Baicalein inhibits the growth of oral squamous cell carcinoma cells by downregulating the expression of transcription factor Sp1

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Abstract. Oral squamous cell carcinoma (OSCC), the most common malignancy of the oral cavity, accounts for >90% of all diagnosed oral cancer cases. Baicalein, a naturally derived compound, has been shown to alter p65 and the nuclear factor (NF)-κB pathway, thus exerting cytotoxic effects on various tumor cell types. However, the mechanism of action of baicalein in OSCC has not been fully elucidated. In the present study, the proliferation of OSCC cells treated with baicalein was examined using a CCK-8 assay. The effects of baicalein on the cell cycle and apoptosis of OSCC cells were determined by flow cytometric analyses. The expression of specificity protein 1 (Sp1), p65 and p50 at the mRNA and protein levels was determined by reverse transcription-quantitative PCR and western blot analysis, respectively. The results of the present study demonstrated that baicalein suppresses the proliferation of OSCC cell lines *in vivo* and *in vitro*. Baicalein also induced apoptosis of OSCC cells and arrested the cell cycle at the G0/G1 phase. Baicalein inhibited the expression of Sp1, p65 and p50 by downregulating the relative mRNA levels. Baicalein reduced the activity of NF-κB in OSCC cells. Knockdown of Sp1 also resulted in reduced expression of p65 and p50. In addition, Sp1 silencing enhanced the effects of baicalein. In conclusion, the present study demonstrated that baicalein suppresses the growth of OSCC cells through an Sp1/NF-κB-dependent mechanism.

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Introduction

There were an estimated ~34,000 new cases of oral cancer and 7,000 oral cancer-related deaths in the United States in 2018 (1). Oral squamous cell carcinoma (OSCC), the most common malignancy of the oral cavity, accounts for >90% of all diagnosed oral cancers (2). Although the 5-year survival rate of OSCC has increased from 56 to 62% due to improvements in treatment, including surgery, radiation and chemotherapy (3), the development of new therapeutic strategies is important.

Baicalein, a bioactive flavonoid present in the dry root of *Scutellariae Radix* (Huang Qin), has been reported to have effects on various malignancies, including lung, breast, hepatocellular, pancreatic and gastric cancer (4-8). In addition, baicalein induces various biological molecular activities by blocking tumor-associated signaling pathways (9). For example, baicalein suppresses the expression of superoxide dismutase and hypoxia-inducible factor-1α, and inhibits lung carcinoma cell proliferation and metastasis (10). Baicalein was previously found to induce apoptosis of pancreatic cancer cells via a myeloid cell leukemia 1-dependent pathway (11). Baicalein also induces apoptosis and autophagy of breast cancer cells by suppressing the phosphatidylinositol 3 kinase/protein kinase B pathway *in vivo* and *in vitro* (12). Importantly, baicalein does not appear to cause mutagenesis in normal cells, which is the major side effect of conventional anticancer drugs (13,14). Previous studies have revealed that baicalein is an effective molecular anticarcinogenic agent against oral cancer (15,16), and that it induces autophagy of oral cancer cells by promoting reactive oxygen species-dependent signaling pathways and arresting the proliferation of oral cancer cells in the G0/G1 phase by enhancing the degradation of cyclin D1 and activating aryl hydrocarbon receptor to decrease retinoblastoma (Rb) phosphorylation (15,16).

Specificity protein 1 (Sp1) is a zinc finger-type transcription factor with a guanine-cytosine-rich binding sequence in the gene promoter (17). Sp1 is involved in multiple aspects of tumor cell behavior, including growth, survival, angiogenesis and apoptosis (18-20). Sp1 expression and activation are considered to be associated with human cancer development and progression (21). Lines of evidence have indicated that targeting Sp1 and its downstream target proteins may be a potential treatment strategy for oral cancer (22). Our previous

study suggested that baicalein inhibits the expression of Sp1 and the downstream protein Epstein-Barr virus nuclear antigen 1 (EBNA1) and induces apoptosis of nasopharyngeal carcinoma (NPC) (23).

The present study examined the effects of baicalein on the proliferation, apoptosis and cell cycle progression of OSCC cells and xenograft tumors *in vivo*, and investigated the underlying mechanism.

Materials and methods

Cell lines and reagents. SCC25, CAL27 and HSC3 cells were kindly provided by Professor Bin Shi (University of Wuhan, China). All cell lines were cultured in RPMI-1640 media (HyClone; GE Healthcare Life Sciences) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C and humidified atmosphere of 5% CO_2. Baicalein (Sigma-Aldrich; Merck KGaA) was dissolved in DMSO at 100 mM as a stock solution, and diluted to a working concentration (1 mM) with PBS prior to use. For the *in vivo* xenograft studies, baicalein was dissolved in a solution containing 80% PBS and 20% DMSO.

Plasmid. For the construction of the Sp1 expression plasmid, the full-length cDNA was amplified and inserted into the *Eco*RI/*Xho*I sites of the pcDNA3.1 vector (Invitrogen; Thermo Fisher Scientific, Inc.). The primers for Sp1 were as follows: Forward, 5'-CCAAAATGCGATCGCATGAGCGACCAAGATCAC-3' and reverse, 5'-GAATCAAGTTTAAACTCAGAAGCCATTGCCACT-3'. The NF-κB activity plasmid was a gift from Professor H. Shu (University of Wuhan). The PRL-TK plasmid was a gift from Professor D. Guo.

Cell viability assay. SCC25, CAL27 and HSC3 cells ($1x10^4$ cells/well) were seeded in 96-well plates and treated with DMSO control (0.01%) or increasing concentrations of baicalein (30, 60 and 120 μM) at 37˚C. After incubation for 24, 48 and 72 h, the cells were treated with 10 μl Cell Counting Kit-8 reagent (Dojindo Molecular Technologies, Inc.) and the plates were incubated at 37˚C for 1 h in the dark. The optical density was measured at an absorbance of 450 nm using an ELx800 microimmunoanalyser (BioTek Instruments, Inc.).

Cell cycle analysis. SCC25, CAL27 and HSC3 cells were seeded in 6-well plates and treated with DMSO (0.01%) or baicalein (60 μM) at 37˚C for 24 h. The process was performed as described previously (24). Briefly, cells were digested and washed twice with cold PBS solution, and then resuspended in cold 75% ethanol. After fixation at -20˚C for 24 h, cells were collected and resuspended in 0.5 ml cold PBS. Cells were mixed with reagent A [Multisciences (Lianke) Biotech Co., Ltd.] and incubated at 4˚C for 30 min in the dark. Cell cycle analysis was performed using a Beckman Coulter system (EPICS Altra II; Beckman Coulter, Inc.).

Cell apoptosis analysis. Apoptosis of OSCC cells was detected using the Annexin V-FITC/propidium iodide (PI) apoptosis detection kit [Multisciences (Lianke) Biotech Co., Ltd.], as instructed by the manufacturer. Briefly, following treatment with baicalein (15, 30 and 60 μM) at 37˚C for 24 h, cells were digested. After three washes with PBS, $2x10^5$ cells were resuspended in 500 μl 1X binding buffer, followed by addition of 5 μl Annexin V-FITC and 10 μl PI, and incubated for 30 min at 20-28˚C in the dark. Cell apoptosis was immediately analyzed using a Beckman Coulter system (EPICS Altra II; Beckman Coulter, Inc.).

Western blot analysis. At 4 h post-transfection, SCC25, CAL27 and HSC3 cells were washed with PBS and treated with DMSO (0.01%) or baicalein (30 or 60 μM). After 48 h of incubation, cells were harvested and dissolved in RIPA lysis buffer (Beyotime Institute of Biotechnology) with 0.5% cocktail protease inhibitor (Roche Diagnostics). Following incubation on ice for 10 min, cell lysates were collected and sonicated for 20 sec. Protein concentration was determined using a BCA assay (BioRad Laboratories, Inc.). Quantified proteins were mixed with 5X loading buffer [250 nM Tris-Hcl (pH 6.8), 0.5% bromophenol blue, 50% glycerol, 10% SDS and 5% β-mercaptoethanol] and boiled for 5 min. The lysates were separated by SDS-PAGE on 10% gels, then subjected to immunoblot analyses. The primary antibodies used were as follows: GAPDH (cat. no. 10494-1-AP; 1:5,000; ProteinTech Group, Inc.); cleaved caspase-3 (cat. no. 9664; 1:1,000; Cell Signaling Technology, Inc.); caspase-9 polyclonal antibody (cat. no. A2636; 1:1,000; ABclonal Biotech Co., Ltd.); cleaved poly(ADP-ribose) polymerase (PARP-1; cat. no. sc-56196; 1:500; Santa Cruz Biotechnology, Inc.); Sp1 (cat. no. 9389; 1:1,000; Cell Signaling Technology, Inc.); p65 (cat. no. ab16502; 1:1,500; Abcam); p-p65 (cat. no. ab86299; 1:1,000; Abcam); and p50 (cat. no. 3035; 1:1,000; Cell Signaling Technology, Inc.). Western blot gray values were determined by ImageJ software (National Institutes of Health).

Reverse transcription-quantitative PCR (RT-qPCR). SCC25, CAL27 and HSC3 cells ($1x10^5$) were placed in 24-well plates. After attaching to the wells, the cells were washed with PBS and treated with DMSO (0.01%) or baicalein (30 or 60 μM). After incubation at 37˚C for 24 h, total RNA was extracted by using TRIzol reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Subsequently, cDNA was synthesized from total RNAs (1 μg) using an RT kit (Takara Bio, Inc.) according to the manufacturer's instructions. The mRNA expression levels of Sp1, p65 and p50 were quantified using the CFX96 Real-Time PCR Detection System with an SYBR Premix Ex Taq kit (Takara Bio, Inc.). The primers were as follows: p65, forward 5'-CGGGATGGCTTCTATGAGG-3' and reverse 5'-CTCCAGGTCCCGCTTCTT-3'; p50, forward 5'-ACCCTGACCTTGCCTATTTG-3' and reverse 5'-AGCTCTTTTTCCCGATCTCC-3'; Sp1, forward 5'-ATGGGGGCAATGGTAATGGTGG-3' and reverse 5'-TCAGAACTTGCTGGTTCTGTAAG-3'; GAPDH, forward 5'-GGTGGCTTCTGACTTCAACA-3' and reverse 5'-GTTGCTGTAGCCAAATTCGTTGT-3'. The mRNA levels were normalized to GAPDH as the reference gene.

Cell transfection. A specific short hairpin RNA (shRNA) targeting Sp1 (5'-GCATATTTGCCACATCCAAGG-3', Sp1-Homo-1828; GenePharma, Co., Ltd.) or a non-specific control (NC; 5'-TTCTCCGAACGTGTCACGT-3'; Shanghai GenePharma Co., Ltd.) were transfected to cells using X-treme

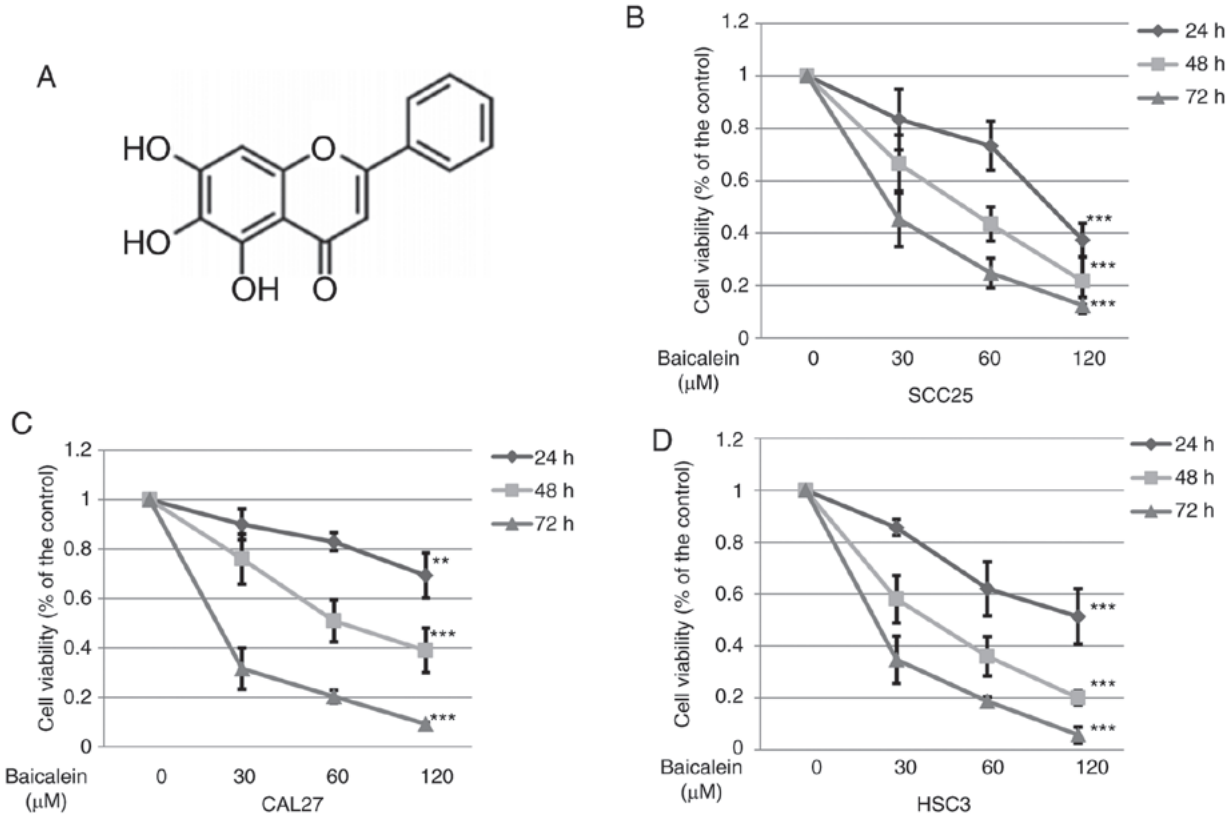

Figure 1. Baicalein inhibits the proliferation of oral squamous cell carcinoma cells. (A) Chemical structure of baicalein (5,6,7-trihydroxyflavone). (B) SCC25, (C) CAL27 and (D) HSC3 cells were treated with 0.01% DMSO or a series of increasing baicalein concentrations (30, 60 and 120 μM) for 24, 48 and 72 h, and the cell viability was detected using a CCK-8 assay. All the experiments were repeated three times. The values shown are the mean ± standard deviation of 3 independent experiments carried out in triplicate. One-way ANOVA was used to compare the experimental and control groups. *P<0.05, **P<0.01 and ***P<0.001.

GENE HP DNA Transfection Reagent (Roche Diagnostics; 40 pmol for each shRNA) according to the manufacturer's instructions. PcDNA3.1-Sp1 or pcDNA3.1 (vehicle control) was transfected to cells using X-treme GENE HP DNA Transfection Reagent. At 4 h post-transfection, cells (SCC25) were washed with PBS and treated with DMSO (0.01%) or baicalein (60 μM). After 48 h, the cells were harvested for western blot analysis. For RT-qPCR, total RNA was collected after 12 or 24 h of incubation. For the dual luciferase reporter assay, cells were harvested after 24 or 48 h of incubation.

Dual luciferase reporter assay. Cells ($1x10^5$) were placed in 24-well plates and incubated at 37˚C overnight prior to transfection. Plasmids were co-transfected into the cells for 24 h. After treatment with baicalein or control for 24 h, cells were collected and analyzed using the Dual Luciferase Reporter Assay system (Promega Corporation) as previously described (25).

Animal studies. The Medical Ethics Committee of Wuhan University approved the animal experimental protocols in the present study (G201725). A total of 10 BALB/c nude mice were obtained from the Animal Biosafety Level-III Laboratory of Wuhan University and housed in a specific pathogen-free environment. SCC25 cells ($5x10^6$/100 μl) were subcutaneously injected into the flanks of the mice when they were 6-7 weeks old. When the tumors became macroscopically visible (~7 days), the mice were randomly divided into two groups (n=5/group). The control group mice were injected with PBS (0.01% DMSO) and baicalein group mice were injected with baicalein (30 mg/kg, three times a week). The mice were sacrificed after 21 days of treatment. The mouse weight and tumor size were measured. Tumor volume was calculated as 0.5 x length x width^2. Tissue samples were fixed in 10% neutral buffered formalin, embedded in paraffin and cut into 3-μm sections).

Immunohistochemical analysis. Immunohistochemistry was performed using antibodies against Sp1 (cat. no. 9389; 1:1,000; Cell Signaling Technology, Inc.), p65 (cat. no. ab16502; 1:1,500; Abcam) and cleaved caspase-3 (cat. no. 9664; 1:1,000; Cell Signaling Technology, Inc.) according to the manufacturer's instructions. The process was performed as described previously (24). Briefly, after deparaffinization, the tissue sections were boiled in citric acid (pH 6.0) for 20 min and immersed in 3% H_2O_2 for 10 min to quench the endogenous peroxidase activity. After blocking in goat serum for 1 h at 20-28˚C, the tissue sections were incubated with primary antibodies overnight at 4˚C. After washing, the tissue sections were incubated with secondary antibody (MaxVision™ Kits; MaxVision Biosciences, Inc.) conjugated to horseradish peroxidase. Subsequently, the tissue sections were incubated with diaminobenzidine for 1 min and lightly counterstained with hematoxylin.

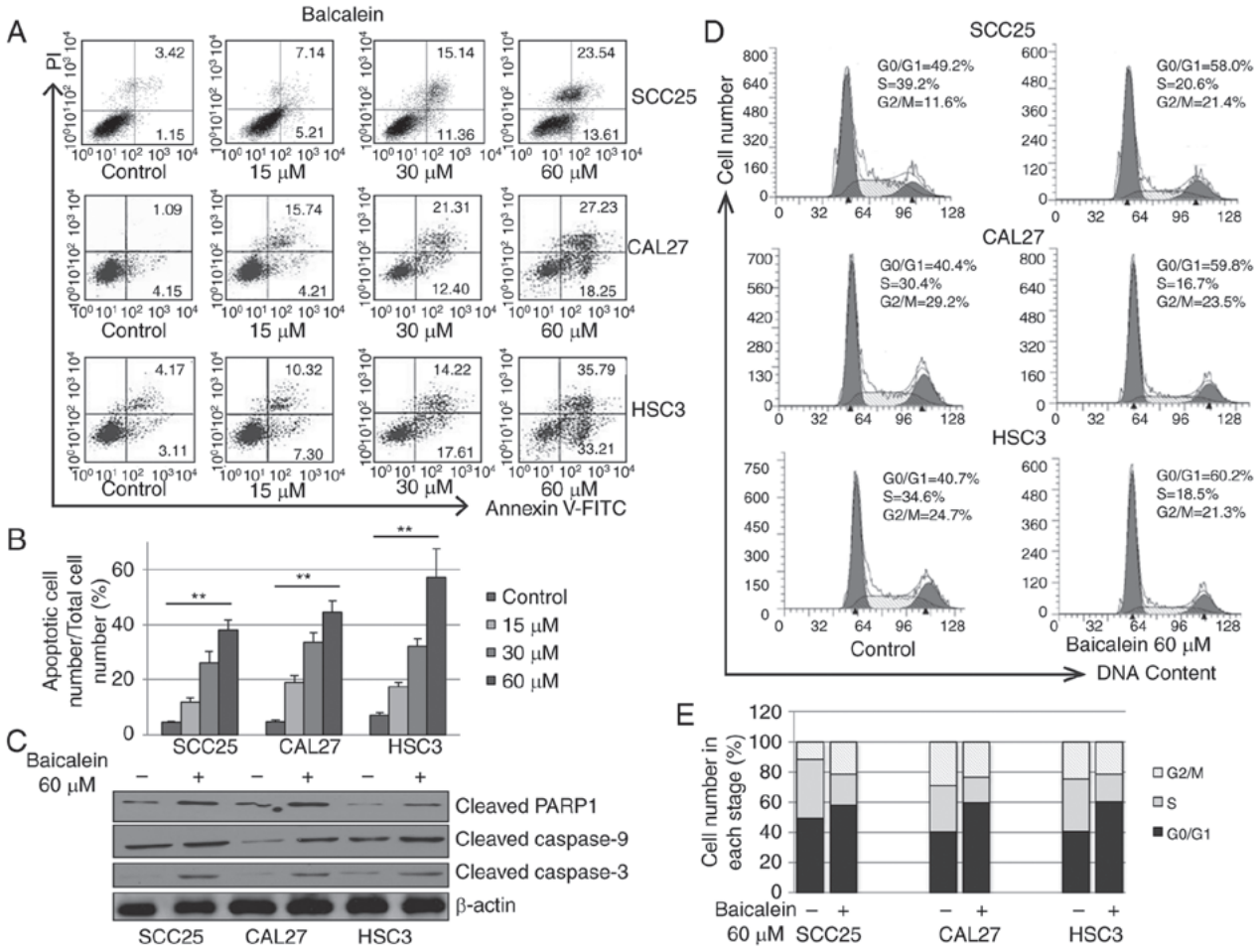

Figure 2. Baicalein arrests the cell cycle and induces apoptosis of oral squamous cell carcinoma cells. (A) SCC25, CAL27 and HSC3 cells were treated with 0.01% DMSO or baicalein (15, 30 and 60 μM) for 24 h. Apoptosis analysis was performed by flow cytometry. (B) Apoptotic cell distribution quantification. (C) Expression of apoptosis-related proteins was determined by western blotting. (D) SCC25, CAL27 and HSC3 cells were treated with 0.01% DMSO or baicalein (60 μM) for 24 h. Cell cycle analysis was performed by flow cytometry. (E) Cell cycle distribution was quantified. All the experiments were repeated three times. The values shown are the mean ± standard deviation of 3 independent experiments carried out in triplicate. One-way ANOVA was used to compare experimental and control groups. *P<0.05, **P<0.01 and ***P<0.001.

Statistical analysis. Data are presented as the mean ± standard deviation of three independent experiments. For the comparison of two groups, Student's t-test was selected. For the comparison of multiple groups, one-way ANOVA followed by the Newman-Keuls post hoc test was carried out. All statistical data were analyzed by using GraphPad Prism for Windows, version 5.0 (GraphPad Software, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

Baicalein effectively suppresses the proliferation of OSCC cells. To determine whether baicalein decreases the viability of OSCC cells, SCC25, CAL27 and HSC3 cells were treated with varying doses of baicalein and for different durations. The chemical structure of baicalein (5,6,7-trihydroxyflavone) is shown in Fig. 1A. Baicalein significantly (P<0.05) reduced the viability of SCC25, CAL27 and HSC3 cells compared with cells treated with DMSO control (Fig. 1B-D). These results suggest that baicalein effectively inhibits the proliferation of different OSCC cell lines.

Baicalein induces apoptosis in OSCC cells. To examine the apoptotic effect exerted by baicalein on OSCC cells, SCC25, CAL27 and HSC3 cells were treated with increasing doses of baicalein for 24 h. As shown in Fig. 2A and B, baicalein significantly induced apoptosis of SCC25, CAL27 and HSC3 cells. To further assess stimulation of the apoptotic pathway, expression of several apoptosis-associated proteins was detected by western blot analysis. Baicalein increased the protein levels of cleaved caspase-9, cleaved caspase-3 and cleaved PARP-1 in SCC25, CAL27 and HSC3 cells (Fig. 2C), suggesting that baicalein induces OSCC cell apoptosis via the mitochondrial apoptotic pathway.

Baicalein arrests the cell cycle in the G0/G1 phase. To determine whether baicalein affects the cell cycle, SCC25, CAL27 and HSC3 cells were treated with vehicle control (0.01% DMSO) or baicalein (60 μM) for 24 h. The distribution of cells in different cell cycle phases was analyzed by flow cytometry. As shown in Fig. 2D and E, baicalein treatment induced cell cycle arrest in the G0/G1 phase. The fraction of SCC25, CAL27 and HSC3 cells in the G0/G1 phase was increased by 18.8, 29.4 and 19.5%, respectively, when treated with baicalein

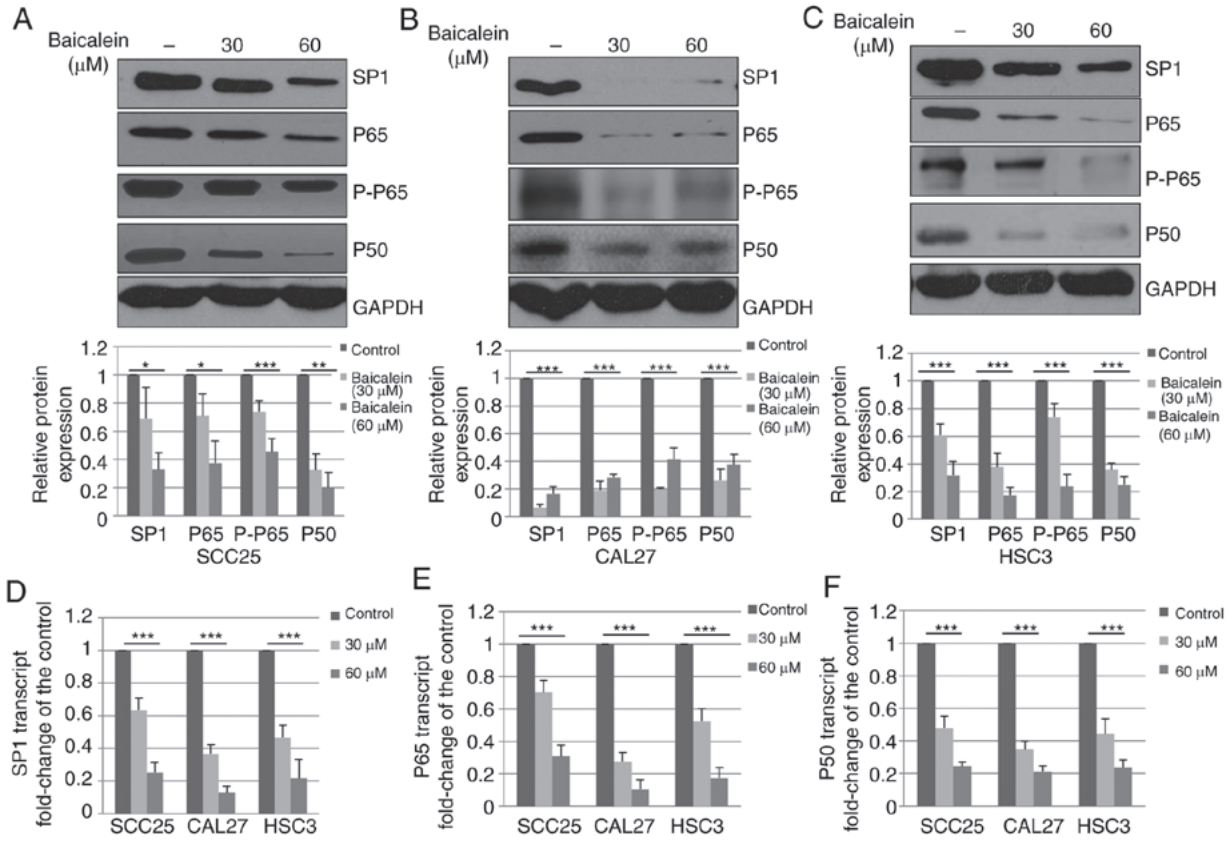

Figure 3. Baicalein inhibits the expression of Sp1, p65 and p50 in oral squamous cell carcinoma cell lines. (A) SCC25, (B) CAL27 and (C) HSC3 cells were treated with 0.01% DMSO or baicalein (30 and 60 μM) for 48 h. The expression of Sp1, p65, p-p65 and p50 was measured by immunoblot analysis as indicated. SCC25, CAL27 and HSC3 cells were treated with 0.01% DMSO or baicalein (30 and 60 μM) for 24 h. The mRNA levels of (D) Sp1, (E) p65 and (F) p50 were examined by reverse transcription-quantitative PCR analysis. All the experiments were repeated three times. The values shown are the mean ± standard deviation of 3 independent experiments carried out in triplicate. One-way ANOVA was used to compare experimental and control groups. *P<0.05, **P<0.01 and ***P<0.001. Sp1, specificity protein 1.

(60 μM). These results demonstrated that baicalein reduces the proliferation of OSCC cells by causing G0/G1 cell cycle arrest.

Baicalein decreases the expression of Sp1, p65 and p50. To determine whether baicalein alters the expression of Sp1 in OSCC cells, three different OSCC cell lines (SCC25, CAL27 and HSC3) were treated with DMSO control or baicalein. As shown in Fig. 3A-C, baicalein significantly (P<0.05) decreased Sp1 expression in SCC25, CAL27 and HSC3 cells. Sp1 has been previously demonstrated to be a transcription factor that regulates the expression of p65 and p50 (26). Therefore, the expression of p65 and p50 was measured, and the levels of p65, p-p65 and p50 were found to be decreased following treatment with baicalein (Fig. 3A-C).

The observed decrease in protein expression may be due to reduced mRNA levels; therefore, the effects of baicalein on Sp1 were also analyzed at the transcriptional level. SCC25, CAL27 and HSC3 were treated with DMSO control or baicalein, and total RNA was collected and subjected to RT-qPCR analysis. The mRNA expression of Sp1 in SCC25, CAL27 and HSC3 cells was found to be decreased by 75.7, 87.0 and 78.8%, respectively, compared with controls at 48 h (Fig. 3D). Additionally, the mRNA levels of p65 and p50 were analyzed. As shown in Fig. 3E and F, the mRNA levels of p65 and p50 were significantly decreased following treatment with baicalein. These results suggest that baicalein decreases the expression of Sp1, p65 and p50 in OSCC cell lines by reducing the mRNA expression.

Baicalein decreases NF-κB activity in OSCC cells. The reduced expression of p65 and p50 may lead to suppression of the NF-κB pathway. To confirm this hypothesis, SCC25, CAL27 and HSC3 cells were treated with DMSO control or baicalein, and the activity of NF-κB was determined using a Dual Luciferase Reporter Assay system. As shown in Fig. 4A-C, the activity of the NF-κB pathway was downregulated to 15.3% (SCC25), 8.6% (CAL27) and 12.0% (HSC3) following treatment with baicalein (60 μM) for 24 h. These results suggest that baicalein markedly inhibits the activation of NF-κB signaling in OSCC cells.

Reduced expression of Sp1 decreases the expression of p65 and p50. To determine whether the decrease in p65 and p50 expression was associated with the expression of Sp1 in OSCC cells, SCC25 and CAL27 cells were transfected with a specific shRNA targeting Sp1. Total proteins were harvested and subjected to western blot analysis. As shown in Fig. 5A and B, silencing of Sp1 significantly reduced the expression of p65 and p50. Additionally, the mRNA levels of p65 were decreased to 44.3 and 29.3% following silencing of Sp1 in SCC25 and CAL27 cells, respectively (Fig. 5C). The mRNA levels of p50 in SCC25 and CAL27 cells with

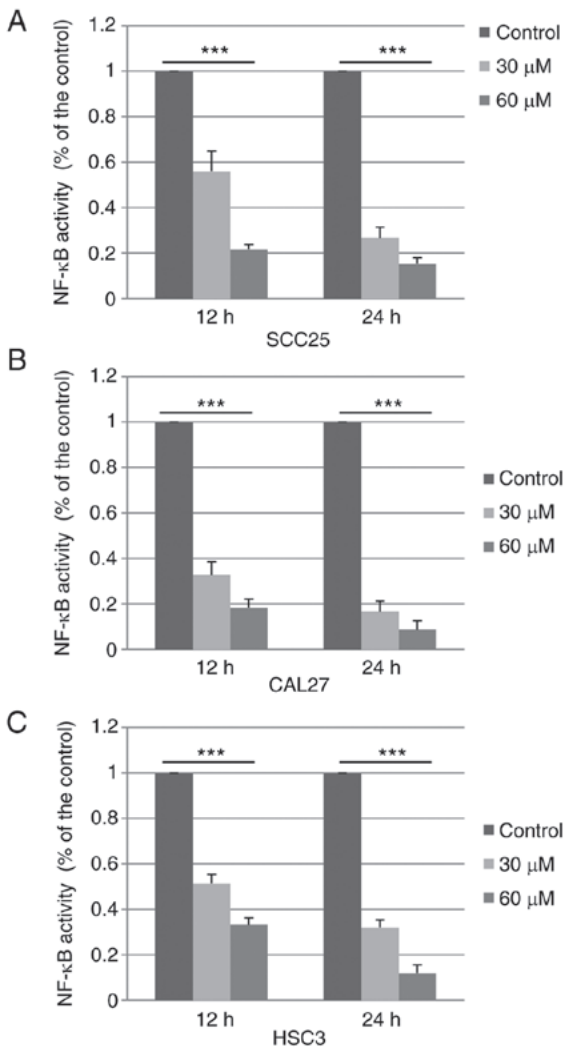

Figure 4. Baicalein inhibits the activity of the nuclear factor (NF)-κB pathway. (A) SCC25, (B) CAL27 and (C) HSC3 cells were treated with 0.01% DMSO or baicalein (30 and 60 μM) for 12 or 48 h. The activity of NF-κB was determined using the Dual Luciferase Reporter Assay system. All the experiments were repeated three times. The values shown are the mean ± standard deviation of 3 independent experiments carried out in triplicate. One-way ANOVA was used to compare experimental and control groups. *P<0.05, **P<0.01 and ***P<0.001.

silenced Sp1 expression were decreased to 40.3 and 25.0%, respectively (Fig. 5D). These results indicate that downregulated expression of Sp1 reduces the expression of p65 and p50 in OSCC cells.

Downregulated expression of Sp1 inhibits NF-κB activity and the viability of OSCC cells. To determine the effect of Sp1 silencing on NF-κB activity and cell viability, SCC25 and CAL27 cells were transfected with Sp1-specific shRNA. The activity of NF-κB was determined using the Dual Luciferase Reporter Assay system. As shown in Fig. 5E, the activity of the NF-κB pathway was reduced to 35 and 39.3% by Sp1-shRNA in SCC25 and CAL27 cells, respectively. The viability of SCC25 and CAL27 cells was decreased to 63.7 and 44.7%, respectively, by specific Sp1-shRNA (Fig. 5F). These results indicate that silencing of Sp1 expression inhibits NF-κB activity and reduces the viability of OSCC cells.

shRNA-Sp1 enhances the effect of baicalein. To determine whether the effects of baicalein on NF-κB activity and cell viability are dependent on Sp1, SCC25 cells were transfected with shRNA-Sp1 or homo-NC, and treated with baicalein or DMSO control. As shown in Fig. 6A, combination with shRNA-Sp1 significantly enhanced the baicalein-induced suppression of Sp1, p65 and p50 expression. In addition, knockdown of Sp1 significantly contributed to the inhibitory effects of baicalein on NF-κB activity (Fig. 6B) and cell viability (Fig. 6C).

Exogenous Sp1 attenuates the effects of baicalein. To determine whether the effects of baicalein on cell viability are primarily caused by the reduced expression of Sp1, SCC25 cells were transiently transfected with pcDNA3.1-Sp1 or vehicle control (pcDNA3.1). The cells were subsequently treated with DMSO or baicalein. As shown in Fig. 6D, pcDNA3.1-Sp1 significantly increased the expression of Sp1, p65 and p50. Compared with Sp1 overexpression alone, combined treatment with baicalein and pcDNA3.1-Sp1 transfection decreased the expression of Sp1, p65 and p50. As shown in Fig. 6E, the NF-κB activity was enhanced by 273.3 and 503.1% following transfection with pcDNA3.1-Sp1 for 12 and 24 h, respectively. Overexpression of Sp1 attenuated the effects of baicalein on NF-κB activity. In addition, Sp1 overexpression increased the viability of SCC25 cells and attenuated the inhibitory effect of baicalein on cell viability (Fig. 6F). These results suggest that baicalein reduces SCC25 cell viability and NF-κB activity, which is mediated by the Sp1 pathway.

In vivo effects of baicalein on BALB/c mice inoculated with SCC25 cells. To determine the effects of baicalein on OSCC *in vivo*, SCC25 cells were used to establish subcutaneous xenograft tumors in immune-deficient BALB/c nude mice. The mice were administered baicalein or DMSO at 7 days after tumor cell inoculation. After treatment with baicalein or DMSO for 21 days, the mice were sacrificed and their tumors were measured. The weight of the mice was also evaluated. As shown in Fig. 7A, tumor volumes were decreased by treatment with baicalein. Additionally, there was no significant difference in the weight of the mice between the two groups (Fig. 7B). Representative images of the tumors are shown in Fig. 7C. To further elucidate the mechanism underlying the effects of baicalein on tumor growth, the expression and distribution of p65, Sp1 and cleaved caspase-3 were determined by immunohistochemical examination of the tumor tissues. As shown in Fig. 7D, the expression p65 and Sp1 was stronger in the sections from the control group compared with the baicalein-treated group. Conversely, cleaved caspase-3 expression was weaker in the control group compared with that in the baicalein-treated group. These results suggest that baicalein reduces the growth of SCC25 OSCC cell xenografts *in vivo*.

Discussion

Accumulating evidence suggests that targeting Sp1 may be a novel therapeutic strategy for cancer treatment, as Sp1 is involved in tumor development, growth and metastasis (27,28). Sp1 is overexpressed in a number of human cancer cells, including oral cancer cells (29), which suggests that Sp1 may

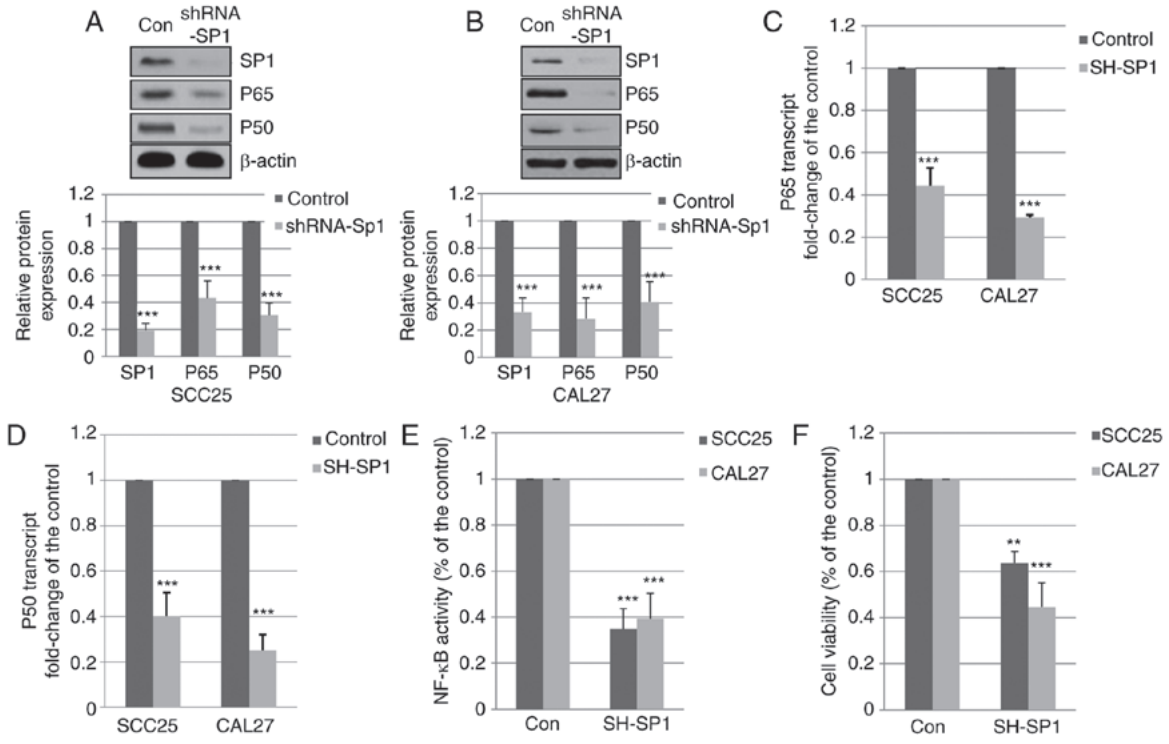

Figure 5. Inhibition of Sp1 reduces the activity of nuclear factor (NF)-κB and the viability of oral squamous cell carcinoma cells. SCC25 and CAL27 cells were transfected with short hairpin RNA-Sp1 for 48 h. (A and B) Total proteins were harvested and subjected to western blot analysis. The mRNA levels of (C) p65 and (D) p50 were determined by reverse transcription-quantitative PCR. (E) The activity of NF-κB was determined using the Dual Luciferase Reporter Assay system. (F) Cell viability was detected using a CCK-8 assay. Student's t-test was used to compare the experimental and control groups. *P<0.05, **P<0.01 and ***P<0.001. Sp1, specificity protein 1.

be associated with cancer cell growth. Our previous study revealed that baicalein inhibits cancer development and expression of EBNA1 in Epstein-Barr virus-positive NPC cells via an Sp1-dependent mechanism (23); thus, it would be of interest to investigate the effects of baicalein in EBV-negative epithelial cancer cells. In the present study, baicalein, a traditional extract used in herbal medicine, effectively suppressed the proliferation of OSCC cell lines. Baicalein induced apoptosis of OSCC cells and arrested the cell cycle in the G0/G1 phase. Baicalein significantly decreased the protein and mRNA expression of Sp1, p65 and p50. Additionally, baicalein reduced NF-κB activity in OSCC cells. Furthermore, knockdown of Sp1 reduced the expression of p65 and p50, and Sp1 silencing enhanced the effects of baicalein. By contrast, overexpression of Sp1 attenuated the inhibitory effects of baicalein on NF-κB activity and cell viability. Furthermore, baicalein reduced the growth of SCC25-induced tumor xenografts *in vivo*.

Baicalein has been attracting increasing attention due to its cytotoxic effects on cancer cells at a low dose (30-33). Our previous study indicated that a low-toxicity dose of baicalein exerted a strong antitumor effect on NPC cells *in vivo* and *in vitro* (23). In this context, the effect of baicalein on OSCC were investigated. Baicalein has been reported to induce apoptosis via both the intrinsic and extrinsic apoptotic pathways in cancer cells. For example, baicalein treatment induces caspase-3 and caspase-9 activation, decreases the expression of Bcl-2 and increases the level of Bax and p53 via the ERK/p38 MAPK pathway in breast cancer (34). In addition to its pro-apoptosis effects, baicalein also regulates the cell cycle. In lung cancer cells, baicalein arrests the cell cycle in the S phase by downregulating the expression of cyclin A (35); however, baicalein induces G0/G1 phase arrest in other cancer types, such as prostate cancer, hepatocellular carcinoma and lung squamous cell carcinoma. Baicalein upregulates the expression of Rb, p53, p21(Cip1) and p27(Kip1), and decreases the expression of cyclin D1, cyclin E, p-Rb and CDK4, which results in an increased percentage of hepatocellular carcinoma cells in the G0/G1 phase (36,37). Baicalein is also reported to reduce the growth of tumor cell-induced xenografts in animal studies, including breast, colon and pancreatic cancer xenografts (12,38,39). In the present study, baicalein induced G0/G1 phase cell cycle arrest, induced apoptosis, and inhibited the growth of OSCC cells *in vitro* and *in vivo*.

Shin *et al* (29) reported that Sp1 is overexpressed in OSCC tissues compared with normal oral mucosal tissues, suggesting that Sp1 may be a valuable molecular target for the treatment of oral cancer (29). Several targeted drugs have exhibited strong cytotoxic effects against OSCC cells, including mithramycin A, an Sp1-specific inhibitor (22,29,40). Our previous study revealed that baicalein reduces the expression of Sp1 in NPC cells (23); thus, it was hypothesized that baicalein may also decrease the expression of Sp1 in OSCC cells. As expected, the expression of Sp1 was reduced by baicalein treatment. Sp1 has been reported to be crucial for the transcription of the NF-κB subunits, p50 and p65, and involved in regulating the activity of the NF-κB pathway. Silencing of Sp1 was shown to reduce the expression of p50 and p65, resulting in reduced activity of the NF-κB pathway in OSCC cells. Knockdown of Sp1 was also found to be associated with reduced cell colony formation, consistently with our current findings (41). In

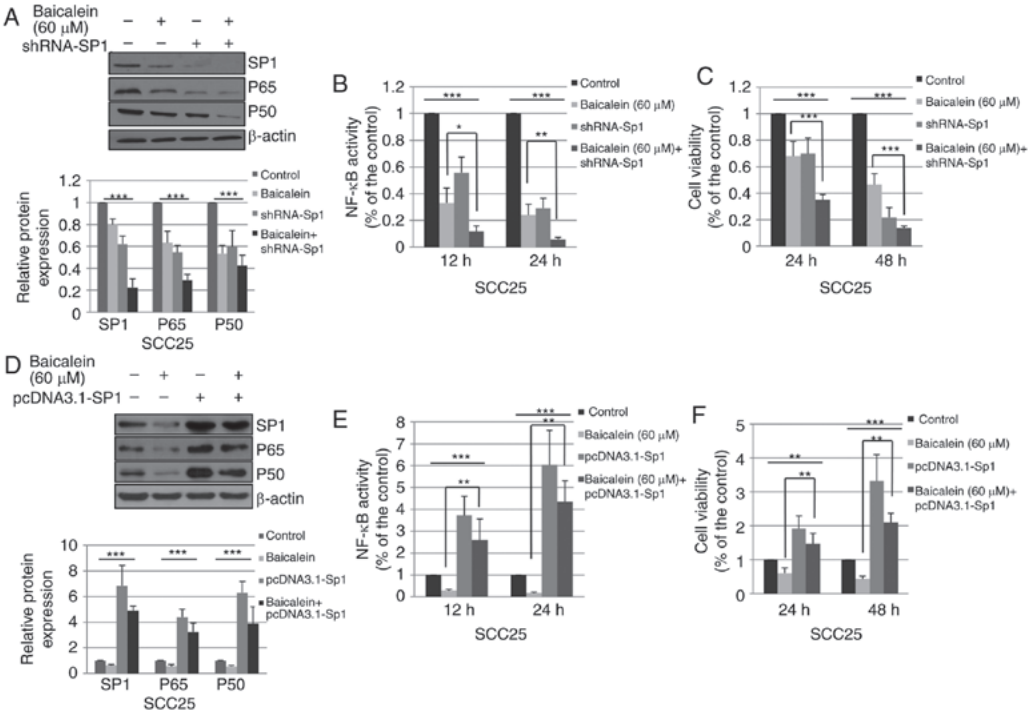

Figure 6. Baicalein inhibits nuclear factor (NF)-κB activity and viability in oral squamous cell carcinoma cells through an Sp1-dependent pathway. SCC25 cells were transfected with short hairpin RNA-Sp1 for 4 h, and treated with baicalein (60 μM) for 48 h. (A) The expression of Sp1, p65 and p50 was detected by western blotting after 48 h of treatment. (B) The activity of NF-κB was determined using a Dual Luciferase Reporter Assay system at 12 or 24 h. (C) Cell viability was detected using a CCK-8 assay. SCC25 cells were transfected with pcDNA3.1-Sp1 for 4 h and treated with baicalein (60 μM). (D) The expression of Sp1, p65 and p50 was detected by western blotting after 48 h of treatment. (E) The activity of NF-κB was determined by the Dual Luciferase Reporter Assay system at 12 or 24 h. (F) Cell viability was detected after 24 or 48 h of treatment using the CCK-8 assay. All the experiments were repeated three times. The values shown are the mean ± standard deviation of 3 independent experiments carried out in triplicate. One-way ANOVA and Newman-Keuls post hoc test were used to compare experimental and control groups. *P<0.05, **P<0.01 and ***P<0.001. Sp1, specificity protein 1.

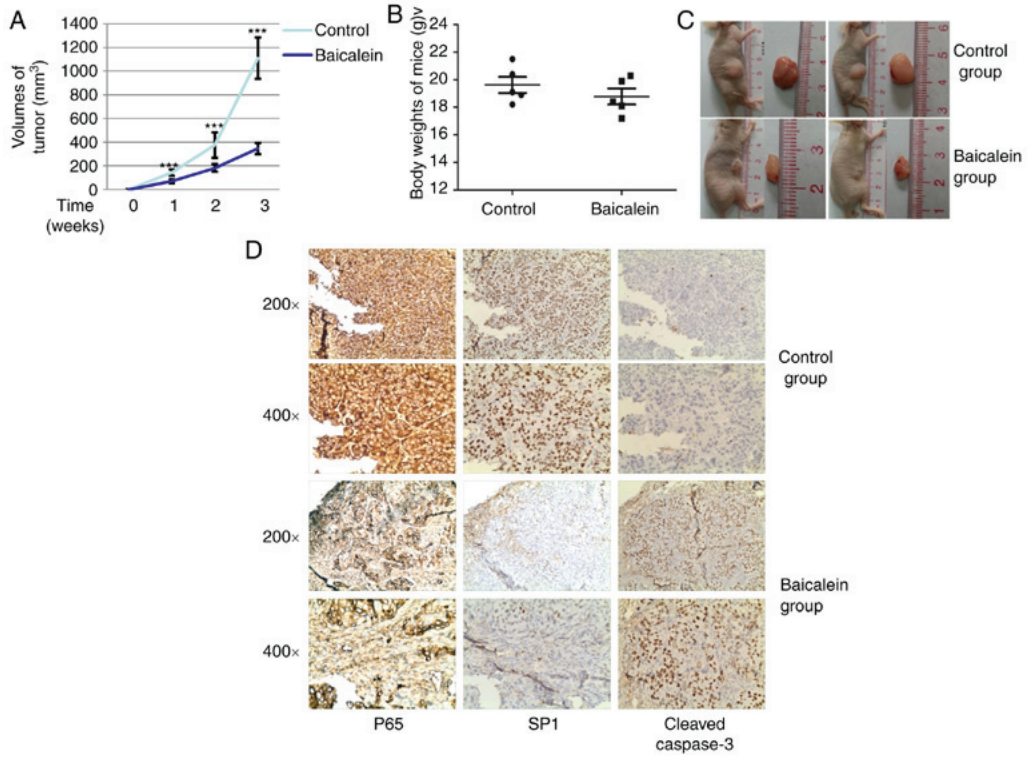

Figure 7. Baicalein inhibits the proliferation of OSCC cell-induced tumors *in vivo*. BALB/c nude mice with SCC25 cell-induced tumors were treated with DMSO (0.01%) or 30 mg/kg/day baicalein (n=5 per group) for 21 days. (A) Tumor volumes and (B) mouse weights were evaluated. (C) Representative photos of the dissected tumors after the mice were sacrificed. (D) The expression of p65, Sp1 and cleaved caspase-3 in tumor tissues was assessed by immunohistochemical analysis. Student's t-test was used to compare the experimental and control groups. *P<0.05, **P<0.01 and ***P<0.001. OSCC, oral squamous cell carcinoma; Sp1, specificity protein 1.

addition, the expression of p65 and p50 in OSCC cells was decreased by baicalein treatment in the present study. Of note, decreased Sp1 expression was reduced at the mRNA level, which suggested that baicalein targets another protein in order to reduce Sp1 expression.

NF-κB is a transcription factor that targets anti-apoptotic genes and can promote tumor development, cell survival and malignant progression in a variety of cancer types, including OSCC (42). Matrix metalloprotease-9, which is synergistically upregulated by pro-inflammatory cytokines and growth factors in an NF-κB-dependent manner, has been reported to be associated with nodal metastasis and reduced survival in oral cancer (43). Chemokine receptor 7, which is associated with metastasis, is also upregulated by NF-κB activation (44). Thus, it may be inferred that the NF-κB pathway is a target for chemoprevention. The results of the present study suggested that Sp1 regulates the activity of NF-κB and the viability of OSCC cells, and attenuates the inhibitory effects induced by baicalein, which indicates that the mechanism of action of baicalein is Sp1/NF-κB-dependent. Of note, a number of studies have reported that baicalein inhibits the activity of NF-κB by reducing the expression of p65, with affects in various diseases and disease models, including cervical cancer, tubular-interstitial nephritis, hepatocellular carcinoma, vascular endothelial injury and Parkinson's disease (45-49). The findings of the present study demonstrated that baicalein decreases the expression of p65 and inhibits the activity of NF-κB via an Sp1-dependent mechanism.

In conclusion, the present study uncovered a novel mechanism to explain how baicalein decreases the viability and the activity of NF-κB in OSCC cells *in vivo* and *in vitro*. Knockdown of Sp1 decreased the expression of p65 and p50 in OSCC cells at the mRNA level, which resulted in reduced cell viability and reduced activity of NF-κB. Baicalein also induced cell apoptosis and arrested the cell cycle in the G0/G1 phase. Notably, baicalein reduced the growth of OSCC cells by blocking an Sp1/NF-κB-dependent pathway.

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Availability of data and materials

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

Authors' contributions

HZ designed the study; ZG performed the experiments; YZ and JL contributed new reagents/analytic tools; ZG analyzed the data; HZ and JL wrote the manuscript. All authors have read and approved the final version of the manuscript for publication.

Ethics approval and consent to participate

All animal experiments were approved by the ABSL-3 animal laboratory at Wuhan University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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