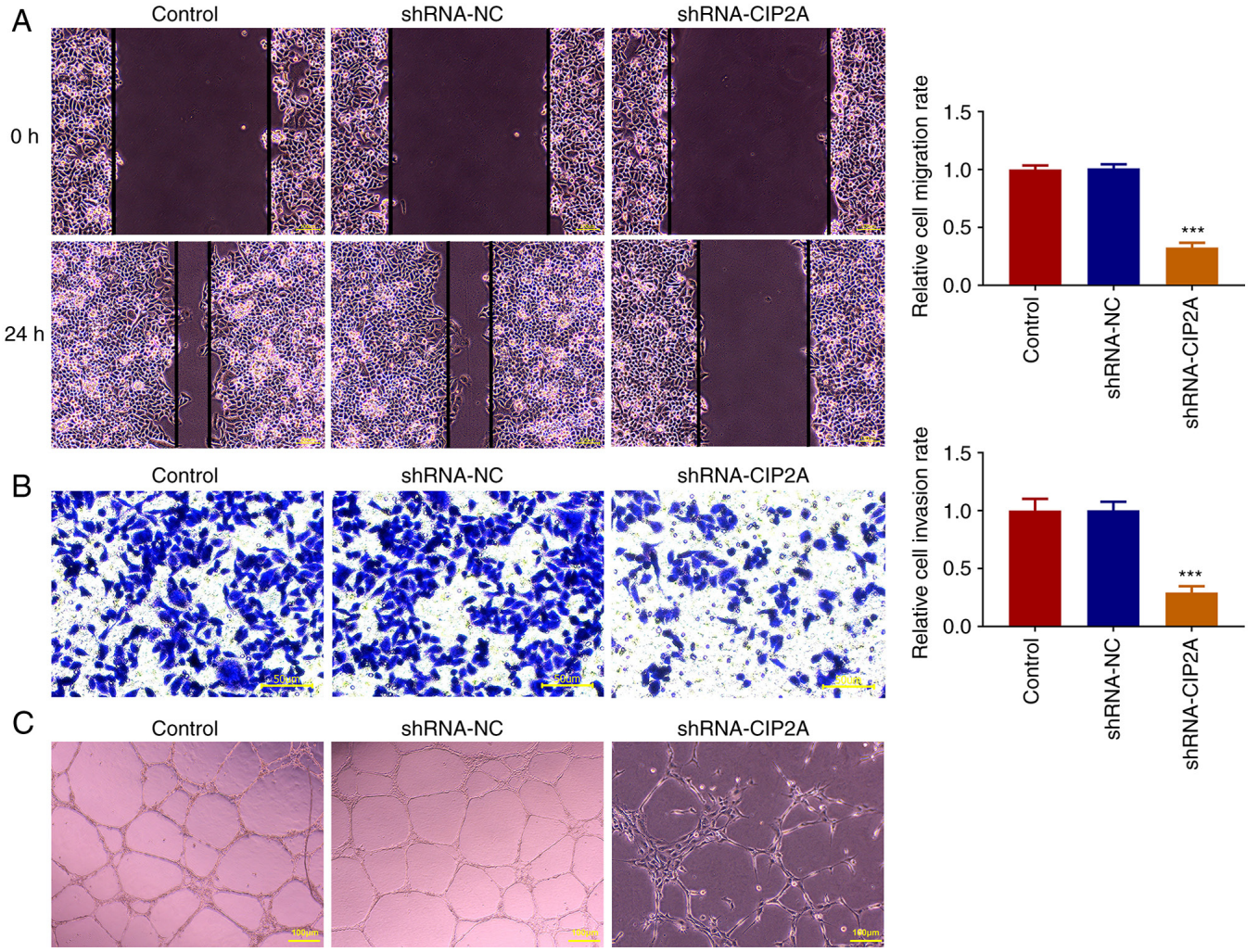

Figure 3. Knockdown of CIP2A alleviates the migration and invasion of oral squamous cell carcinoma cells, and restricts angiogenesis of human umbilical vein endothelial cells. (A) Migration of CAL-27 cells was assessed by wound healing assay. (B) Transwell assay was used to assess the invasion of CAL-27 cells. (C) Tube formation was detected using a tube formation assay. ***P<0.001 vs. shRNA-NC. CIP2A, cancerous inhibitor of protein phosphatase 2A; NC, negative control; shRNA, short hairpin RNA.

Knockdown of CIP2A alleviates the migration and invasion of OSCC cells, and restricts angiogenesis of HUVECs. The wound healing assay was used to evaluate the migration of CAL-27 cells after transfection with shRNA-CIP2A. CIP2A silencing significantly reduced the relative cell migration rate compared with the shRNA-NC group, with an apparent decrease in wound closure (Fig. 3A). The Transwell assay also revealed the same trend in cell invasion as that of cell migration (Fig. 3B). Additionally, tube formation was significantly restricted, with a decreased number of nodes in the CIP2A-silenced group compared with that in the shRNA-NC group (Fig. 3C). Together, these data indicated that knockdown of CIP2A may attenuate the migration and invasion of OSCC cells, and restrict the angiogenesis of HUVECs.

AKT1 can interact with CIP2A in OSCC cells. To explore the potential mechanism of CIP2A in the regulation of CAL-27 cells, the STRING database was used to predict the proteins that could interact with CIP2A, and AKT1 was found to interact with CIP2A (Fig. 4A). Compared with in HOK cells, AKT1 expression was significantly elevated in the HN-4, SCC-9 and CAL-27 cell lines (Fig. 4B). Co-IP assay confirmed the interaction between AKT1 and CIP2A in CAL-27 cells (Fig. 4C).

AKT1 overexpression reverses the inhibitory effects of CIP2A silencing on the proliferation, migration and invasion of OSCC cells, and angiogenesis of HUVECs. Subsequently, AKT1 was overexpressed to analyze its effects on malignant phenotypes of CAL-27 cells transfected with shRNA-CIP2A. As shown in Fig. 5A, AKT1 expression was significantly increased in the Ov-AKT1 group compared with that in the Ov-NC group. The results of the CCK-8 assay suggested that AKT1 overexpression enhanced the viability of CAL-27 cells compared with that in the shRNA-CIP2A + Ov-NC group (Fig. 5B). Furthermore, the EdU fluorescence intensity was strengthened and TUNEL fluorescence intensity was reduced after AKT1 overexpression in CIP2A-silenced CAL-27 cells (Fig. 5C and D). Bcl2 expression was downregulated, whereas Bax expression was upregulated in the shRNA-CIP2A + Ov-AKT1 group compared with the shRNA-CIP2A + Ov-NC group (Fig. 5E). The results of wound healing and Transwell invasion assays suggested that AKT1 overexpression elevated

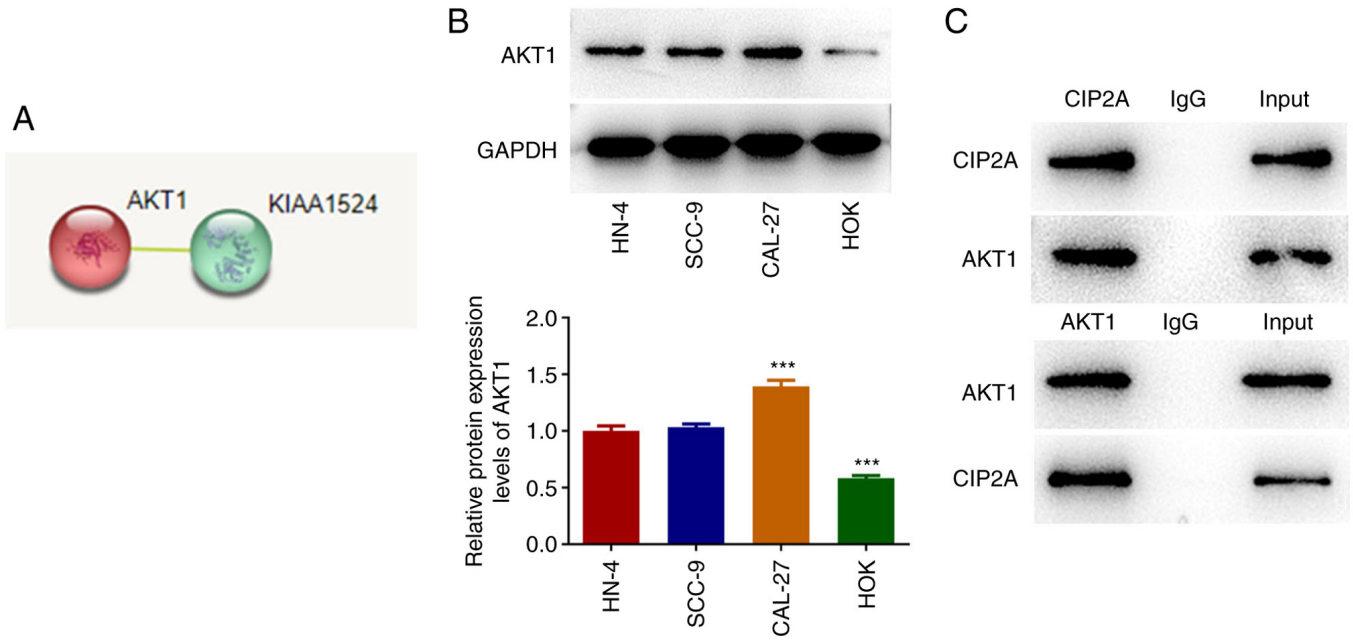

Figure 4. AKT1 can interact with CIP2A in OSCC cells. (A) STRING database predicated that AKT1 could interact with KIAA1524, also known as CIP2A. (B) AKT1 expression in OSCC cells and HOK cells was evaluated by western blotting. ***P<0.001 vs. HOK. (C) Interaction between AKT1 and CIP2A in CAL-27 cells was measured using co-immunoprecipitation assay. CIP2A, cancerous inhibitor of protein phosphatase 2A; HOK, human oral keratinocyte; OSCC, oral squamous cell carcinoma.

the migration and invasion of CAL-27 cells when compared with the shRNA-CIP2A + Ov-NC group (Fig. 6A and B). Furthermore, tube formation was markedly increased with an elevated number of nodes in the Ov-AKT1 group as compared with that in the shRNA-CIP2A + Ov-NC group (Fig. 6C). These data indicated that AKT1 overexpression may relieve the effects of CIP2A silencing on the viability, proliferation, migration and invasion of OSCC cells, and angiogenesis of HUVECs.

AKT1 overexpression alleviates inactivation of the GSK-3β/β-catenin signaling induced by CIP2A knockdown in OSCC cells. To further clarify the downstream mechanism of CIP2A and AKT1 in the regulation of OSCC malignant biological behaviors, the expression levels of proteins in the GSK-3β/β-catenin signaling pathway were detected by western blotting. As shown in Fig. 7, CIP2A knockdown significantly reduced the expression levels of p-GSK-3β and β-catenin compared with that in the shRNA-NC group. However, AKT1 overexpression alleviated inactivation of the GSK-3β/β-catenin signaling pathway induced by CIP2A knockdown in CAL-27 cells.

Discussion

OSCC is the most common malignant tumor of the oral cavity. Due to the high invasiveness of OSCC, a large number of patients often present with the advanced stages of the disease at the time of diagnosis, and therefore face complex surgical procedures and poor prognosis (26). Research aimed at understanding the molecular mechanism underlying distant metastasis of OSCC has garnered attention, with the aim of identifying potential target molecules and achieving targeted therapy. The present study detected upregulation of CIP2A in OSCC cell lines. Furthermore, knockdown of CIP2A markedly attenuated the proliferation, migration and invasion of OSCC cells, and the angiogenesis of HUVECs. Notably, it was revealed that AKT1 could interact with CIP2A to reverse the effects of CIP2A knockdown on the malignant biological behaviors of OSCC cells. Considering these results, CIP2A/AKT1 could be developed as potent antitumor agents targeting OSCC.

Abnormal and accelerated proliferation of cells is a characteristic of tumors. Malignant tumors require nutrients and oxygen to survive and proliferate; therefore, they need to grow near blood vessels to enter the bloodstream. The more neovascularization in the tumor, the higher the malignant degree (27). A critical event contributing to this metastasis is tumor-related angiogenesis (28). Tumor cell proliferation, invasion and metastasis are achieved through angiogenesis (29). Continuous angiogenesis is increasingly being recognized as having a crucial role in the growth and metastasis of solid tumors, including OSCC, as it can support tumor growth as well as the metastasis of malignant cells from the primary tumor site to distant organs (30,31). As a well-known oncoprotein, the expression of which is elevated in multiple human solid tumor types, abnormal CIP2A expression in cancer has been demonstrated in previous studies (32). For example, CIP2A expression has been reported to be higher in renal cell carcinoma (RCC) cells compared with that in renal tubular epithelial cells (HK-2 cells), and CIP2A gain-of-function can strengthen the proliferation and invasion of RCC cells (33). By enhancing the proliferative capacity and restraining the apoptotic ability, CIP2A has also been considered a

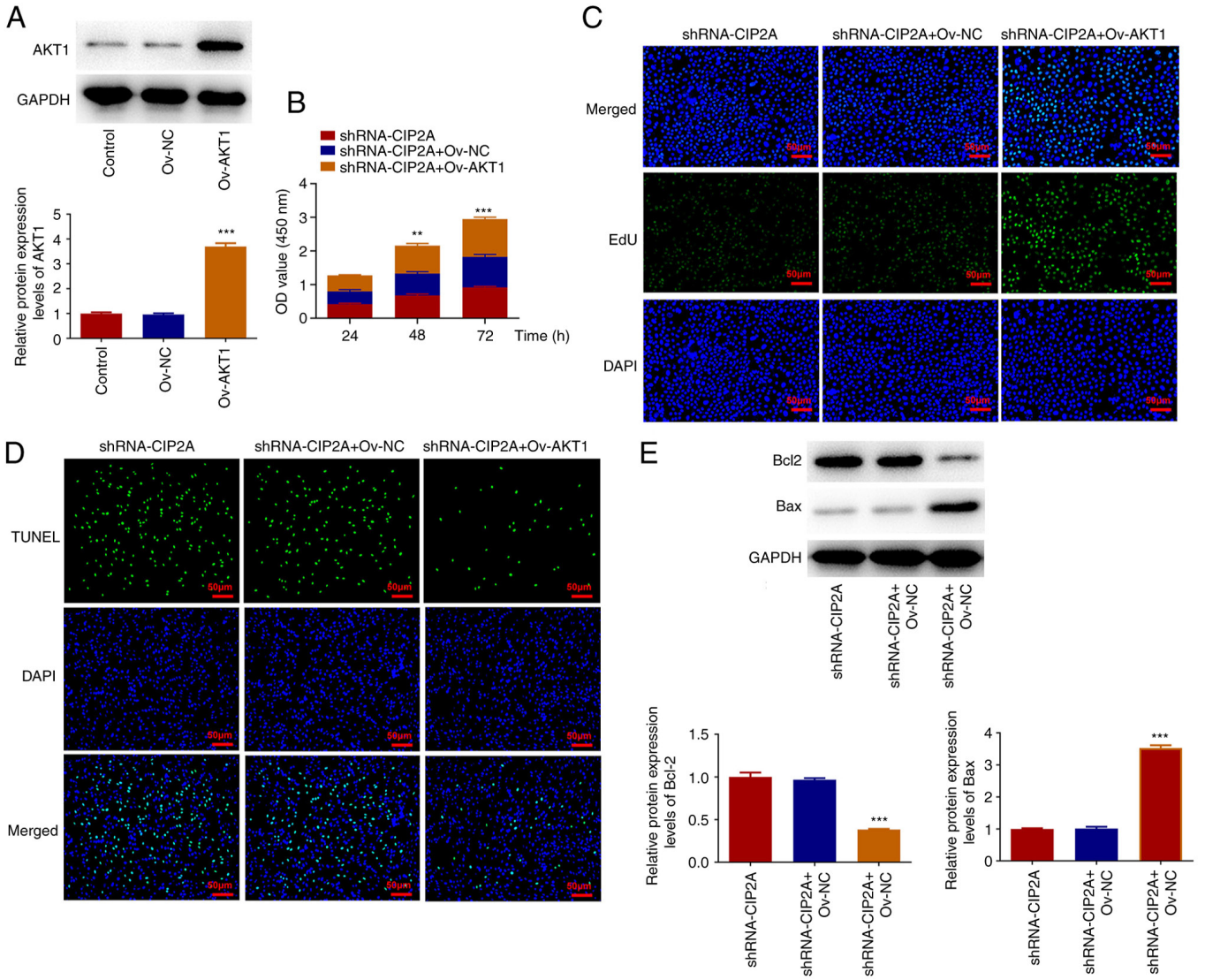

Figure 5. AKT1 overexpression reverses the inhibitory effects of CIP2A silencing on the proliferation of oral squamous cell carcinoma cells. (A) AKT1 expression in CAL-27 cells was detected by western blotting post-transfection with Ov-AKT1. ***P<0.001 vs. Ov-NC. (B) Cell Counting Kit-8 assay was employed to analyze the proliferation of CAL-27 cells. (C) Proliferation of CAL-27 cells after CIP2A knockdown and AKT1 overexpression was measured by EdU staining. (D) TUNEL staining was used to evaluate the apoptosis of CAL-27 cells. (E) Expression of Bcl2 and Bax was assessed using western blotting. **P<0.01, ***P<0.001 vs. shRNA-CIP2A + Ov-NC. CIP2A, cancerous inhibitor of protein phosphatase 2A; EdU, 5-ethynyl-2'-deoxyuridine; NC, negative control; Ov, overexpression; shRNA, short hairpin RNA.

promising therapeutic strategy for patients with multiple myeloma (34). Significantly elevated CIP2A expression has also been found in osteosarcoma, endometrioid adenocarcinoma and laryngeal carcinoma, in which CIP2A can promote the malignant biological behaviors of these tumor cells (35-37). Notably, CIP2A is highly expressed in OSCC tissues, and high CIP2A expression has been reported to be related to poor prognosis and short survival time (9,10). Our previous study indicated that CIP2A is implicated in the regulation of tumor angiogenesis (11). Similarly, the present study revealed that the expression of CIP2A was significantly elevated in OSCC cell lines, and silencing of CIP2A exerted inhibitory effects on the proliferation, migration and invasion of OSCC cells, and the angiogenesis of HUVECs.

To assess the mechanisms underlying the regulatory effects of CIP2A on OSCC progression, the STRING database was used to predict the proteins that could interact with CIP2A. AKT1 was revealed to interact with CIP2A in the present study. CIP2A-mediated AKT activation serves a role in bortezomib-induced apoptosis in head and neck squamous cell carcinoma cells (38). Furthermore, inhibition of CIP2A and its downstream AKT/mTOR signaling cascade can potentiate the chemosensitivity of A549/cisplatin cells to cisplatin by enhancing apoptosis (39). A number of studies have suggested that AKT1, which is the predominantly expressed and best-characterized isoform of AKT in numerous types of cancer, serves as the target protein of a number of genes to exert carcinogenic effects in various types of cancer by accelerating malignant progression, including breast cancer, gastric cancer and papillary thyroid cancer (40-42). A previous study demonstrated that AKT1 is responsible for the growth and survival of endothelial cells, which serve a crucial role in the angiogenesis (43).

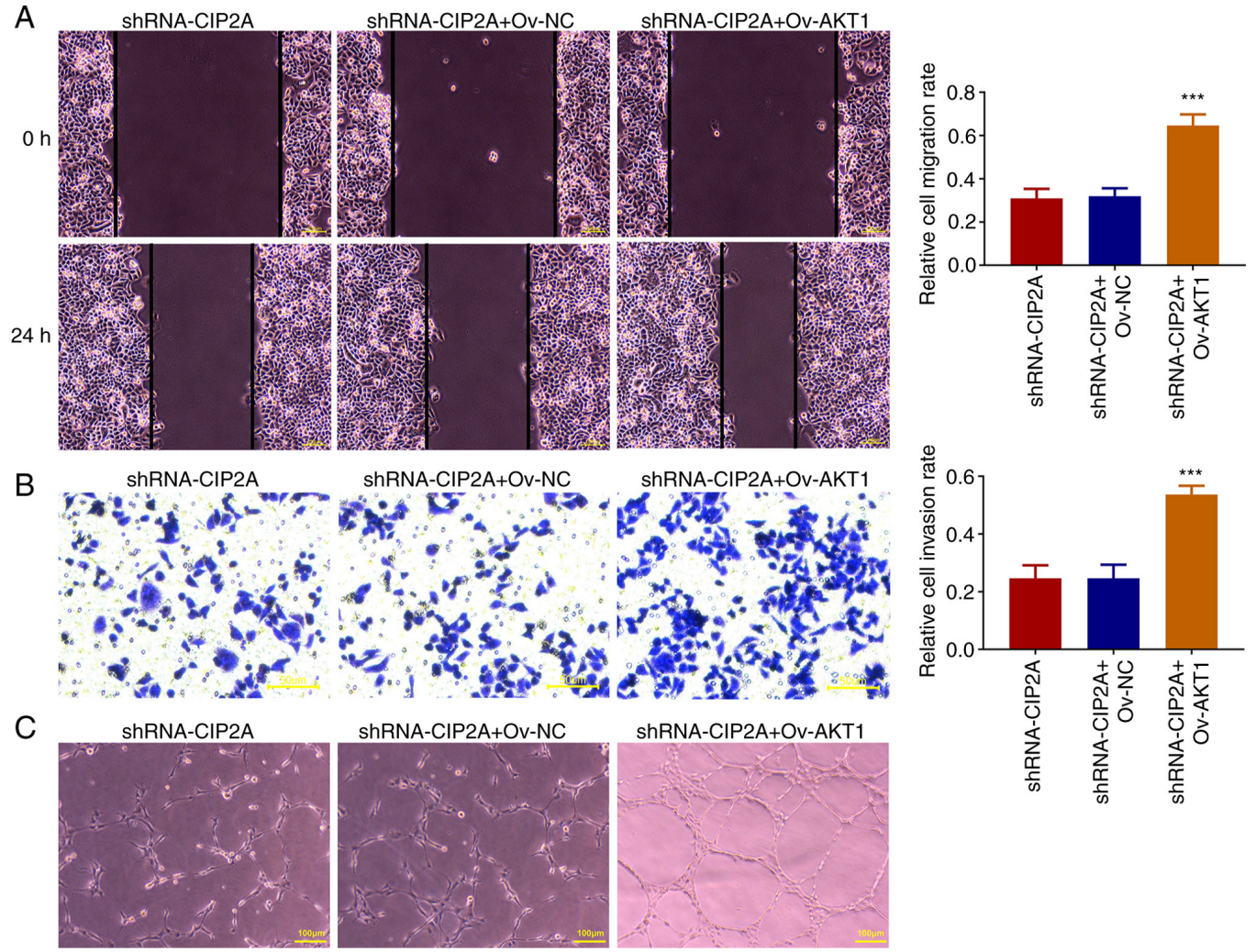

Figure 6. Ov-AKT1 attenuates the effect of CIP2A silencing on the migration and invasion of oral squamous cell carcinoma cells, and angiogenesis of human umbilical vein endothelia cells. (A) Migration of CAL-27 cells was determined by wound healing assay. (B) Transwell assay was used to assess the invasion of CAL-27 cells. (C) Tube formation was detected using tube formation assay. ***P<0.001 vs. shRNA-CIP2A + Ov-NC. CIP2A, cancerous inhibitor of protein phosphatase 2A; NC, negative control; Ov, overexpression; shRNA, short hairpin RNA.

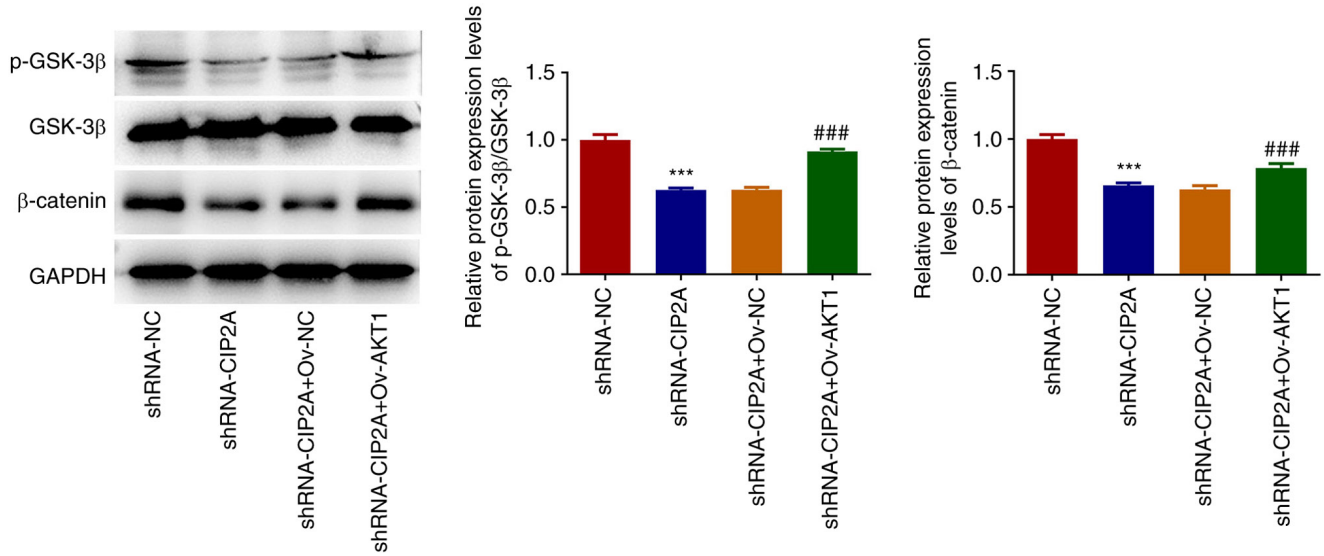

Figure 7. Ov-AKT1 alleviates the inactivation of GSK-3β/β-catenin signaling induced by CIP2A knockdown in oral squamous cell carcinoma cells. Western blot analysis was employed to measure the expression levels of proteins in the GSK-3β/β-catenin signaling pathway in CAL-27 cells. ***P<0.001 vs. control; ###P<0.001 vs. shRNA-CIP2A + Ov-NC. CIP2A, cancerous inhibitor of protein phosphatase 2A; GSK-3β, glycogen synthase kinase-3β; NC, negative control; Ov, overexpression; p-, phosphorylated; shRNA, short hairpin RNA.

Notably, AKT1 expression has been shown to be significantly elevated in OSCC tissues (21). Similar to the previous results reported in the aforementioned literature, the present study also revealed that AKT1 expression was markedly upregulated in OSCC cells. Notably, it was demonstrated that AKT1 overexpression reversed the inhibitory effects of CIP2A knockdown on the proliferation, migration and invasion of OSCC cells, and angiogenesis of HUVECs. These findings suggested the importance of the CIP2A/AKT1 axis in regulation of the malignant biological behaviors of OSCC.

To further investigate the downstream signaling pathway that could be regulated by CIP2A/AKT1, the expression levels of proteins in the GSK-3β/β-catenin signaling pathway were evaluated by western blotting in CAL-27 cells transfected with shRNA-CIP2A and Ov-AKT1. β-catenin acts as a central regulator in multiple physiological processes, and overexpression of β-catenin has been reported to be associated with various types of cancer, and to have an important role in cancer metastasis and angiogenesis (44-46). Degradation of β-catenin is mediated by the phosphokinase activity of GSK-3β, a serine/threonine kinase (47-49). Evidence has documented that the GSK-3β/β-catenin pathway is implicated in various types of cancer, such as hepatocellular carcinoma, breast cancer and colorectal cancer (50-52). Notably, inhibiting the GSK-3β/β-catenin signaling has been demonstrated to suppress the proliferation and promote the apoptosis of OSCC cells (15). By modulating the GSK-3β/β-catenin signaling pathway, the microRNA-203/SNAI2 axis can regulate prostate tumor growth, migration and angiogenesis (13). Galectin-3 favors tumor angiogenesis in hepatocellular carcinoma via activation of the AKT/GSK-3β/β-catenin signaling cascade (46). Moreover, SAMD9 promotes tumor stemness and angiogenesis of esophageal squamous cell carcinoma by stimulating MYH9-mediated GSK3β/β-catenin signaling (53). Notably, AKT1 has been reported to regulate GSK-3β/β-catenin signaling to accelerate the progression of gastric cancer, colorectal cancer and pancreatic carcinoma (49,54,55). In the present study, CIP2A knockdown markedly downregulated p-GSK-3β and β-catenin expression, which was reversed by the overexpression of AKT1 in CAL-27 cells. These results demonstrated that CIP2A/AKT1 could promote the malignant behaviors of OSCC by activating the GSK-3β/β-catenin pathway.

In conclusion, to the best of our knowledge, the present study is the first to demonstrate that CIP2A knockdown suppresses the proliferation, migration and invasion of OSCC cells, and the angiogenesis of HUVECs. Mechanistically, CIP2A can interact with AKT1 to promote the malignant biological behaviors of OSCC by upregulating the GSK-3β/β-catenin pathway. These findings may provide a new insight into the mechanism underlying OSCC and provide potential therapeutic targets for OSCC. Nevertheless, the present study has a limitation; the regulatory effects of CIP2A and AKT1 were only discussed on the progression of OSCC cells. Further *in vivo* experiments involving transgenic animals will be further explored in future investigations.

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Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YC and XW designed the study, and drafted and revised the manuscript. HZ and HL analyzed the data and searched the literature. YC and XW confirm the authenticity of all the raw data. All authors performed the experiments. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The Ethics Committee of Aerospace Center Hospital (Beijing, China) waived the requirement for ethics approval for using the purchased human normal oral keratinocytes and human umbilical vein endothelial cells.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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