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Original Article

γ-Synuclein promotes proliferation and inhibits apoptosis of oral squamous cell carcinoma via JAK2/STAT5b signaling pathway

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Abstract: γ-Synuclein (SNCG) has various biological functions associated with tumorigenesis. However, the role of SNCG in oral squamous cell carcinoma (OSCC) remains unknown. In this study, we found that SNCG expression is associated with the malignancy of OSCC. We showed that SNCG promotes cell proliferation and inhibits apoptosis in OSCC. Mechanistically, we demonstrated for the first time, that SNCG interacts with ERK1/2 and promotes its phosphorylation leading to activation of the JAK2/STAT5b signaling pathway. Subsequent experiments with STAT5b interference and ERK1/2 inhibitor treatment reversed the effects of SNCG on OSCC cell proliferation, apoptosis and cell cycle progression. Our findings suggest that SNCG functions as an oncogene in OSCC by targeting the JAK2/STAT5b axis and thus may be a potential new prognostic marker and therapeutic target in OSCC.

Keywords: SNCG, OSCC, JAK2/STAT5b, proliferation, apoptosis

Introduction

Oral squamous cell carcinoma (OSCC) is one of the most common types of cancer and a leading cause of cancer-related deaths worldwide, accounting for 64%-89% of oral malignant tumors. Approximately 500,000 new cases are diagnosed every year worldwide, and the incidence of OSCC is increasing [1, 2]. Advances in therapeutic techniques have greatly improved the quality of life of OSCC patients. However, the long-term survival rate of OSCC patients has not increased significantly. This may be partly attributed to the development of tumor metastasis. Despite significant advances in the treatment of OSCC, most patients die of tumor metastasis and recurrence. The 5-year survival rate of OSCC patients with lymph node metastasis is about 50%, whereas that of patients with distant metastasis is only about 20% [3]. Diagnosis of the disease at advances stages contributes to the poor prognosis and high mortality of this malignancy. Therefore, it is crucial to explore the molecular mechanisms underlying the progression of OSCC and to identify new therapeutic targets, which will help guide the design of effective treatments for OSCC.

Synucleins are small soluble proteins that belong to the synuclein family, which includes α-synuclein, β-synuclein and γ-synuclein (SNCG) [4]. SNCG was first detected in a human breast cancer cDNA library and was later shown to be abnormally expressed in breast cancer [5]. The function of SNCG in cancer has been studied extensively. SNCG increases endometrial cancer cell proliferation, migration and invasion through the PI3K/AKT/ERK signaling pathway, and upregulates the expression of N-cadherin and vimentin [6]. SNCG interference in gastric cancer cells reduces the phosphorylation of AKT and ERK, blocks the G0/G1 phase of the cell cycle, promotes gastric cancer cell apoptosis, and inhibits the occurrence of gastric cancer [7]. These results indicate that SNCG has

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multiple biological functions involved in tumorigenesis. However, the role of SNCG in OSCC remains largely unknown.

The occurrence and development of tumors is a complex molecular process. The JAK-STAT pathway is a widely distributed signal transduction system *in vivo* and is considered to be one of the key pathways affecting cell growth, proliferation and survival in many human tumors [8]. STAT5 is a member of the STAT family, which is involved in regulating cell proliferation, apoptosis and invasion in multiple tumors [9]. In addition, STAT5 is a downstream regulator of JAK2 in HPV-positive cervical cancer cells [10]. STAT5 has two subtypes, STAT5a and STAT5b, which are highly homologous at the amino acid level, and are closely associated with the occurrence, development and poor prognosis of various tumors. For example, STAT5b promotes the proliferation, migration and invasion of pancreatic cancer cells, inhibits apoptosis, and increases the resistance to chemotherapy in pancreatic cancer [11]. JAK2 regulates a wide range of signaling molecules and transcription factors. Furthermore, the STAT family of transcription factors is the main substrate of JAK2. JAK2 causes tyrosine phosphorylation and activation of STAT5b under hypoxic conditions [12].

In this study, we found that SNCG was significantly upregulated in OSCC patients and associated with shorter survival times. Overexpression of SNCG promoted the proliferation of OSCC cells, whereas knockdown of SNCG had the opposite effect. We showed that SNCG modulates the proliferation of OSCC cells by regulating the JAK2/STAT5b signaling pathway. Collectively, our findings identify a novel mechanism underlying OSCC progression, which may be beneficial in the development of OSCC treatments.

Materials and methods

Patient sample collection

Ninety-four tissue samples were collected from OSCC patients with complete clinical data and pathological diagnosis at the Zhanjiang Central Hospital of Guangdong Medical University from 2019 to 2022. All diagnoses were evaluated independently by two pathologists. Tumors were classified in line with the staging criteria in the proposed AJCC Cancer Staging Manual. This study was performed in accordance with the Declaration of Helsinki, and the study protocol was approved by The Human Investigation and Ethical Committees of Zhanjiang Central Hospital of Guangdong Medical University.

Immunohistochemistry (IHC)

IHC was performed using methods reported previously [13].

Cell culture

Human normal oral keratinocytes (HOKs) were purchased from the BeNa Culture Collection (Beijing, China). The human OSCC cell lines CAL27, SCC15, SCC9, and SCC15 were purchased from the American Tissue Culture Collection Center (ATCC). All cell lines were cultured in DMEM medium (Gibco) supplemented with 10% fetal bovine serum (FBS, HyClone), penicillin and streptomycin (Thermo Scientific). Cells were cultured in an atmosphere of 5% CO_2 at 37°C.

Small interfering RNAs, short hairpin RNAs, plasmids and transfection

Small interfering RNAs (siRNAs; listed in Table S1) were synthesized by GenePharma (Shanghai, China). The targeted sequence for short hairpin RNA-induced silencing of STAT5b 5'-GGACACAGAGAAUGAGUUATT-3' was inserted into the psilencer2.1-U6/neo plasmid. The control short hairpin RNA sequence was 5'-UUCUCCGAACGUGUCACGUTT-3'. The SNCG overexpression plasmid and anti-SNCG monoclonal antibody were a gift from Professor Chengchao Shou of Peking University. CAL27 and SCC15 cells were transfected with plasmids and siRNA using Lip2000 transfection reagent (Invitrogen) according to the manufacturer's instructions. The transfection efficiency was evaluated using qRT-PCR and western blotting. Stably transfected cells were screened 72 hrs after transfection with 5 μg/mL puromycin.

Silence of SNCG by lentiviral infection

To generate OSCC cells stably ablating endogenous SNCG, the lentiviral vectors LV-shSNCG and LV-NC (as control) were constructed, packed, and purified by GenePharma (Shanghai, China). CAL27 cells (5×10^4/well) were seeded into 6-well plates and infected with these vec-

tors at a multiplicity of infection (MOI) of 200 for 24 h. After 24 h, the medium was replaced with 2 mL complete culture medium. After a further 72 h, protein expression was examined by Western blotting. Stably infected cell lines were selected and maintained in the presence of 5 μg/mL puromycin.

Quantitative real-time polymerase chain reaction

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized using the PrimeScript™ RT reagent Kit (TaKaRa, Glen Burnie) following the manufacturer's instructions. Quantification was performed with the SYBR Green qPCR Master Mix (TOYOBO). The fold change in gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method and normalized with *GAPDH*. The primers are listed in Table S2.

Western blot analysis

Total protein was collected from cells using RIPA lysis buffer (Sigma) containing 1% protease inhibitors. Proteins were separated by 10% SDS-polyacrylamide gel electrophoresis, then transferred to PVDF membranes. Membranes were blocked in 5% skim milk (in PBS) for 2 h, then incubated with primary antibodies overnight at 4°C. Membranes were washed three times with TBST for 10 min, then incubated with the corresponding secondary antibody for 2 h at room temperature. Finally, antibody-reacting bands were exposed to ECL luminous fluid (34094; Pierce). The antibodies used are listed in Table S3.

Cell counting kit-8 (CCK8) assay

Cell viability assay was measured using CCK8 Cell Viability Kits (Biosharp, China) according to the manufacturer's instructions. Each group of pretreated cells were seeded in 96-well plates at a density of 2000 cells/well, then incubated with CCK8 reagent for 2 h at 37°C. The OD value of each sample was detected at 450 nm at the indicated times (0, 1, 2, 3 and 4 days) with a microplate reader.

Colony formation assays

Cells in the logarithmic growth phase were seeded into 6-well plates (1500 cells/well) and cultured at 37°C in an atmosphere of 5% CO_2 for 14 days. During this period, the medium was replaced with fresh and complete DMEM. After 14 days, colonies were rinsed twice in PBS and fixed with cold methanol for 30 min. Colonies were stained by incubating with 1 ml of 0.5% crystal violet for 20 min at room temperature. The crystal violet dye was then washed and dried, and images of each well were taken with an inverted fluorescence microscope. Colonies with more than 50 cells were counted.

Cell cycle analysis

Transfected OSCC cells (CAL27 and SCC15) were collected, fixed with 75% cold ethanol for 24 h at -20°C, then hydrated in 5 ml precooled PBS for 15 min. Cells were incubated with 1 ml DNA staining solution (Multi Sciences, Hangzhou, China) at room temperature in the dark for 30 min. Cell cycle progression was analyzed by flow cytometry (Beckman, Fullerton, CA, USA).

Apoptosis assay

The Annexin V-FITC/PI Apoptosis Kit (Multi Sciences, Hangzhou, China) was used to detect cell apoptosis. Transfected OSCC cells were collected, and 1×10^6 cells/ml were suspended in 1 × binding buffer. Cells were incubated with 5 μl Annexin V FITC and 10 μl PI at room temperature in the dark for 5 min. Samples were analyzed and quantified by flow cytometry (Beckman).

Bioinformatics prediction

Proteins that may interact with SNCG were predicted using bioinformatics data obtained from the Inbio Discover (http://www.intomics.com) and PPI Finder (http://www.genelab-bch.com.cn/tm/) websites. All data on this website can be obtained and used for free.

Immunoprecipitation test

Cells were lysed in cell lysis buffer (BL509A, Biosharp). Lysates (1 mg total protein) were incubated with 2 μg anti-SNCG or anti-ERK1/2 antibody for 16 h at 4°C. Mouse IgG was used as the negative control. The beads were washed with lysis buffer three times. Immunopreci-

pitates were examined by western blotting with anti-SNCG or anti-ERK1/2 antibodies.

Glutathione-S-transferase (GST) pull-down assay

The human full-length SNCG cDNA sequence was cloned into the pGEX-4T-1 vector, Recombinant GST-SNCG protein was expressed in *Escherichia coli* and purified with Glutathione Sepharose 4B beads. 2 μg of His-ERK1/2 (ProteinTech Group) was incubated with 2 μg of either GST-SNCG or GST plus glutathione beads in binding buffer overnight at 4°C, then washed three times with the same buffer. Proteins were boiled in SDS loading buffer and analyzed by western blotting with anti-His and anti-GST antibodies.

In vivo tumorigenesis in nude mice

Female BALB/c nude mice, 6 weeks old, were purchased from the Beijing Laboratory Animal Center (Beijing, China). CAL27 cells with stably silenced SNCG were generated by lentiviral infection. For nude mouse tumorigenicity assays, 2×10^7 of the indicated cells were injected into 6-week-old BALB/c female nude mice. The length (L) and width (W) of each tumor mass were measured by calipers once a week. Tumor volume was calculated according to the following formula: tumor volume = $(L \times W^2)/2$. The nude mice were humanely killed, and tumor tissues were weighed and removed for further experiments. The animal study was approved by the Biomedical Ethical Committee of Zhanjiang Central Hospital of Guangdong Medical University and carried out in accordance with established National Institutes of Health guidelines for the care and use of animals concordant with the United States guidelines.

Statistical analysis

Statistical analyses were performed using SPSS 20.0 software. Patient pathological data were preliminarily studied by using descriptive analysis. One way ANOVA was used to compare multiple groups of data. Data were presented as means ± SD for mRNA and protein expression levels, cell proliferation rate and apoptosis rate. $P < 0.05$ was considered statistically significant.

Results

SNCG expression is closely associated with OSCC tumor differentiation

IHC was used to examine the expression of SNCG in the tissue samples of 94 OSCC patients. A representative image showing IHC staining of SNCG in OSCC tissues is shown in **Figure 1A**. The correlation between SNCG expression levels and the degree of OSCC malignancy was determined by IHC detection of SNCG in OSCC tumor tissues of different histopathological grades. The highest SNCG expression levels were observed in poorly differentiated tumors, while moderate upregulation of SNCG expression was found in moderately differentiated tumors (**Figure 1B**, **1C**). Specifically, a significantly higher percentage of SNCG-positive cells and significantly increased staining intensity were observed in poorly differentiated cancer tissues than in high and moderately differentiated cancer tissues (**Figure 1B**, **1C**). Our data confirm that increased SNCG expression is correlated with the degree of malignancy of OSCC.

SNCG promotes proliferation and inhibits apoptosis of OSCC cells

Next, we used qRT-PCR and western blot analyses to examine SNCG expression levels in normal HOK and OSCC cell lines including CAL27, SCC9, SCC15 and SCC25 (**Figure 2A**, **2B**). We found that SNCG expression was significantly higher in OSCC cells than HOK cells, further confirming an association between SNCG and OSCC.

To determine the biological role of SNCG in OSCC, we assessed the effects of SNCG knockdown in CAL27 and SCC15 cells. The siSNCG2 construct achieved the highest knockdown efficiency as determined by qRT-PCR ($P < 0.001$, **Figure 2C**) and western blot analysis (**Figure 2D**). Next, CAL 27 and SCC-15 cells with stably silenced SNCG were generated by lentiviral infection of shRNA targeting SNCG, whose target was identical to that of siSNCG-2. CCK8 and colony formation assays revealed that knockdown of SNCG markedly attenuated the viability of CAL27 and SCC15 cells compared with LV-shNC treatment ($P < 0.001$) (**Figure 2G-I**). The effects of SNCG overexpression were next

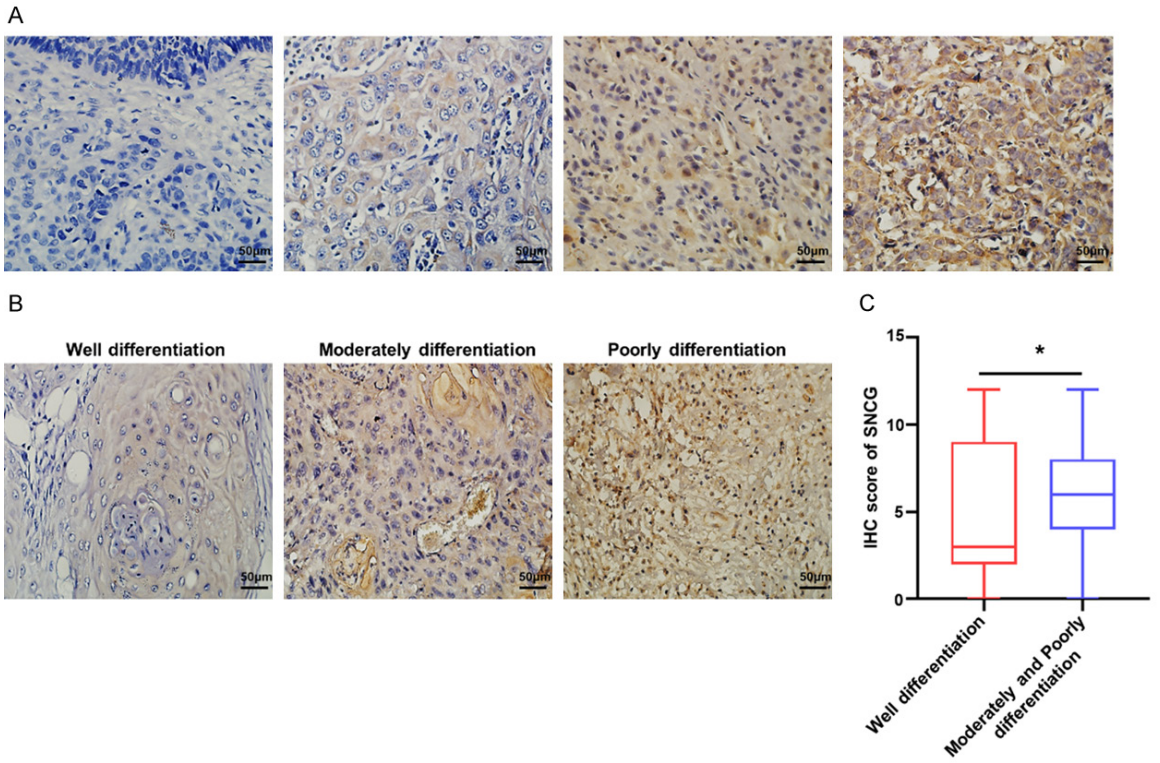

Figure 1. SNCG immunohistochemistry staining for different grades of OSCC tissues. A. Representative Immunohistochemistry (IHC) staining of SNCG in the adjacent normal tissues and different grades of OSCC tissues. B. Representative images of the expression of SNCG in well differentiated, moderately differentiated and poorly differentiated of OSCC tissues. C. The percentages and relative intensity of SNCG-positive cells in the different grades of OSCC tissues. Scale bar, 50 μm. Values shown are mean ± SEM, $*P < 0.05$.

examined by transfecting OSCC cells with the pcDNA3-SNCG plasmid. The transfection efficiency of the pcDNA3-SNCG plasmid is shown in **Figure 2E**, **2F**. Overexpression of SNCG was found to increase the proliferation of CAL27 and SCC15 cells ($P < 0.001$, **Figure 2H**, **2J**), suggesting that SNCG promotes OSCC proliferation.

The effect of SNCG on cell cycle progression and apoptosis were assessed by flow cytometry. Knockdown of SNCG significantly increased the proportion of CAL27 and SCC15 cells in the G1 phase with a concomitant decrease in those in S phase (**Figures 3A**, S1A), whereas ectopic expression of SNCG promoted the G1-S transition in the cell cycle (**Figures 3B**, S1B). Taken together, these data indicate that SNCG promotes the proliferation of OSCC cells by promoting the G1/S transition. To determine whether SNCG is involved in the regulation of OSCC cell death, we next examined the effects of SNCG knockdown or overexpression on OSCC cellular apoptosis by flow cytometry. We found that the frequency of cells in the middle and late stages of apoptosis was significantly higher in the SNCG knockdown group than in the control group (**Figures 3C**, S1C), whereas SNCG overexpression had the opposite effect (**Figures 3D**, S1D). These findings indicate that SNCG promotes the growth and proliferation of OSCC cells by inhibiting late apoptosis. Western blot analysis revealed knockdown of SNCG in CAL27 and SCC15 cells significantly downregulated the expression of proliferation-related protein kinase PKC and the anti-apoptotic protein Bcl2, and significantly upregulated expression of the apoptosis-related protein c-PARP, while SNCG overexpression had the opposite effects (**Figure 3E**). Taken together, these data indicate that SNCG promotes proliferation and inhibits the apoptosis of OSCC cells.

SNCG activates the JAK2/STAT5b signaling pathway in OSCC cells

The JAK/STAT signaling pathway is an important signal transduction system that is widely

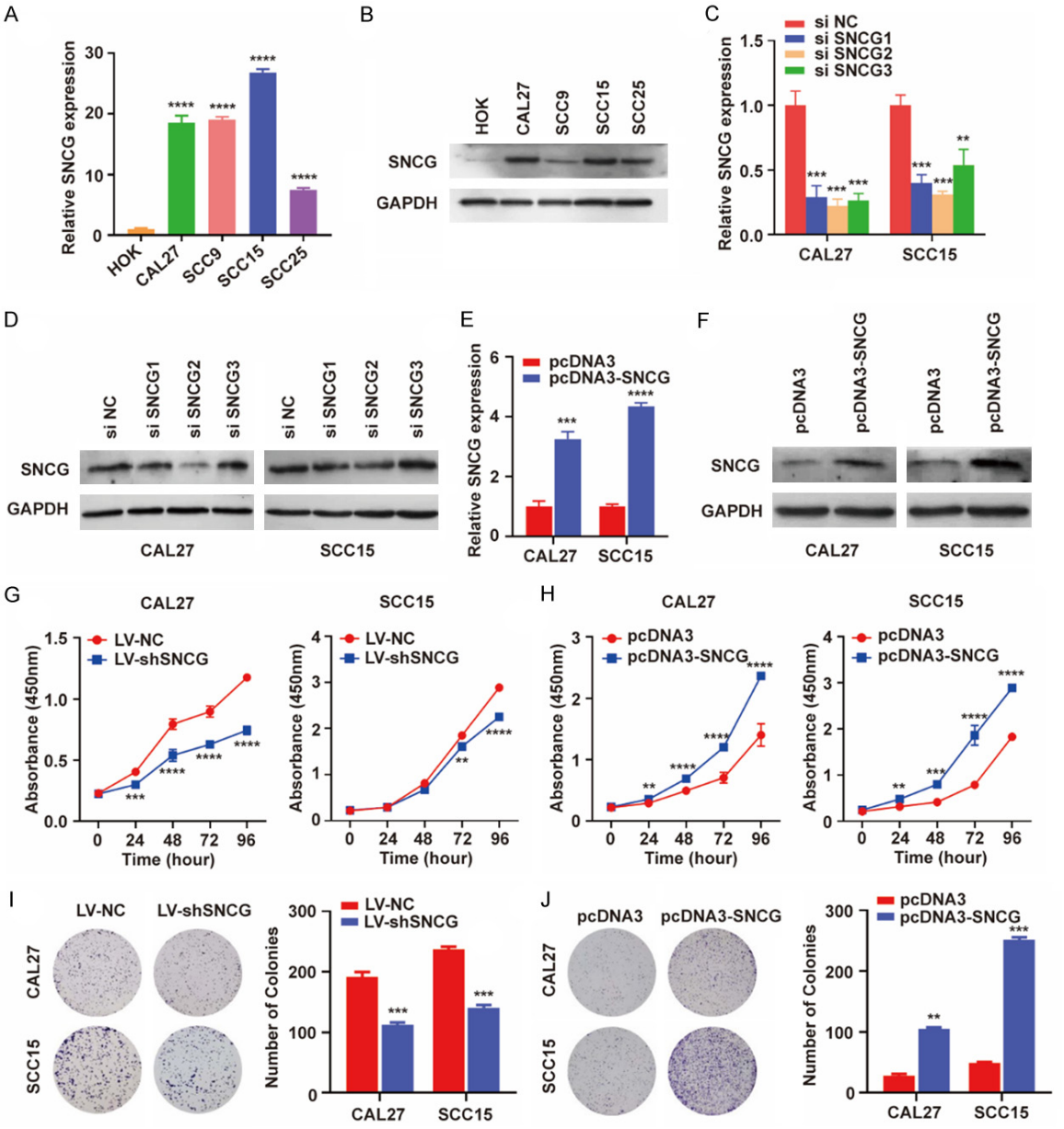

Figure 2. SNCG promotes proliferation and inhibits apoptosis of OSCC cells. A, B. Expression levels of SNCG were determined by qRT-PCR and Western blotting in normal oral keratinocytes (HOKs) and OSCC cell lines. C-F. Verification of SNCG knockdown or overexpression in CAL27 and SCC15 cells after transfected with siSNCG or pcDNA3-SNCG plasmid by qRT-PCR and western blotting. G, H. The effect of SNCG knockdown or overexpression on cell viability was detected by cell counting kit-8. I, J. Colony formation of LV-shSNCG group or pcDNA3-SNCG group in CAL27 and SCC15 cells. Representative images and statistical analysis were showed. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

distributed in organisms, and is considered to be one of the key pathways affecting cell growth, proliferation and survival in many human tumors [8, 14]. To examine the relationship between SNCG and the JAK/STAT signaling pathway in OSCC, we used the gene expression omnibus (GEO) microarray database to analyze the correlation between the mRNA levels of SNCG and JAK/STAT signaling-related molecules in OSCC. We found that the SNCG and JAK1 mRNA expression levels were negatively correlated in OSCC (**Figure 4A**), while the mRNA expressions levels of SNCG and JAK2, STAT2, STAT3, STAT5b and STAT6 were positively correlated in OSCC (**Figure 4B-F**). Among them, SNCG and STAT5b showed the

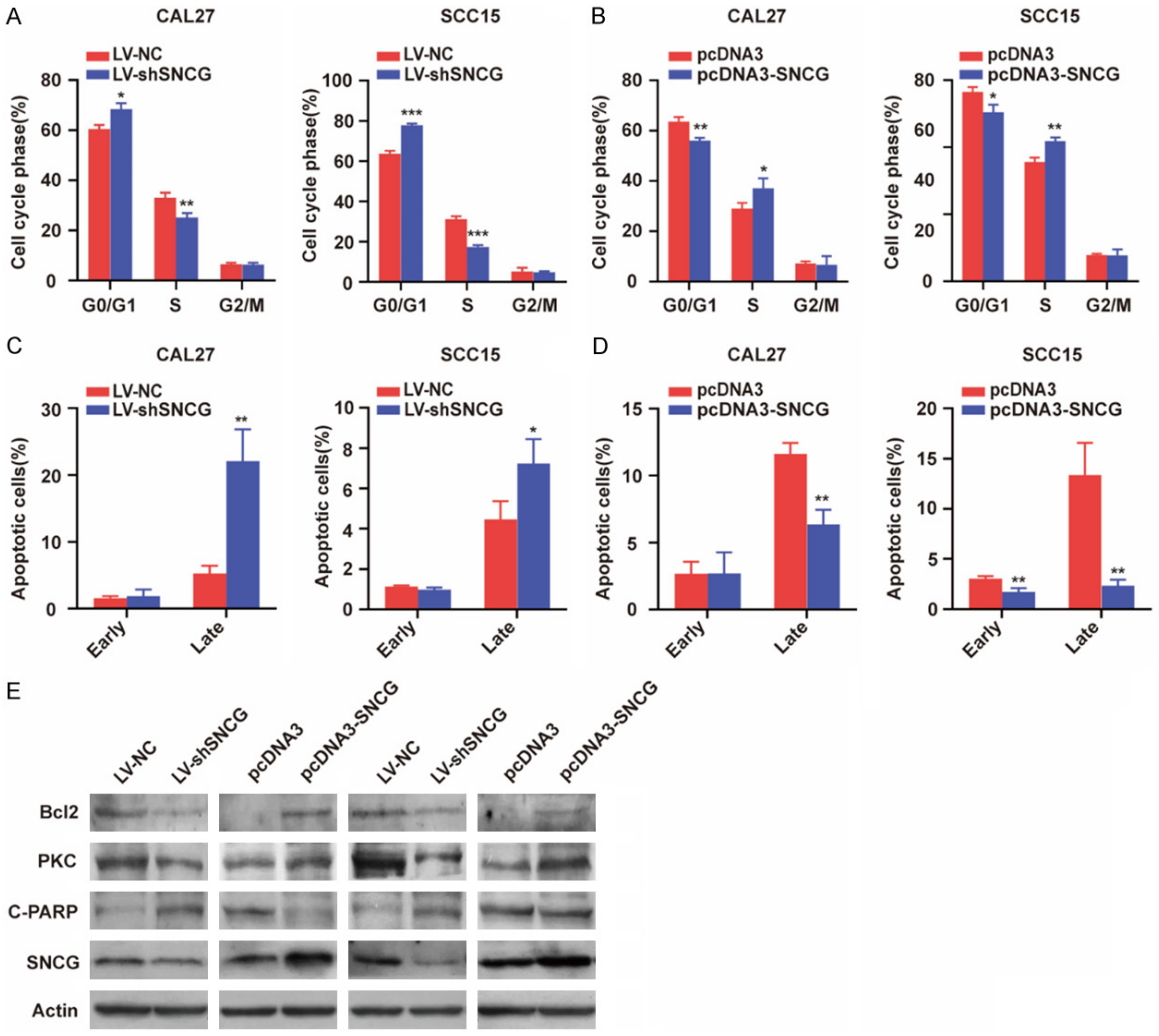

Figure 3. The effects of SNCG on OSCC cell apoptosis and cell cycle distribution. A, B. Knockdown of SNCG blocked the cell cycle at G0/G1 phase in CAL27 and SCC15 cells, while upregulation of SNCG induced the opposite effects in CAL27 and SCC15 cells. Cell cycle changes were detected by flow cytometry. C, D. After transfected with LV-shSNCG or pcDNA3-SNCG, cell apoptosis rates of CAL27 and SCC15 cells were performed by flow cytometry. Quantification of early apoptosis and late apoptosis/necrosis cells in CAL27 and SCC15 cells. E. Western blotting was used to quantitatively analyze the levels of proliferation-related and apoptosis-related proteins PKC, Bcl2 and c-PARP, after treatment with LV-shSNCG or pcDNA3-SNCG in CAL27 and SCC15 cells. Student's t-test was used to evaluate values. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

highest correlation in OSCC. Western blot analysis of JAK/STAT signaling pathway protein expression levels in OSCC cells showed that p-STAT1, p-STAT3, p-STAT5a, p-STAT5b and JAK2 were significantly downregulated after SNCG knockdown and significantly upregulated following SNCG overexpression, with p-STAT5b showing the most significant changes. The expression levels of the STAT family molecules, p-STAT2 and p-STAT6 did not change significantly after knockdown or overexpression of SNCG (**Figure 4G**). STAT5 is a downstream regulator of JAK2 in HPV-positive cervical cancer cells. Taken together, the GEO database and western blot analysis results confirm the involvement of the JAK2/STAT5b signaling pathway in OSCC tumorigenesis and suggest that SNCG activates JAK2/STAT5b signaling.

SNCG promotes proliferation and inhibits apoptosis through the JAK2/STAT5b signaling pathway in OSCC cells

Next, we sought to determine whether SNCG plays a role in OSCC tumorigenesis through regulation of the JAK2/STAT5b signaling path-

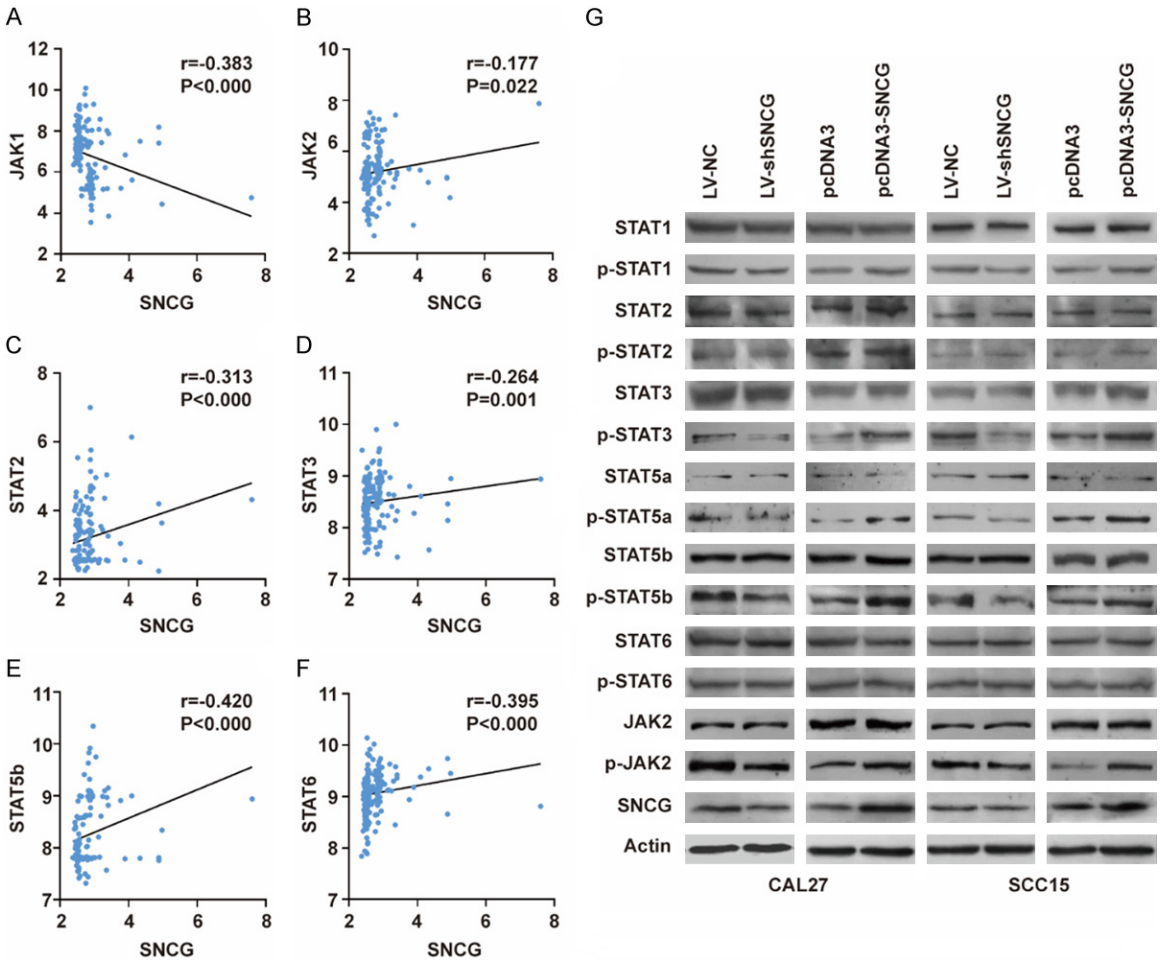

Figure 4. SNCG activates JAK2/STAT5b signaling pathway in OSCC cells. A-F. The correlation of SNCG mRNA expression with JAK1, JAK2, STAT2, STAT3, STAT5b, and STAT6 analyzed by the Pearson correlation analysis. G. Western blot analysis of JAK2/STAT5b pathway, and downstream target genes in CAL27 and SCC15 cells.

way. First, cells were transfected with shSTAT5b, and the transfection efficiency was confirmed by qRT-PCR and western blotting (**Figure 5A-C**). Knockdown of STAT5b in CAL27 and SCC15 cells was found to have no effect on SNCG protein expression levels compared with the shNC group (**Figure 5C**), suggesting that STAT5b does not regulate SNCG expression. The effect of SNCG on regulating p-STAT5b in OSCC cells was partially reversed by STAT5b knockdown in cells transfected with shSTAT5b and pcDNA3-SNCG (**Figure 5D**). Next, we examined whether the JAK2/STAT5b signaling pathway is required for SNCG-induced OSCC proliferation and apoptosis. We found that the increase in cell proliferation induced by overexpression of SNCG was partially reversed by knockdown of STAT5b (**Figure 5E-G**). Similarly, the effect of SNCG overexpression on cell cycle progression and apoptosis were partially reversed by STAT5b knockdown (**Figures 5H**, **5I**, S2A, S2B). In addition, the effects of SNCG overexpression on the expression of proliferation- and apoptosis-related factors were partially reversed by the knockdown of STAT5b (Figure S2C). Together, these results suggest that SNCG promotes proliferation and inhibits apoptosis through the JAK2/STAT5b signaling pathway in OSCC cells.

SNCG interacts with and activates ERK1/2 leading to activation of the JAK2/STAT5b signaling pathway

Although our results suggested that activation of the JAK2/STAT5b signaling pathway is involved in the SNCG-mediated promotion of OSCC cellular proliferation and control of apoptosis, the underlying mechanism required fur-

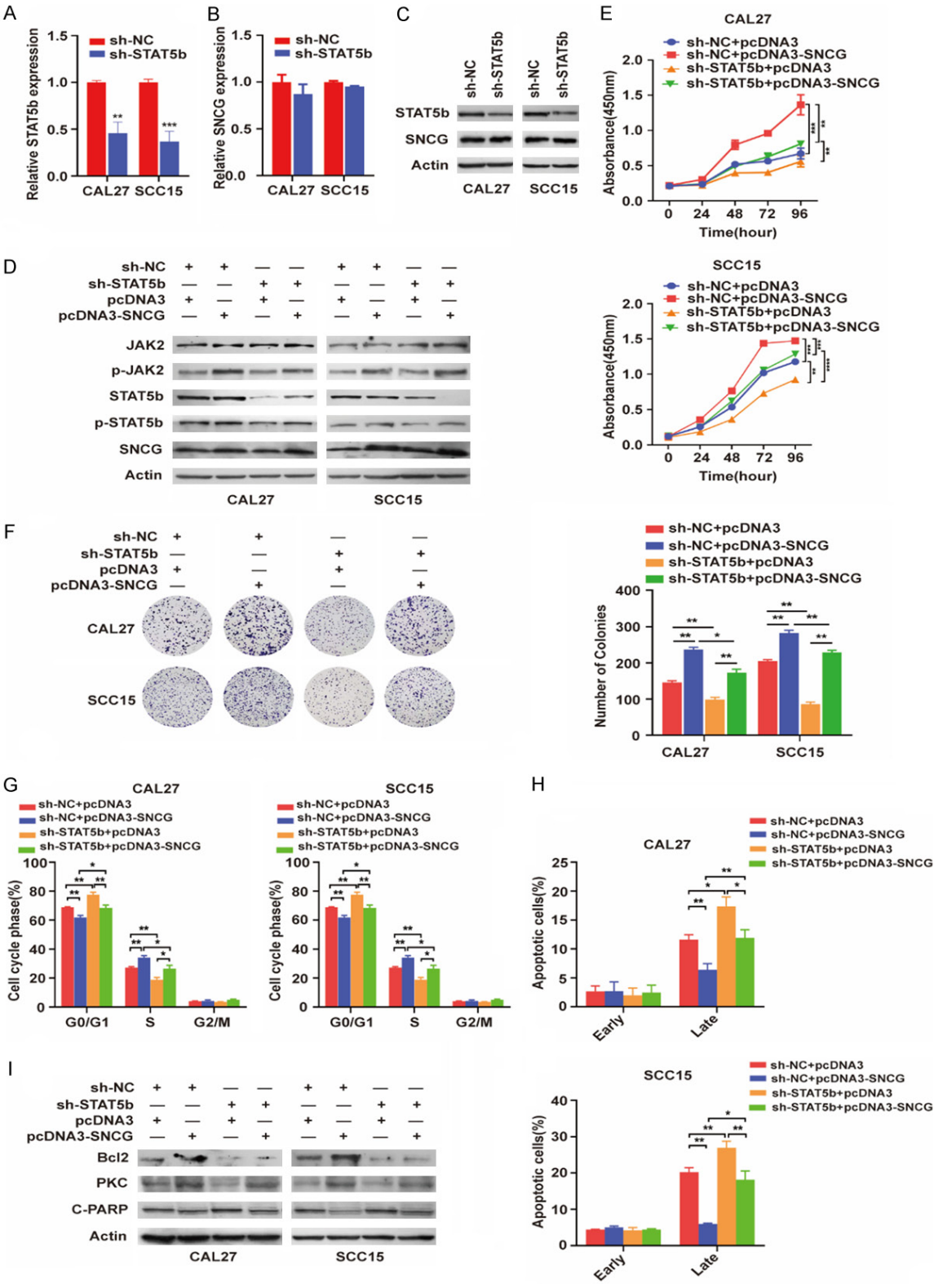

Figure 5. The effect of SNCG elevated on proliferation, cell cycle and apoptosis can be reversed by STAT5b knockdown in CAL27 and SCC15 cells. (A-C) The knockdown efficiency of sh-STAT5b in CAL27 and SCC15 cells were detected by qRT-PCR (A, B) and western blotting (C). CAL27 and SCC15 cells were divided into four groups: shNC + pcDNA3, shNC + pcDNA3-SNCG, shSTAT5b + pcDNA3, and shSTAT5b + pcDNA3-SNCG. (D) Western blot assay was applied to detect the expression levels of JAK2/STAT5b signaling pathway. (E) The viability of OSCC cells was de-

tected by Cell counting kit-8. (F) Colony formation was performed to evaluate cell proliferation. (G) Cell cycle changes were detected by flow cytometry. (H) Cells were treated as above and examined the apoptosis rate by flow cytometry, and quantification of early apoptosis and late apoptosis/necrosis cells. (I) Western blot analysis of the levels of proliferation-related and apoptosis-related proteins PKC, Bcl2 and c-PARP. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

ther elucidation. The Inbio Discover website and PPI Finder predicted that ERK1/2 may interact with SNCG (Figure S3). Therefore, we performed co-immunoprecipitation and GST pulldown experiments, and confirmed that SNCG interacted with ERK1/2 (**Figure 6A-C**). In addition, we found that ERK1/2 phosphorylation levels were significantly reduced after SNCG knockdown, but significantly increased after SNCG overexpression in OSCC cells (**Figure 6D**). To determine whether ERK1/2 activates the JAK2/STAT5b signaling pathway, we treated OSCC cells with the ERK1/2 inhibitor U0126 and examined the dose- and time-dependent effects. We found that treatment of CAL27 cells with 40 μM U0126, and SCC15 cells with 50 μM U0126 for 12 h significantly downregulated the expression of p-ERK1/2 with no effect on ERK1/2 expression levels (**Figure 6E**, **6F**).

Treatment of SNCG-overexpressing OSCC cells with the ERK1/2 inhibitor U0126 revealed that SNCG expression levels remained unchanged after ERK1/2 inhibition, indicating that ERK1/2 did not regulate SNCG expression. However, the effects of SNCG overexpression on p-JAK2 and p-STAT5b was reversed in the presence of U0126 (**Figure 6G**). Together, these findings indicate that SNCG activates the JAK2/STAT5b signaling pathway by interacting with and activating ERK1/2, thereby promoting proliferation and inhibiting apoptosis.

Knockdown of SNCG attenuates tumorigenesis in vivo

To determine whether the effects of SNCG on regulating tumorigenesis are similar *in vivo* and *in vitro*, a subcutaneous xenograft tumor model was established using CAL27 cells infected with LV-shSNCG and Vector (LV-shNC) (**Figure 7A**, **7B**). We found that the tumor volume and weight were significantly higher in the LV-shNC group than in the LV-shSNCG group (**Figure 7C-E**). The structural features of the tumors and the expression of related proteins were examined by hematoxylin & eosin (H&E) and IHC staining. Cells in the LV-shSNCG group had more nuclear fragmentation than those in the LV-shNC group. SNCG protein expression levels were downregulated in the LV-shSNCG group. SNCG knockdown led to a significant reduction in Ki-67, Bcl2 and PKC protein expression levels (**Figure 7F**). Taken together, these data indicate that SNCG knockdown suppresses xenograft tumor growth rates and is associated with changes in the expression of Ki-67, Bcl2 and PKC.

Discussion

SNCG was first identified in a human breast cancer cDNA library and later confirmed to be abnormally expressed in breast cancer [5]. Overexpression of SNCG has been reported in a variety of malignant solid tumors, including ovarian and colorectal cancer [15, 16], and is especially overexpressed in advanced tumors, suggesting that SNCG has lost its original tissue specificity during carcinogenesis. Increased SNCG expression levels in primary tumors have been positively correlated with distant metastasis or tumor recurrence in breast, colon and pancreatic cancer patients, and are associated with poor prognosis in many human cancers [17-19]. We have previous reported that SNCG is highly expressed in the saliva, serum, and tissues of OSCC patients and proposed that SNCG could be a potential novel diagnostic marker for OSCC [20, 21]. We have also shown that upregulation of SNCG contributes to poor prognosis in OSCC patients [13].

In this study, we found that the expression of SNCG was closely related to the differentiation of OSCC tumors, indicating that the upregulation of SNCG expression is associated with the malignancy of OSCC. SNCG is a multifunctional protein that can promote the proliferation, survival and metastasis of cancer cells, weaken cell cycle checkpoints, and mediate resistance to chemotherapy drugs [22, 23]. However, the role and molecular mechanism of SNCG in OSCC proliferation and apoptosis remain unclear. The results of this study indicate that SNCG is upregulated in OSCC cells and promotes OSCC proliferation by inducing cell cycle

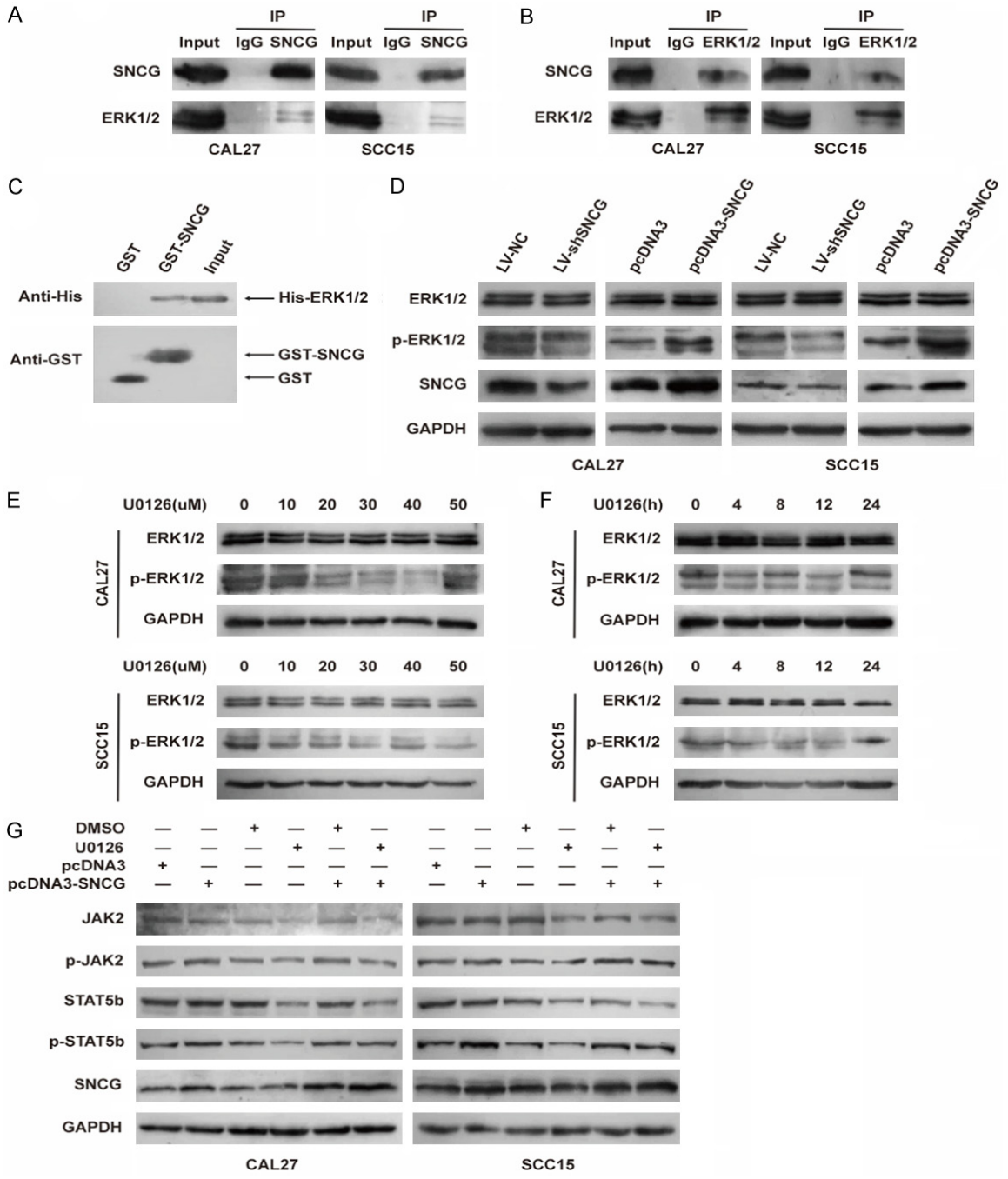

Figure 6. SNCG interacts with and activates ERK1/2 leading to activation of the JAK2/STAT5b signaling pathway. A, B. The endogenous interactions between SNCG and ERK1/2 in CAL27 and SCC15 cells were demonstrated by Co-immunoprecipitation experiments. C. Binding assay of His-ERK1/2 with GST or GST-SNCG. D. LV-shSNCG or pcDNA3-SNCG were transfected into CAL27 and SCC15 cells, respectively. The ERK1/2 and p-ERK1/2 were detected by western blotting. All experiments were carried out in triplicate. E. CAL27 and SCC15 cells were treated for 4 h with elevated concentrations of ERK1/2 inhibitor U0126 (10, 20, 30, 40 and 50 μM), and the ERK1/2 and p-ERK1/2 were determined by western blotting. U0126 treatment attenuated the content of phosphorylated ERK1/2 in a dose-dependent manner with maximum effect at 40 μM in CAL27 and 50 μM in SCC15 cells, respectively. F. CAL27 was treated with 40 μM U0126, and SCC15 was treated with 50 μM, U0126 respectively at different time intervals (4, 8, 12 and 24 h), and ERK1/2 activation was determined by western blotting. U0126 treatment attenuated the content of phosphorylated ERK1/2 in a time-dependent manner with best effect at 12 h. G. Representative western blotting showing the effects of ERK1/2 inhibitor U0126 on the phosphorylation levels of JAK2 and STAT5b induced by SNCG in CAL27 and SCC15 cells.

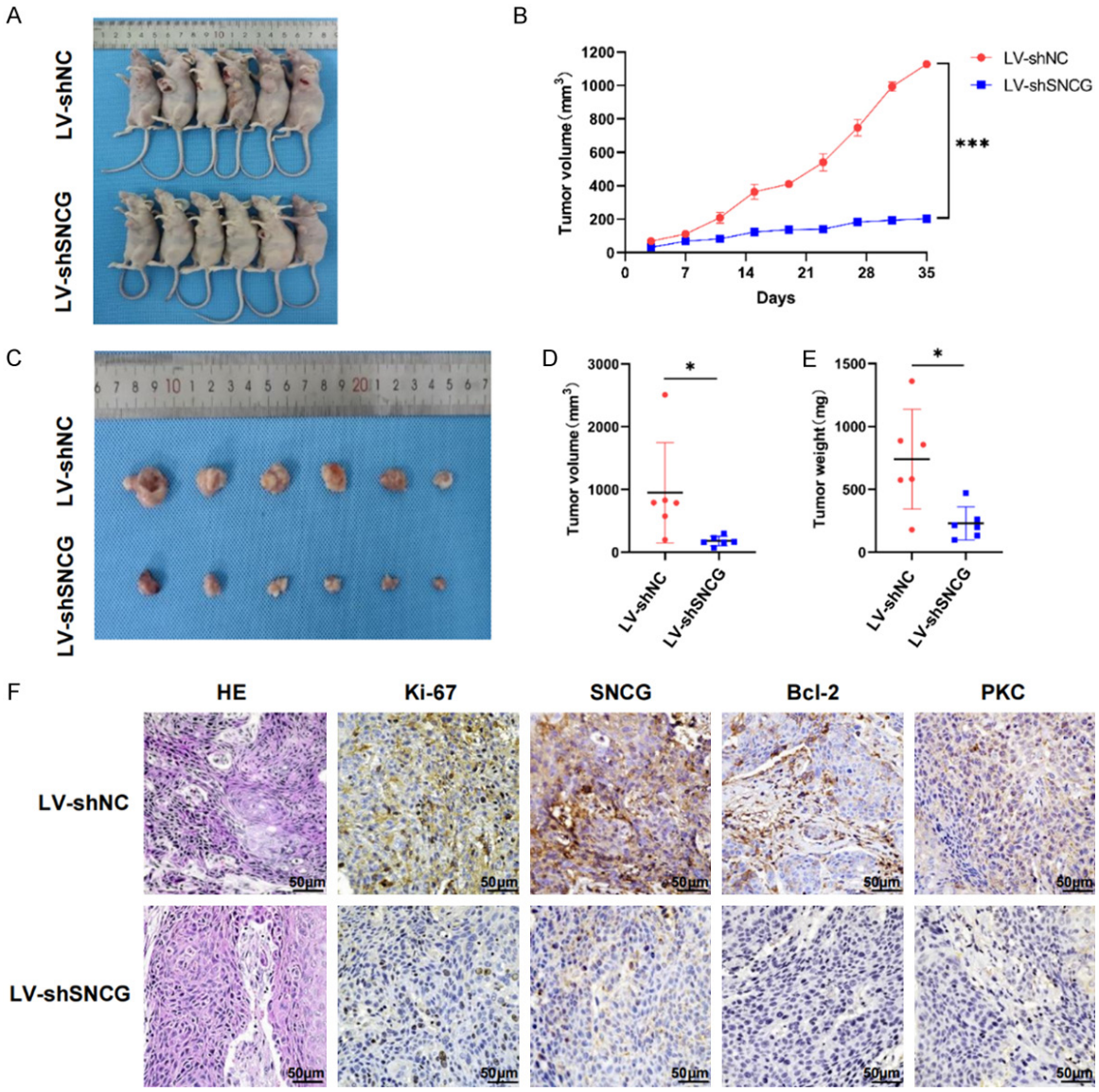

Figure 7. Knockdown of SNCG attenuates tumorigenesis in vivo. A. Images of subcutaneous xenograft tumors harvested at the end of the experiment. B. The tumor volume recorded at 7 days interval in LV-shNC group and LV-shSNCG group. C-E. Macrograph, volume and weight of tumors in both two groups. Six mice were analyzed in each group. F. Representative images of HE staining and immunohistochemical expression analysis of Ki-67, SNCG, Bcl2 and PKC, scale bar, 50 μm. *$P < 0.05$, ***$P < 0.001$.

progression and decreasing apoptosis. These findings are consistent with previous studies in human cervical cancer cells, which demonstrated that SNCG promotes the proliferation of cervical cancer cells and inhibits apoptosis by promoting cell cycle progression [24]. We also detected the expression of proliferation- and apoptosis-related proteins and found that SNCG regulates the expression of PKC, c-PARP and Bcl2. Our *in vivo* experiments using a nude mouse xenograft tumor model showed that the tumor volume and size were lower in the SNCG knockdown group than the control group. Thus, the current study provides the first evidence that SNCG promotes the proliferation of OSCC cells and inhibits apoptosis by regulating cell cycle- and apoptosis-related proteins *in vitro* and *in vivo*.

The JAK/STAT pathway is an important signal transduction system that is widely distributed in organisms, and a key pathway affecting cell growth, proliferation and survival in many human tumors [8, 14]. We used the GEO data-

base to examine the relationship between SNCG and the JAK/STAT signaling pathway in OSCC. We found that SNCG was significantly positively correlated with STAT5b. Western blot analysis revealed that p-STAT1, p-STAT3, p-STAT5a and p-STAT5b expression levels were significantly decreased after knockdown of SNCG, and significantly increased following SNCG overexpression, with p-STAT5b displaying the most significant changes. In addition, we showed that SNCG can activate JAK2. JAK2 activates downstream STAT5 in HPV-positive cervical cancer cells [10]. The role of STAT5 in tumors is extremely complex and context dependent. STAT5 has been shown to act as either a tumor suppressor or an oncogene in various cancers [25], indicating that STAT5 exerts different effects depending on the type of tumor. The specific role of the JAK2-STAT5 signaling pathway in OSCC proliferation and apoptosis is still unclear. The present data indicate, for the first time, that SNCG activates the JAK2/STAT5b signaling pathway in OSCC. We also found that knockdown of STAT5b had no effect on SNCG mRNA and protein expression levels, suggesting that STAT5b does not regulate SNCG, and confirming that SNCG is upstream of STAT5b. JAK2/STAT5b signaling is involved in cell proliferation, differentiation and immune regulation *in vivo* [26] and STAT5b is closely related to the proliferation process of tumor cells [27]. We performed a rescue test and found that STAT5b interference reversed the effects of SNCG on the proliferation and apoptosis of OSCC cells, as well as the expression of proliferation- and apoptosis-related factors including PKC, c-PARP and Bcl2. The expression of STAT5b has been shown to be significantly higher in glioblastoma than the normal cortex and low-grade astrocytomas. Silencing STAT5b in a glioblastoma cell line was found to cause cell cycle arrest and inhibit cell growth and tumor cell invasion. Silencing STAT5b has also been shown to downregulate the expression of downstream signaling molecules including Bcl2, focal adhesion kinase and vascular endothelial growth factor [28]. Therefore, SNCG plays an important role in the proliferation and apoptosis of OSCC by activating the JAK2/STAT5b signaling pathway.

Activation of the JAK2/STAT5b signaling pathway is involved in the SNCG-mediated promotion of OSCC proliferation and control of apoptosis, although the underlying mechanism remains unclear. Using the Inbio Discover website and PPI Finder, we predicted that ERK1/2 may interact with SNCG. ERK1/2 has previously been shown to activate the JAK2/STAT5 signaling pathway [29], while SNCG knockdown reportedly decreases the proliferation, colony forming ability and migration of lung cancer cells induced by high glucose, and inhibits the expression of ERK1/2 and p-ERK1/2 [30]. Thus, we speculated that SNCG may activate the JAK2/STAT5b signaling pathway by interacting with ERK1/2. Co-immunoprecipitation and GST pulldown experiments showed that SNCG interacted with ERK1/2 and promoted ERK1/2 phosphorylation. However, the mechanism by which SNCG regulates ERK1/2 phosphorylation remains unclear. ERK is a serine/threonine protein kinase that transmits the mitogen signal [31]. ERK is composed of ERK1 (also known as MAPK3) and ERK2 (also known as MAPK1), and phosphorylated ERK1/2 is the important active form. RAF directly regulates mitogen/extracellular protein kinases (MEK1 and MEK2), eventually leading to the phosphorylation of downstream ERK1/2, thereby forming the Raf-MEK-ERK pathway [32]. Phosphorylated ERK1/2 can activate downstream target genes in the nucleus and cytoplasm, many of which are transcription factors necessary for cell proliferation and survival [33]. Thus, ERK1/2 plays an important role in tumor progression. In this study, we showed that SNCG can directly interact with ERK1/2 and activate ERK1/2 in OSCC cells. Whether SNCG depends on the RAF-MEK-ERK phosphorylation pathway to promote ERK1/2 phosphorylation has not been reported and needs further confirmation. However, our evidence indicates that SNCG plays a key role in regulating the MAPK/ERK and JAK2/STAT5b signaling pathways. The effects of SNCG on p-JAK2 and p-STAT5b expression were partially reversed by U0126, indicating that SNCG can activate the JAK2/STAT5b signaling pathway by interacting with and activating ERK1/2, thereby playing a role in promoting proliferation and inhibiting apoptosis. Further, SNCG expression did not change after inhibition of ERK1/2, indicating that ERK1/2 did not regulate SNCG expression. However, we cannot rule out the possibility that JAK2/STAT5b signaling pathway is activated through a different pathway, which requires further research.

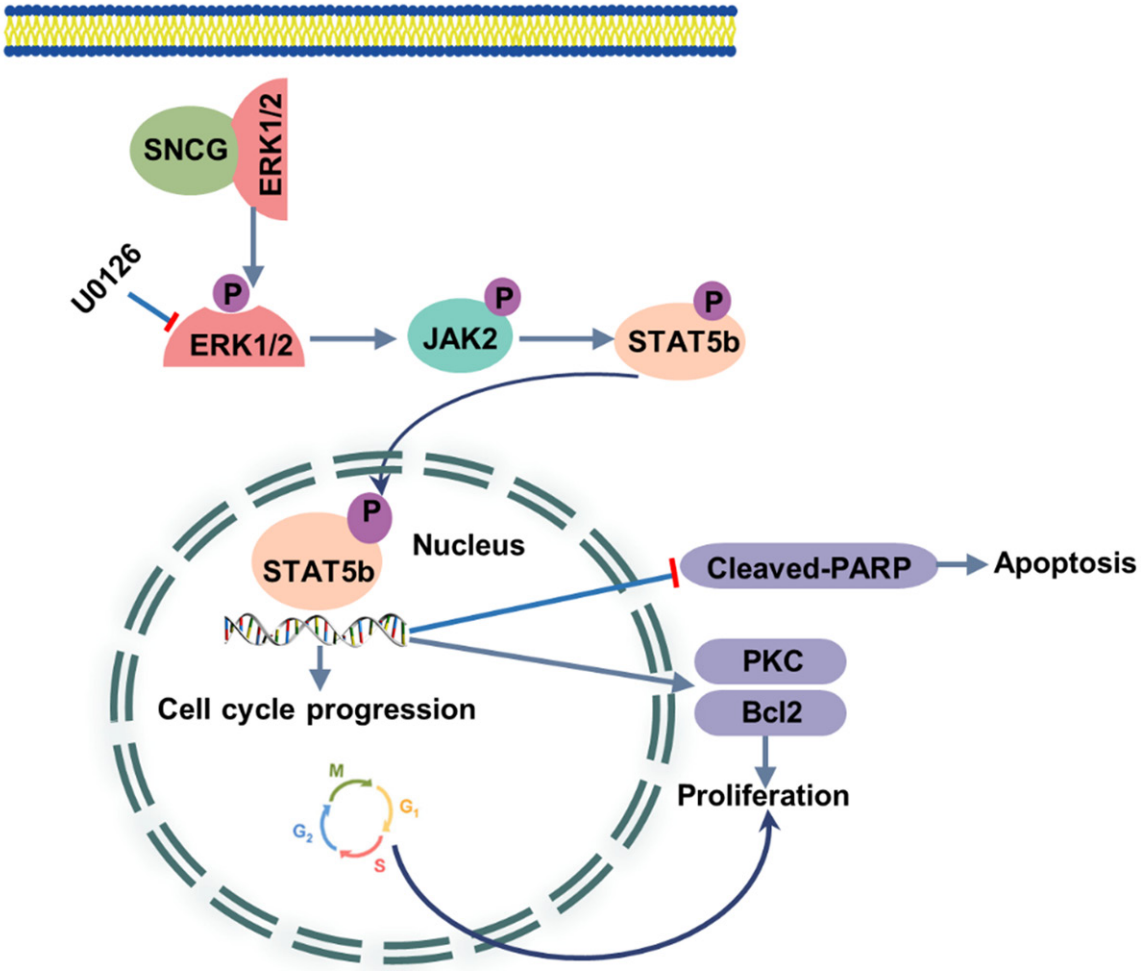

Figure 8. Schematic representation depicting the effects of SNCG on OSCC proliferation and apoptosis. SNCG, by interacting with ERK1/2 and promoting ERK1/2 phosphorylation, activates the JAK2/STAT5b signaling pathway. Thereby STAT5b interference and treatment with an ERK1/2 inhibitor U0126 reverses the effects of SNCG on the proliferation, apoptosis and cell cycle of OSCC cells, which leads to the downregulation of PKC and Bcl2, and up-regulation of c-PARP. Therefore, SNCG play the role of promoting the proliferation and inhibiting the apoptosis of OSCC cells.

In summary, the results of this study indicate that high SNCG expression is associated with the malignancy of OSCC. SNCG promotes OSCC proliferation and inhibits apoptosis *in vitro and in vivo*. We further demonstrate for the first time that SNCG interacts with ERK1/2 and promotes ERK1/2 phosphorylation, thereby activating the JAK2/STAT5b signaling pathway. We show that STAT5b interference and treatment with an ERK1/2 inhibitor U0126 reversed the effects of SNCG on the proliferation, apoptosis and cell cycle of OSCC cells (as shown in **Figure 8**). Taken together, these findings provide new insights into the mechanism of SNCG and may guide the future development of OSCC treatment strategies.

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Disclosure of conflict of interest

None.

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Table S1. The sequences of siRNAs

siRNA	Forward	Reverse
si SNCG-1	AGCAGCUGAGAAGACCAAGTT	CUUGGUCUUCUCAGCUGCUUC
si SNCG-2	UGACGGAAGCAGCUGAGAATT	UUCUCAGCUGCUUCCGUCATT
si SNCG-3	CCAAGGAGAAUGUUGUACATT	UGUACAACAUUCUCCUUGGUC
si NC	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT

Table S2. The sequences of primers

Primer	Forward	Reverse
GAPDH	GCACCGTCAAGGCTGAGAAC	ATGGTGGTGAAGACGCCAGT
SNCG	CAAGAAGGGCTTCTCCATCGCCAAGG	CCTCTTTCTCTTTGGATGCCACACCC

Table S3. Antibodies used in this study

Antibody	Manufacturer	Catalogue No	WorkingConcentrations
JAK2	CST	3230T	WB: 1:1000
p-JAK2	CST	4406T	WB: 1:1000
p-STAT5b	Bioss	bs-5703R	WB: 1:200
STAT5B	BOSTER	BM4716	WB: 1:300
ERK1/2	CST	4695S	WB: 1:1000
p-ERK1/2	Wanleibio	M03021512	WB: 1:300
Cleaved-PARP	Bioss	bsm-33138M	WB: 1:300
BCL2	Bioss	bs-4563R	WB: 1:300
PKC	Bioss	bs-23716R	WB: 1:300

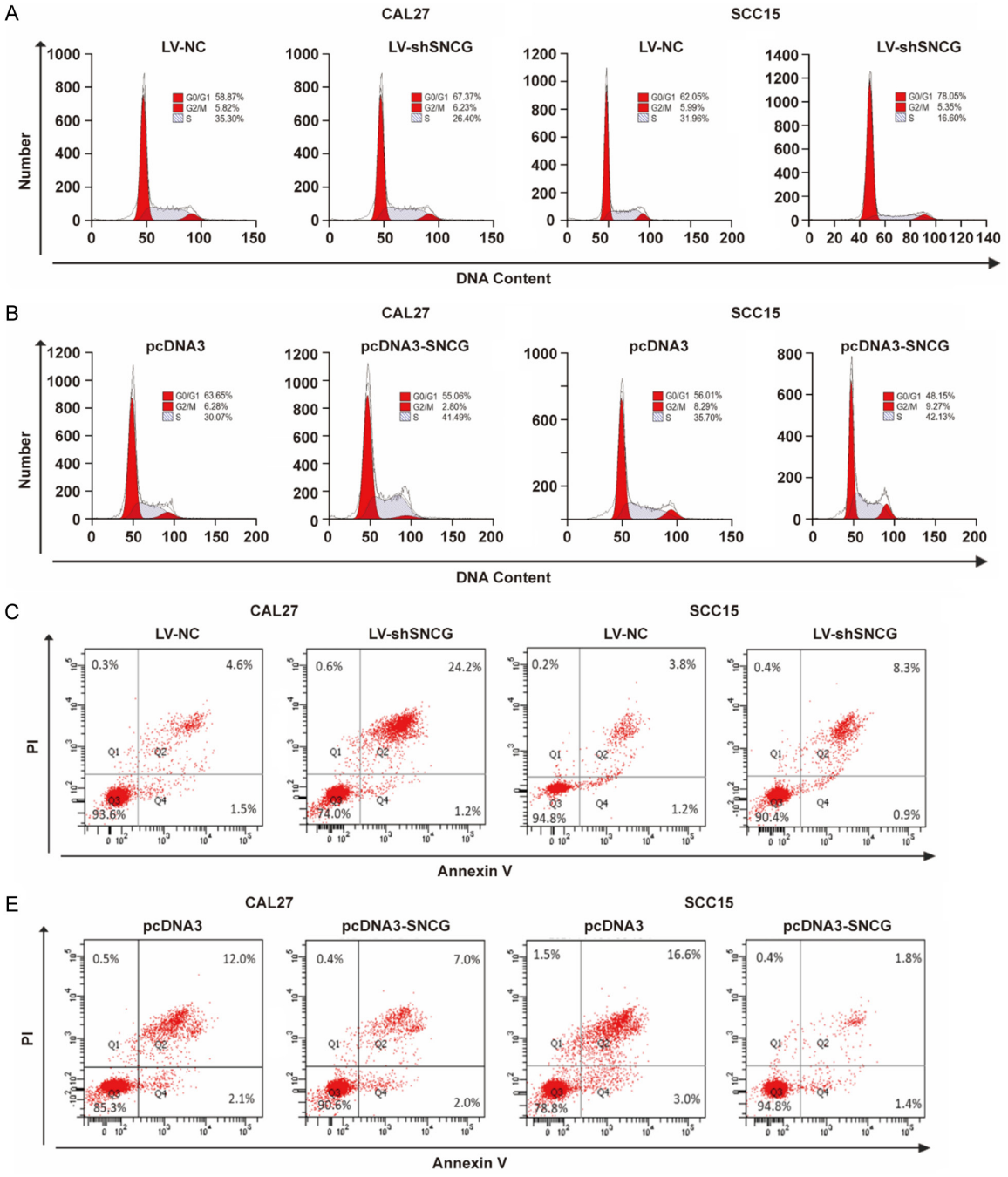

Figure S1. The effects of SNCG on OSCC cell cycle distribution and cell apoptosis. Distributions of cell cycles detected by flow cytometry in CAL27 and SCC15 cells after treatment with LV-shSNCG (A) and pcDNA3-SNCG (B). After treated with LV-shSNCG (C) and pcDNA3-SNCG (D), cell apoptosis rates of the CAL27 and SCC15 cells were performed by flow cytometry.

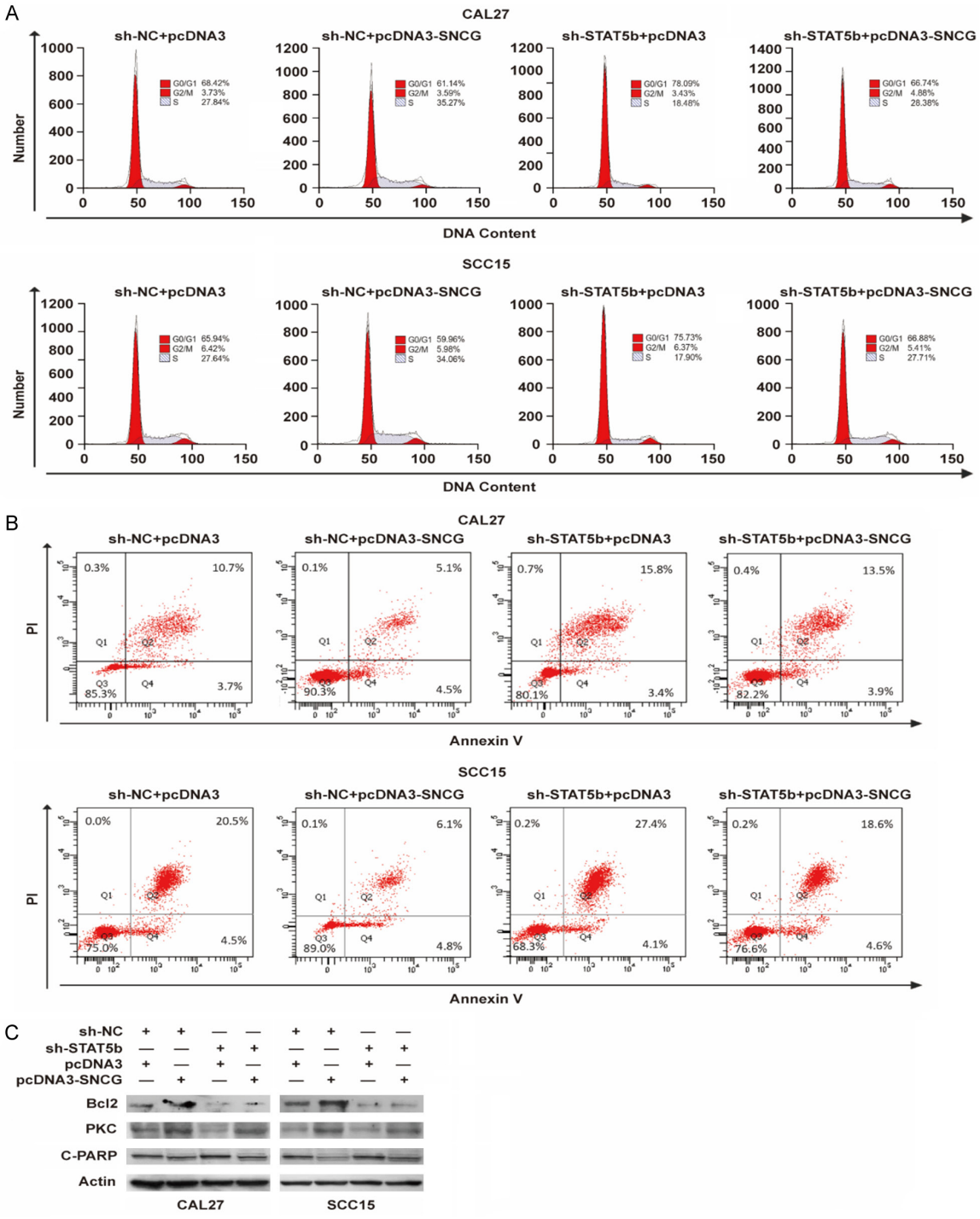

Figure S2. The effect of SNCG elevated on cell cycle and apoptosis can be reversed by STAT5b knockdown in OSCC cells. CAL27 and SCC15 cells were divided into four groups: shNC + pcDNA3, shNC + pcDNA3-SNCG, shSTAT5b + pcDNA3, and shSTAT5b + pcDNA3-SNCG. A. Cell cycle changes were detected by flow cytometry. B. Cells were treated as above and examined the apoptosis rate by flow cytometry. C. Western blot assay was applied to detect the levels of proliferation-related and apoptosis-related proteins PKC, Bcl2 and c-PARP.

A

B

SNCG synuclein, gamma (breast cancer-specific protein 1) [*Homo sapiens*]

Gene Info

GeneID	6623	**Organism**	Homo sapiens
Official Symbol	SNCG	**Official Full Name**	synuclein, gamma (breast cancer-specific protein 1)
Also known as	BCSG1; SR; breast cancer-specific protein 1; persyn; synoretin; synuclein, gamma (breast cancer-specific gene 1)	**Description**	synuclein, gamma (breast cancer-specific protein 1)
Gene type	protein-coding	**Genomic context**	chromosome: 10; Location: 10q23.2-q23.3
Locus tag	-	**See related**	*PubMed*; HGNC:11141; MIM:602998; HPRD:04297
Gene Ontology	. protein binding(IPI) - *PubMed*	None Available	. cytoplasm(IEA) . centrosome(IEA) . spindle(IEA)

MAPK1	1	HPRD(in vivo) - *PubMed* BioGRID(Invivo) - *PubMed*	. nucleotide binding(IEA) . phosphotyrosine binding(IEA) . protein serine/threonine kinase activity(EXP) - *PubMed* . protein serine/threonine kinase activity(IEA) . MAP kinase activity(TAS) - *PubMed* . protein binding(IPI) - *PubMed* . ATP binding(IEA) . transferase activity(IEA)	. MAPKKK cascade(IEA) . protein amino acid phosphorylation(IEA) . induction of apoptosis(TAS) - *PubMed* . chemotaxis(TAS) - *PubMed* . response to DNA damage stimulus(IEA) . cell cycle(IEA) . Ras protein signal transduction(EXP) - *PubMed* . synaptic transmission(TAS) - *PubMed* . organ morphogenesis(IEA) . cytosine metabolic process(IEA) . lipopolysaccharide-mediated signaling pathway(IEA) . response to lipopolysaccharide(IEA) . response to exogenous dsRNA(IEA) . interspecies interaction between organisms(IEA) . negative regulation of cell differentiation(IEA)	. nucleus(IEA) . nucleoplasm(EXP) - *PubMed* . cytoplasm(IEA) . cytosol(EXP) - *PubMed*

Figure S3. Bioinformatics predicts that SNCG may interact with ERK1/2. The Inbio Discover website (A) and PPI Finder (B) predicted that ERK1/2 may interact with SNCG.