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ORIGINAL RESEARCH

# Scutellarein Inhibits Osteosarcoma Growth by Targeting the TLR4/TRAF6/NF-κB Pathway

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**Purpose:** Osteosarcoma (OS) is the most common malignant tumor associated with poor patient outcomes and a limited availability of therapeutic agents. Scutellarein (SCU) is a monomeric flavone bioactive compound with potent anti-cancer activity. However, the effects and mechanisms of SCU on the growth of OS remain unknown.

**Methods:** The Cell Counting Kit-8, colony formation assay and 5-ethynyl-2′-deoxyuridine (EdU) incorporation assays were used to analyze cell proliferation ability in vitro. TLR4/TRAF6/NF-κB signaling transduction was investigated by RNA sequencing analysis, quantitative real-time polymerase chain reaction, Western blotting, NF-κB luciferase reporter assay, immunofluorescent staining, and immunoprecipitation. Molecular docking and cellular thermal shift assay were employed to confirm the binding interaction between SCU and TLR4. The effects of SCU and TLR4 overexpression on OS growth were analyzed using a xenograft tumor model and immunohistochemical staining.

**Results:** SCU was found to significantly inhibit OS cell proliferation, and RNA sequencing analysis suggested that the NF-κB pathway is closely associated with this process. Further studies revealed that SCU inhibited the canonical NF-κB pathway through its binding with TLR4, which disrupted the interaction of TLR4 and TRAF6. Moreover, SCU also repressed NF-κB signal transduction by inhibiting TLR4 expression. Furthermore, SCU was revealed to suppress OS cell proliferation by targeting TLR4 in vitro and in vivo.

**Conclusion:** SCU exhibited a dual impact by inhibiting TLR4 expression and disrupting TLR4–TRAF6 interaction, resulting in NF-κB inactivation, thereby blocking OS growth.

**Keywords:** scutellarein, osteosarcoma, TLR4, NF-κB pathway, molecular modeling

## Introduction

Osteosarcoma (OS) is the most common malignant tumor originating from bone. It most often occurs in adolescents aged 10 to 20 years and in older people more than 60 years old, and frequently affects the distal femur, proximal tibia and proximal humerus.[1,2] To date, the standard treatment strategy for OS is surgery combined with neoadjuvant chemotherapy, but the chemotherapy drugs have associated toxic effects and have a considerable impact on quality of life. Consequently there has been no significant improvement in the 5-year survival rate of OS patients over the past few decades.[3] Accordingly, the 5-year survival rate for patients without metastasis or recurrence is about 60%, but this rate drops to less than 30% for patients with metastasis or recurrence.[1,4] There is therefore an urgent need to identify novel chemotherapy drugs to restrict OS carcinogenesis.

Natural products have been shown to exert versatile chemotherapeutic benefits, which make them indispensable drugs for malignant cancer therapy.[5] Scutellarein (SCU) is a monomeric flavone active compound extracted from Chinese traditional herbs including *Scutellaria baicalensis* Georgi.[6,7] Previous studies have suggested that SCU has bioactive

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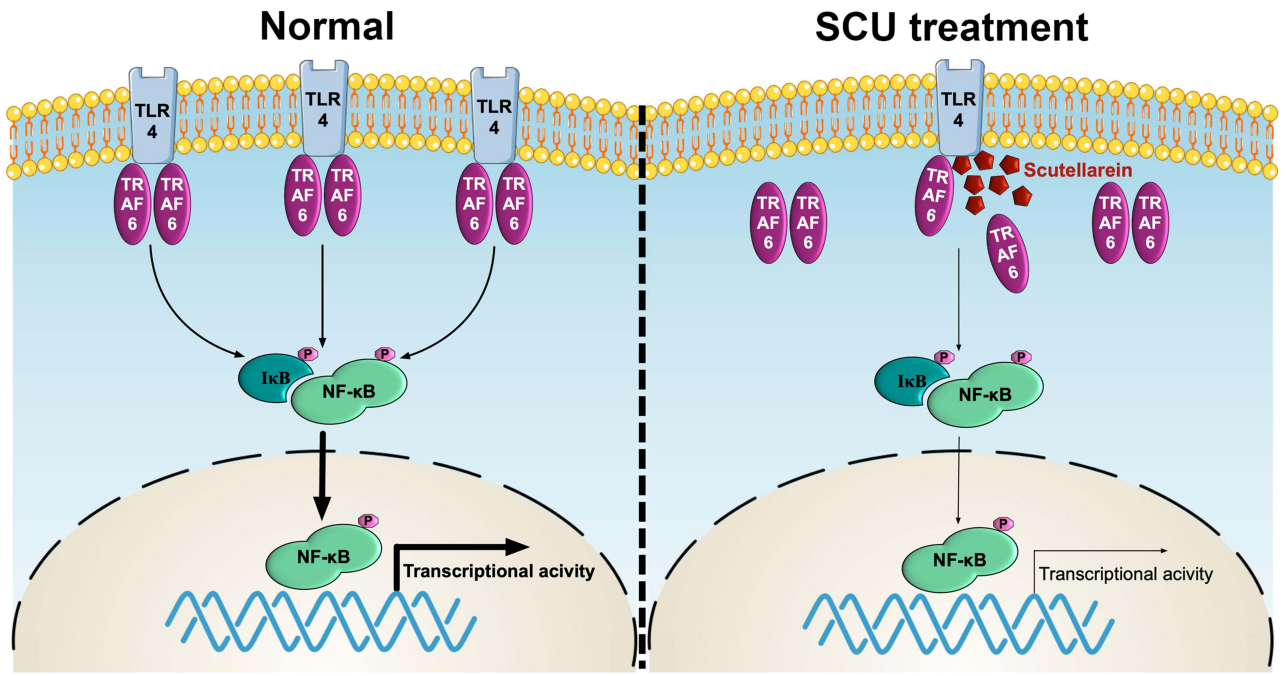
**Graphical Abstract**




properties including anti-tumorigenic activities.[8–11] SCU has also been found to show potent cytotoxic effects on cancer cells but not on normal somatic cells,[10] indicating that SCU is a promising anti-cancer agent with unique characteristics and safety. However, the anti-cancer effect and mechanism of SCU on OS are still unknown.

The regulatory effects of herbal monomer components on the nuclear factor-κB (NF-κB) pathway is of considerable and growing interest.[12,13] NF-κB is an important transcription factor family that has five members, p65 (RelA), RelB, c-Rel, NF-κB1 (p105/p50), and NF-κB2 (p100/p52). These members predominantly mediate NF-κB signaling through canonical and noncanonical pathways.[14,15] In the canonical NF-κB pathway, NF-κB p65 and p50 normally form a heterodimer and bind to IκB, the inhibitor of NF-κB, while upon stimulation including by inflammatory cytokines and stress signals, IκB kinase (IKK) phosphorylates downstream IκB leading to its degradation, releasing the NF-κB heterodimer which then translocates from the cytoplasm into the nucleus and triggers transcription of its target genes.[15,16] Accumulating studies have revealed that NF-κB activation aggravates the progression of OS,[17–19] and consequently elucidating the upstream molecular pathway of NF-κB activation will help to construct an OS therapy schedule.

Toll-like receptors (TLRs) are important upstream regulators of NF-κB and play a prominent role primarily in inflammation and cancer.[20] TLR4 is the only TLR that can trigger both myeloid differentiation factor-88 adaptor protein (MyD88)-dependent pathways and the toll/interleukin 1 receptor domain-containing adaptor inducing the interferon-β (TRIF) pathway for NF-κB activation.[21,22] TLR4 participates in the regulation of an intricate network of protein interactions and signal transduction, and TLR4 turns out to be an important anti-tumor target. However, the role of TLR4 in OS progression is controversial and remains to be revealed,[23,24] thus, elucidating the role and mechanism of TLR4 on OS oncogenesis can provide useful insights into the development of OS therapeutic biomarkers and treatment options.

In this study, we investigated the anti-cancer effect of SCU and unraveled the role of TLR4 on OS growth, with the aim of providing alternative treatment strategies for OS.

# Materials and Methods

## Chemicals and Reagents

Scutellarein (SCU) was obtained from Chengdu Biopurify Phytochemicals Ltd. (Chengdu, Sichuan, China). Dimethyl sulfoxide (DMSO) was obtained from Sigma-Aldrich (St. Louis, MO, USA). DMEM, FBS, 0.25% trypsin/EDTA, 100 U $mL^{-1}$ penicillin and 100 μg $mL^{-1}$ streptomycin were purchased from Gibco (Carlsbad, CA, USA). The 5-ethynyl-20-deoxyuridine (EdU) incorporation kit, Protein A/G Agarose, cell counting kit-8 (CCK-8) and crystal violet were obtained from Beyotime (Beijing, China). The NF-κB luciferase reporter, pRL-TK Renilla luciferase construct and dual-luciferase assay system were provided by Promega (Madison, WI, USA). pLVX-TLR4-FLAG plasmids were purchased from PPL (Nanjing, China), psPAX2 vector and pCMV-VSV-G vector were obtained from Miao-LingBio (Wuhan, China). Lipofectamine 2000 was obtained from Invitrogen (Carlsbad, CA, USA).

Anti-p-p65 Ser536 (93H1), anti-p-IκBα Ser32 (14D4), anti-IκBα (L35A5) and anti-TRAF6 (D21G3) were supplied by Cell Signaling Technology (CST; Danvers, MA, USA). Anti-p65 (66,535-1-Ig), anti-α-Tubulin (66031-1-Ig), anti-TLR4 (66,350-1-Ig), anti-Ki67 (27,309-1-AP) and anti-Lamin B1 (12,987-1-AP) were purchased from Proteintech (Wuhan, Hubei, China). Anti-TLR4 (AF7017) was from Affinity Biosciences Ltd., (Cincinnati, OH, USA). Anti-FLAG (F1804) was obtained from Sigma-Aldrich, anti-p-p65 Ser536 (ab86299) was purchased from Abcam (Cambridge, UK), anti-TRAF6 (sc-8409) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Corresponding secondary antibodies were supplied by ZSGB Biological Technology Co. Ltd. (Beijing, China).

## Cell Culture and Construction of Stable Cell Lines

U2OS, MG63, 143B and HEK293T cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM containing 10% FBS, supplemented with 100 U $mL^{-1}$ penicillin and 100 μg $mL^{-1}$ streptomycin. The culture medium was changed every two days. The cells were incubated in a humidified atmosphere with 5% $CO_2$ at 37°C. When indicated, different concentrations of SCU were added following the manufacturer's specifications.

To establish 143B osteosarcoma cells stably overexpressing TLR4 (TLR4 OE), pLVX-TLR4-FLAG plasmids, psPAX2 packing vector, and pCMV-VSV-G envelope vector were transfected into HEK293T cells to obtain supernatant containing lentivirus. Following viral infection in the presence of 10 μg/mL polybrene, transduced 143B cells were selected by culture with 3 μg/mL puromycin.

## Western Blotting and Immunoprecipitation Analysis

For co-immunoprecipitation, cells were lysed in E1A buffer (pH = 7.4, 50 mm HEPES, 250 mm NaCl, 0.1% NP-40, 1 mm EDTA, 1 mm DTT and protease inhibitors). Cell lysates were incubated with 4 μg antibody overnight at 4°C, followed by incubation with agarose beads for 4 h. Subsequently, the complex was washed five times before being subjected to Western blotting (WB). To avoid the influence of IgH, especially for the TRAF6 immunoprecipitation assay, we used antibodies obtained from different species to enrich or show the target protein. WB was performed as described previously.[25]

For nuclear and cytoplasmic protein extraction, after cells were harvested, the cell pellet was resuspended in five pellet volumes of cytoplasmic extract (CE) buffer (pH = 7.9, 10 mm HEPES, 10 mm KCl, 1.5 mm $MgCl_2$, 0.2 mm EDTA, 0.05% (v/v) NP40, and 1× protease inhibitors). Through incubation on ice and centrifugation, the supernatant was collected as the cytoplasmic extract. Then, the nuclear pellet was carefully washed with CE buffer without detergent. Nuclear extract (NE) buffer (20 mm Tris Cl, 420 mm NaCl, 1 mm EDTA, 0.3% (v/v) NP40, 1× protease inhibitors and 25% (v/v) glycerol, adjusted to pH 7.9) was used to extract nuclear proteins. An equal volume of NE buffer was added to the nuclear pellet, and after vortexing the nuclear pellet vigorously and centrifuging, the supernatant was saved as the nuclear extract.

## Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

OS cells were maintained with different concentrations of SCU for the indicated times. Total RNA was obtained using Trizol reagent and was reverse transcribed into cDNA using a PrimeScript™ RT reagent kit (Takara Bio Inc., Shiga, Japan). cDNA was amplified using the UltraSYBR Mixture (CWBIO, Jiangsu, China). The whole procedure was performed as follows: pre-denaturation at 95°C for 5 min, followed by 40 cycles at 95°C for 10s, 65°C for 30s and 72°C for 50s. The relevant target genes were normalized to GAPDH. Relative amounts of mRNA were calculated using the $2^{-\Delta\Delta CT}$ method. The sequences of primers were as follows: TLR4 (Forward, 5′-TTTCACCTGATGCTTCTTGCT-3′, Reverse, 5′-TCCTTACCCAGTCCTCATCCT-3′); TRAF6 (Forward, 5′-TTTGCTCTTATGGATTGTCCCC-3′, Reverse, 5′-CATTGATGCAGCACAGTTGTC-3′); *IκB* (Forward, 5′-CTCCGAGACTTTCGAGGAAATAC-3′, Reverse, 5′-GCCATTGTAGTTGGTAGCCTTCA-3′); *P65* (Forward, 5′-AGCTCAAGATCTGCCGAGTG-3′, Reverse, 5′-ACATCAGCTTGCGAAAAGGA-3′); *GAPDH* (Forward, 5′-AGAAGGCTGGGGCTCATTTG-3′, Reverse, 5′-AGGGGCCATCCACAGTCTTC-3′).

## Dual-Luciferase Reporter Assay

MG63 and U2OS cells were seeded into 24-well plates at a density of 40,000 cells per well. The NF-κB luciferase reporter and pRL-TK Renilla luciferase construct were provided by Promega. After transfection with Lipofectamine 2000, MG63 and U2OS cells were treated with 100 μM SCU. After a further 24 hours, the luciferase activity was measured using the Dual-Luciferase Assay System following the manufacturer's instructions. The relative luciferase activity was determined by normalizing firefly luciferase to Renilla luciferase.

## Laser Confocal Fluorescence Imaging and Immunofluorescent Staining

OS cells were seeded onto sterilized coverslips and allowed to attach for 16 h. After treatment with different concentrations of SCU for 24 h, the cells were fixed in 4% paraformaldehyde for 15 minutes at room temperature (RT). Next, 0.2% Triton X-100 in PBS was used to permeabilize the cells on ice for 30 minutes. After blocking with 5% goat serum, cells were incubated with primary antibodies against TLR4 (1:200, Proteintech), TRAF6 (1:100, CST) and NF-κB p65 (1:100, Proteintech) at 4°C overnight, then the cells were incubated with fluorescein-conjugated secondary antibody. Nuclei were stained with DAPI for 3 minutes at RT. NF-κB p65 was visualized by laser confocal microscopy (Zeiss LSM 800, Carl Zeiss Microscopy GmbH, Jena, Germany), while TLR4 and TRAF6 images were captured using a fluorescence microscope (IX73, Olympus, Tokyo, Japan).

## Cell Proliferation Assays

Briefly, after treatment with SCU at the indicated concentrations, cells were incubated with 20 μL EdU for 2 hours following the manufacturer's instructions. Cell nuclei were stained with DAPI for 3 minutes in the dark and images were captured using a fluorescence microscope (IX73, Olympus, Tokyo, Japan). For the colony formation assay, the indicated OS cells were seeded at 800 cells per well and treated with different concentrations of SCU for 12 days, then the colonies were stained with crystal violet. In addition, the stable TLR4-overexpressing 143B cells and wild-type 143B cells were treated with DMSO or 100 μM SCU for 48 h, then the cells were dissociated using 0.25% trypsin solution and resuspended in culture medium for cell counting. The CCK-8 assay was carried out following the manufacturer's instructions. Cell viability was calculated using the following formula: cell viability (%) = (As − Ab) / (Ac − Ab) × 100%, where As, Ab, and Ac represent the absorbance of the sample, blank, and control, respectively.

## RNA Sequencing (RNA-Seq)

Briefly, after treatment with DMSO or SCU for 48 h, total RNA was isolated from 143B cells using Trizol reagent. Poly-T oligo-attached magnetic beads were used to purify mRNA from total RNA. To obtain target cDNA fragments, the library fragments were purified using an AMPure XP system (Beverly, CA, USA). The sequencing library was analyzed on the NovaSeq 6000 platform (Illumina) by Beijing Qinglian Biotech Co., Ltd.

## Molecular Docking Analysis

To evaluate whether SCU could bind directly to TLR4, we performed molecular docking analysis of SCU with TLR4. The structure of TLR4 protein was downloaded from Protein Data Bank (PDB, 1LB6, http://www.rcsb.org/), and the structure of SCU was obtained from PubChem (CID:5281697, https://pubchem.ncbi. nlm.nih.gov/). The docking of SCU against TLR4 protein was simulated by AutoDocktools (v1.5.6). The alignment between target objects was measured by the Pymol2.3.0 system, and binding energy was used to predict the possibility of direct binding.

## Cellular Thermal Shift Assay (CETSA)

Cellular thermal shift assay was performed to further confirm that SCU targeted TLR4. OS cells were pretreated with 100 μM SCU or DMSO, then divided into six equal portions in PCR tubes, and heated at 45, 55, 60, 65, 70 and 75°C for 4 min. The cells were then cooled to 4°C and lysed by freeze-thawing. After centrifuging at 14,000 × *g* for 20 min, the samples were collected and subjected to WB analysis.

## Animal Xenograft Experiments

The effects of SCU and TLR4 overexpression on OS growth were investigated using a nude mouse model. All animal experiments were performed in accordance with the welfare and ethics guidelines set by the Ethics Committee of the Affiliated Hospital of Jining Medical University for the care and use of animals, following a protocol reviewed and approved by the Ethics Committee of the Affiliated Hospital of Jining Medical University (Approval Number: 2022–11-B001). The mice were housed in specific pathogen-free conditions (temperature of 22°C–25°C, 12-hour light/dark cycle) and were allowed free access to water and food. All efforts were made to alleviate any discomfort during the experimental procedures. Twenty-eight female BALB/c nude mice (6 weeks old, 18 ± 2 g) were obtained from Ji'nan Pengyue Laboratory Animal Breeding Co., Ltd. Fourteen nude mice were injected with TLR4-overexpressing stable 143B cells ($1 \times 10^6$ in 200 μL PBS) and another fourteen mice were injected with wild-type 143B cells ($1 \times 10^6$ in 200 μL PBS). When the tumor volume reached about 60 $mm^3$, the mice were randomly assigned to one of four groups: (1) the TLR4 OE group, subcutaneously injected with stably overexpressing TLR4 143B cells; (2) the TLR4 OE + SCU group, subcutaneously injected with stably overexpressing TLR4 143B cells and treated with SCU; (3) the control group, subcutaneously injected with wild-type 143B cells; (4) the SCU group, subcutaneously injected with wild-type 143B cells and treated with SCU. SCU (0.5 μg/g) or the same volume of DMSO was injected intraperitoneally every 2 days. The diameter of the tumors and the body weight of the mice were monitored every 4 days, and tumor volume was determined using the formula: 1/2(length × $width^2$), this formula was selected based on a previously reported study.[26] Lastly, tumors were collected for immunohistochemical staining with antibodies against p-p65 Ser536 (Abcam) and Ki67 (Proteintech).

## Statistical Analysis

Data are presented as the means ± standard deviation (SD). Statistical comparisons between two groups were analyzed by Student's *t*-tests. * $P < 0.05$; ** $P < 0.01$ were considered statistically significant. GraphPad Prism 9 (San Diego, CA, USA) was adopted to analyze the data. All experiments were conducted independently at least five times.

# Results

## SCU Inhibits the Growth of OS Cells

SCU is a flavonoid monomer (Figure 1A), a preliminary experiment was conducted to investigate the toxicity of SCU on OS cells and its effect on their viability. Using the OS cell lines 143B and MG63, we found that SCU decreased cell viability in a dose-dependent manner (Figure 1B). To further verify the effect of SCU on OS cell proliferation, we conducted an EdU assay. As shown in Figure 1C and D, compared with the 0 μM group, the number of fluorescent cells decreased significantly in the SCU group ($P < 0.01$). Similar results were observed in the colony-formation assay (Figure 1E), where SCU decreased the colony number. Collectively, these results suggest that SCU retarded the growth of OS cells.

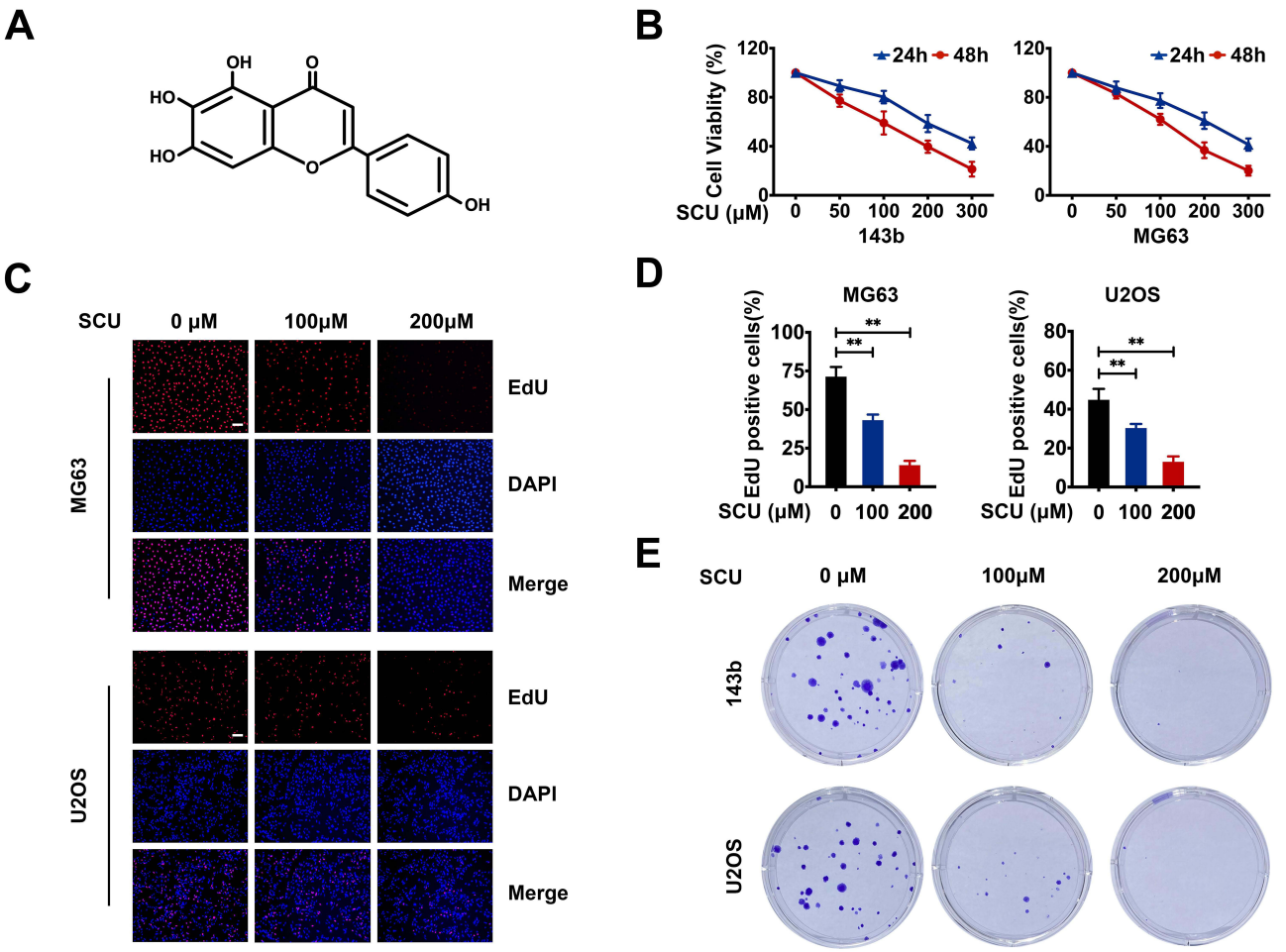



**Figure 1** SCU inhibits the growth of OS cells. (**A**) The chemical structure of scutellarein. (**B**) CCK8 assay was performed to measure the viability of 143B and MG63 cells at different concentrations of Scutellarein (SCU; 0–300 μM). (**C**) The EdU incorporation assay was used to analyze the proliferation of osteosarcoma (OS) cells following SCU treatment. Scale bars, 100 μm. (**D**) Quantification of OS cell proliferation shown in (**C**). (**E**) Colony formation assay was applied to investigate proliferation of 143B and MG63 cells under SCU treatment. Data are shown as mean ± SD (n=5). $^{**}P < 0.01$ compared with the 0 μM group.

## SCU Suppresses the Canonical NF-κB Pathway

To gain insight into the mechanism by which SCU inhibits OS cell growth, we performed RNA-seq on DMSO- or SCU-treated 143B cells. Among the pathways illustrated in the KEGG pathway enrichment of RNA-seq data, we noticed that the NF-κB pathway, which is important for inflammatory regulation and OS progression, was affected by SCU treatment (Figure 2A). Considering the role of SCU in inflammatory regulation, we next focused on the NF-κB signaling pathway. As illustrated in Figure 2B and C, SCU had no impact on the protein or mRNA levels of NF-κB p65 or its kinase IκB, while the levels of p-p65 Ser536 and p-IκB Ser32 decreased dramatically following SCU treatment, which indicated that SCU inhibits the canonical NF-κB pathway. In the canonical NF-κB pathway, NF-κB p65 translocates from the cytoplasm into the nucleus once it is phosphorylated, and then NF-κB p65 binds to the promoter region of its target genes and triggers transcription of these genes.[15,16] To further confirm the regulatory role of SCU on the canonical NF-κB pathway, we investigated the subcellular location of NF-κB p65. As depicted in Figure 2D and E, SCU significantly inhibited NF-κB p65 nuclear translocation in 143B cells. NF-κB luciferase assay was carried out to further identify the transcriptional activity of NF-κB, and the result revealed that the transcriptional activity of NF-κB was inhibited by SCU (Figure 2F). These observations strongly suggest that SCU suppresses the canonical NF-κB pathway.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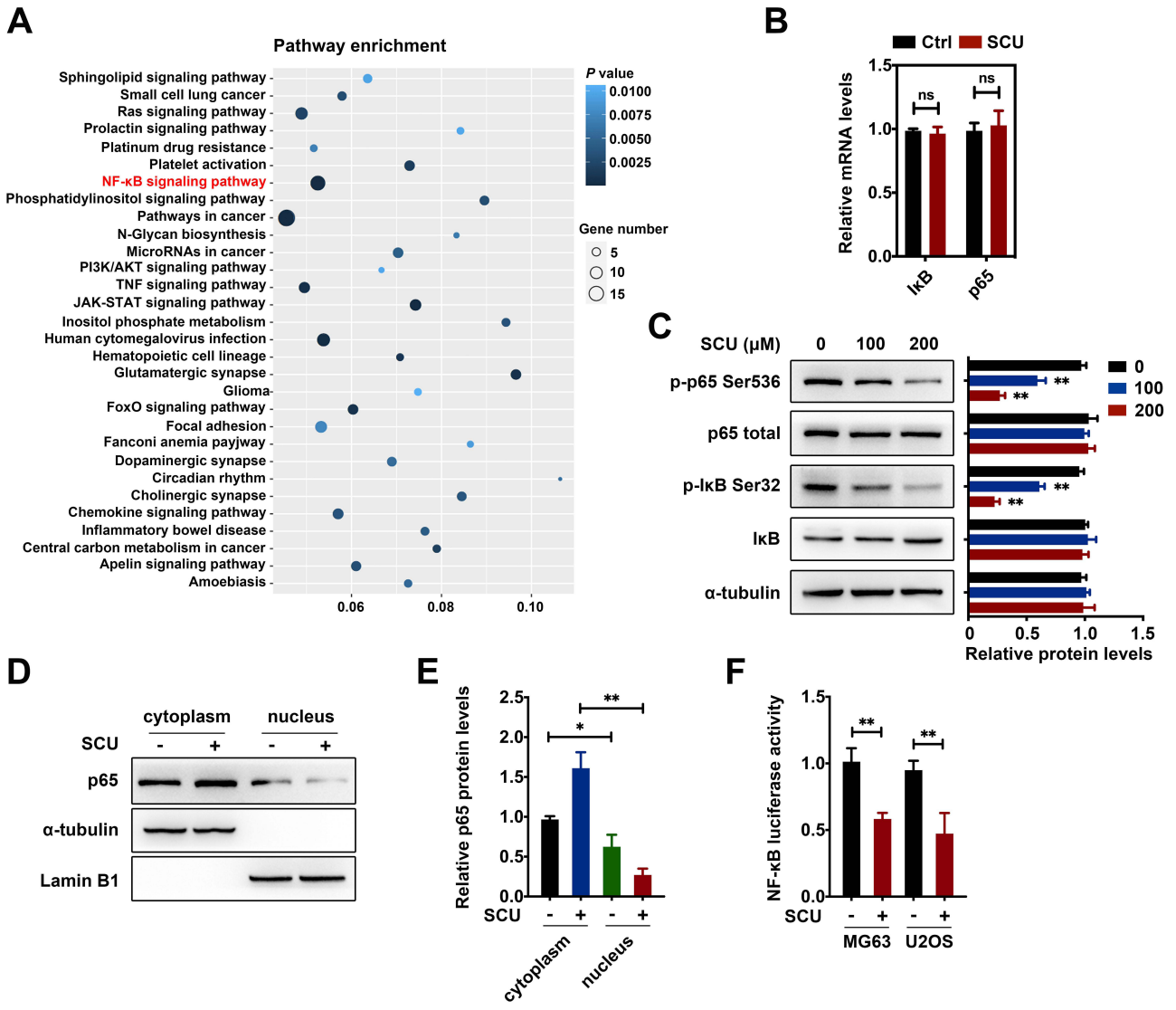


**Figure 2** SCU suppresses the canonical NF-κB pathway. (**A**) KEGG pathway enrichment analysis of the differentially expressed genes between control and SCU groups based on RNA-seq data. (**B**) Analysis of mRNA levels of indicated genes from the groups treated with DMSO and SCU (100 μM) for 48 h. GAPDH served as the loading control. (**C**) Western blot (WB) analysis of the canonical NF-κB pathway of OS cells treated with different concentrations of SCU for 48 h. Bar graphs show the quantified results of WB showing the relative expression of indicated proteins. (**D**) Sublocation of NF-κB p65 was analyzed by WB. α-tubulin and Lamin B1 were used as internal markers for the cytoplasm and nucleus, respectively. (**E**) Quantification and normalization of p65 bands shown in (**D**) were performed relative to corresponding internal markers. (**F**) MG63 and U2OS cells were transfected with NF-κB p65-responsive luciferase reporters and then treated with SCU, after which cells were collected for dual-luciferase activity assay. Data are expressed as the mean ± SD (n=5). ns, no significant difference; **P* < 0.05; ***P* < 0.01 compared with the control group.

## SCU Inhibits the Canonical NF-κB Pathway by Inhibiting TLR4 Expression and Attenuating the Interaction of TLR4 and TRAF6

To investigate how SCU downregulates the canonical NF-κB signaling pathway, we measured the upstream modulators of NF-κB. Wang et al reported that traditional Chinese herbal medicine can suppress the NF-κB signaling pathway by negatively regulating TLR4 and TRAF6 expression.[27] In the present work, we found that SCU barely affected the expression of TRAF6, but the expression of TLR4 decreased dramatically (Figure 3A and B). Considering the positive regulatory effect of TLR4 on NF-κB activation, we hypothesized that SCU downregulates NF-κB signaling in a TLR4-dependent manner. To elucidate this, first, we investigated whether SCU interacts with TLR4. As depicted in Figure 3C and D, MG63 cells maintained with SCU or DMSO, then subjected to a CETSA heat pulse, notably revealed that TLR4 undergoes dramatic thermal stabilization under SCU treatment. In addition, molecular docking analysis clarified that SCU directly interacts with TLR4 protein (Figure 3E and F). These results suggested that SCU bound to TLR4 directly.


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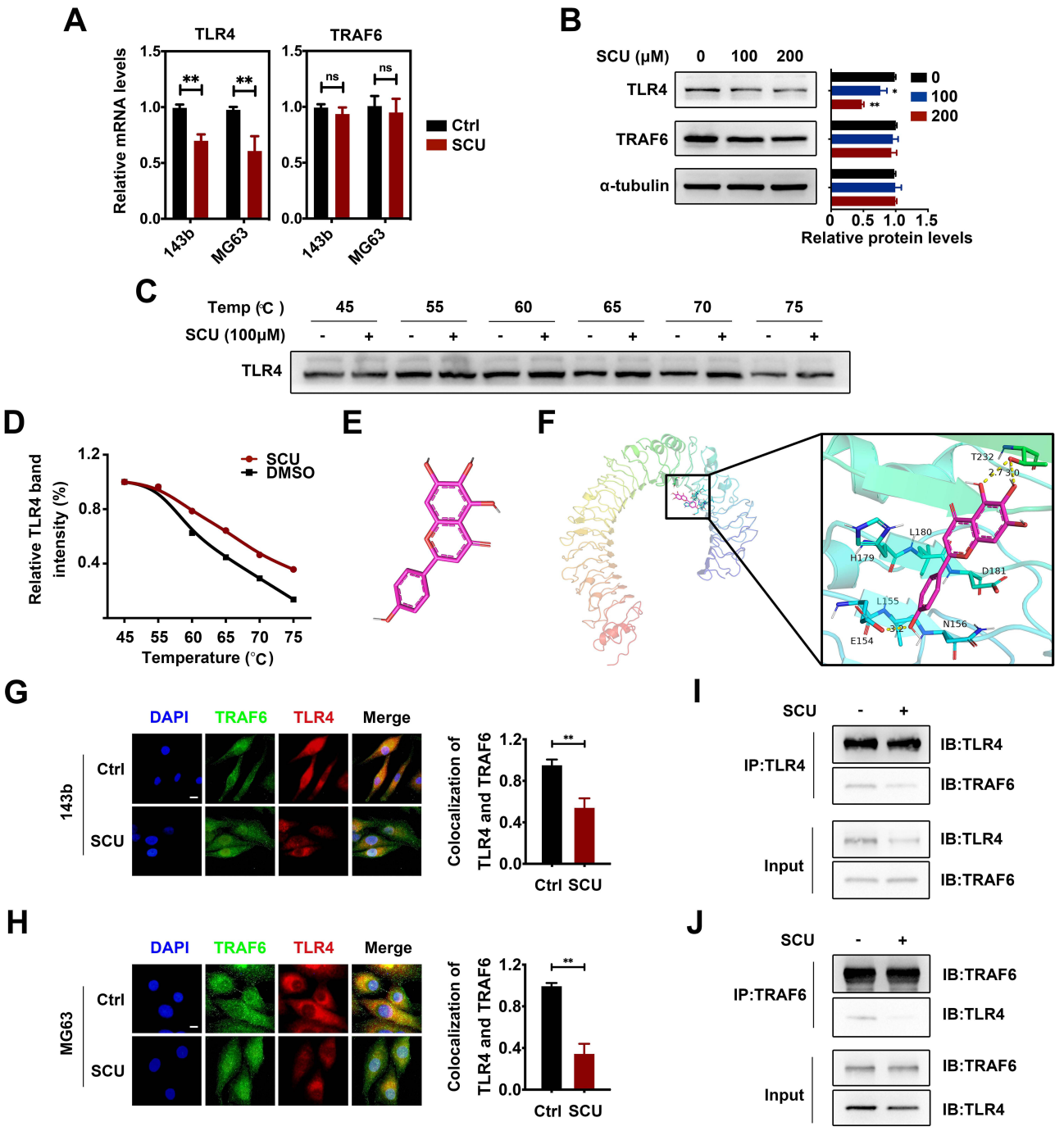


**Figure 3** SCU inhibits the canonical NF-κB pathway by inhibiting TLR4 expression and attenuating the interaction of TLR4 and TRAF6. (**A**) qRT-PCR was used to evaluate the mRNA levels of the indicated genes in OS cells treated with DMSO or SCU (100 μM) for 48 h. GAPDH served as the loading control. (**B**) WB analysis of TLR4 and TRAF6 in cells treated with different concentrations of SCU for 48 h. Bar graphs are the results of quantification of WB, showing the relative expression of the proteins. (**C**) Cellular thermal shift assay (CETSA)-WB analysis of the interaction between SCU and TLR4. (**D**) The relative intensity of TLR4 bands in (**C**) were analyzed. (**E**) The structure of SCU. (**F**) Biomolecular interaction of SCU and TLR4 predicted by the PyMOL system. The 3D structure of TLR4 bound efficiently with SCU with the interacting amino acids being T232, E154, N156, D181, L180, H179 and L155. The cell lines 143B (**G**) and MG63 (**H**) were maintained with 100 μM SCU, then TRAF6 (green) and TLR4 (red) colocalization was determined by immunofluorescence. Scale bar, 20 μm. The graph represents the quantified results of the colocalization of TRAF6 and TLR4. (**I**) SCU treatment decreased the interaction of endogenous TLR4 and endogenous TRAF6. Endogenous TLR4 proteins were immunoprecipitated from either SCU- or DMSO-treated 143B cells. (**J**) The endogenous interaction between TRAF6 and TLR4 in MG63 cells was attenuated by SCU. Data are presented as the mean ± SD (n=5). ns, no significant difference; **P* < 0.05; ***P* < 0.01 compared with the control group.

Secondly, we investigated the molecular mechanism via which TLR4 influences NF-κB activation. TRAF6 is an effector of TLR4, and Lin et al previously reported that lipopolysaccharide (LPS) can affect the interaction of TLR4 and TRAF6.[28] Considering that SCU binds to TLR4 directly, we hypothesized that SCU may intervene in the interaction

between TLR4 and TRAF6, resulting in the inactivation of the NF-κB signaling pathway. As displayed in Figure 3G, under SCU treatment, the co-localization of TLR4 and TRAF6 in 143B cells decreased compared with the control group. Similar results were obtained in MG63 cells (Figure 3H). To further confirm the effect of SCU on the interaction between TLR4 and TRAF6, we separately immunoprecipitated TLR4 and TRAF6. As shown in Figure 3I, SCU counteracted the interaction between endogenous TLR4 and TRAF6 in 143B cells. When we immunoprecipitated TRAF6 with rabbit anti-TRAF6, the result revealed that TLR4 band intensity in MG63 cells treated with SCU was dramatically weakened compared with that in the control group (Figure 3J). These data suggest that SCU significantly decreased the expression of TLR4 and the TLR4–TRAF6 interaction, thus hindering activation of the NF-κB pathway.

## SCU Inhibition of NF-κB Activation is Dependent on TLR4

Next, we implemented a rescue assay to determine whether SCU downregulation of canonical NF-κB signaling is dependent on TLR4. First, stable pLVX-TLR4-FLAG overexpressing cells were established, then TLR4 mRNA levels were measured by qRT-PCR assay, and the endogenous and exogenous TLR4 protein levels were assessed by WB. The results indicated that stable pLVX-TLR4-FLAG overexpressing 143B cells were established successfully (Figure 4A and B). Both wild-type and TLR4 stably-expressing 143B cells were treated with DMSO or SCU, as shown in Figure 4B and C, and SCU was found to reduce the expression of both endogenous and exogenous TLR4, which further indicated that SCU downregulated TLR4 expression, moreover, TLR4 overexpression prevented the decrease of p65 phosphorylation induced by SCU. Similarly, the results of laser confocal scanning electron microscopy suggested that SCU treatment dramatically inhibited p65 nuclear translocation, indicating that NF-κB was inactivated; however, this phenomenon was attenuated by TLR4 overexpression

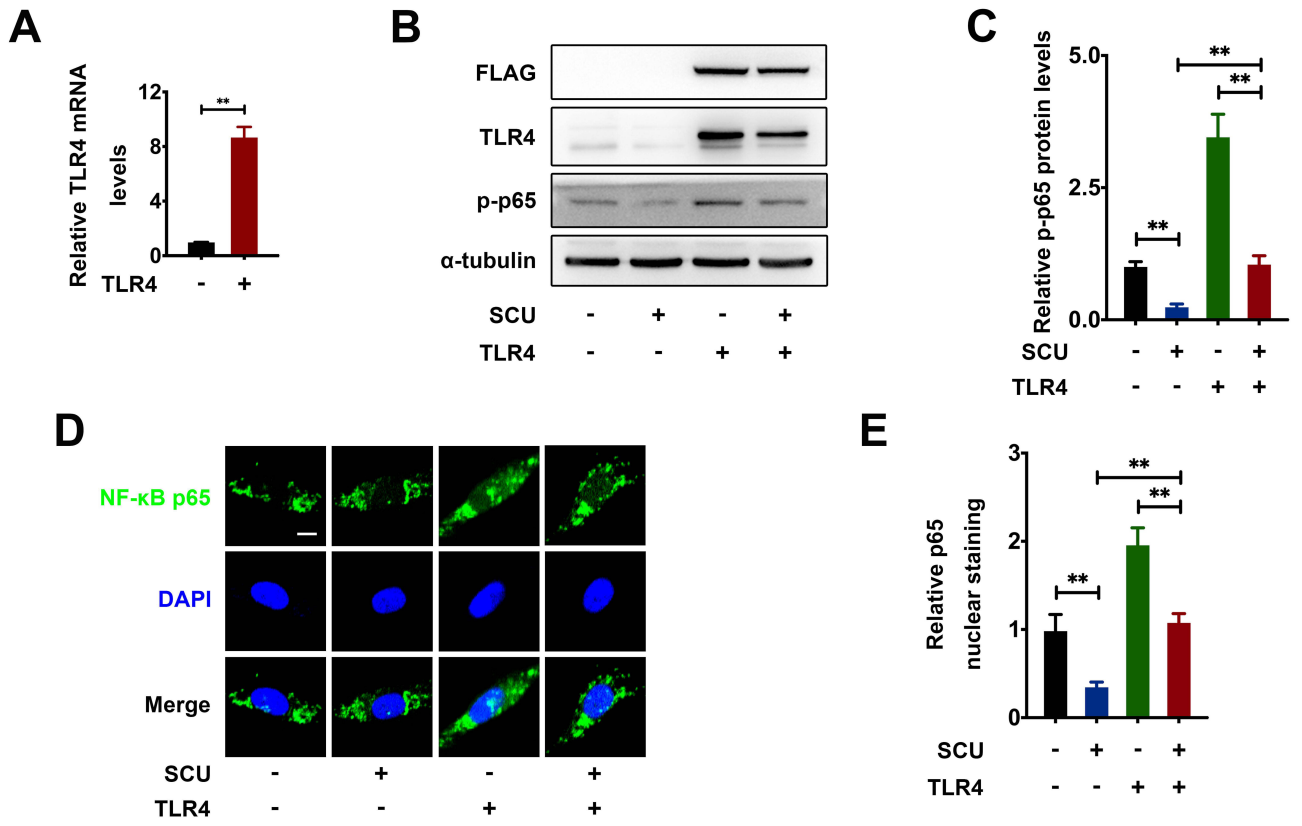


**Figure 4** SCU inhibition of NF-κB activation is dependent on TLR4. (**A**) Stable 143B cell lines overexpressing pLVX-TLR4-FLAG were established, qRT-PCR was used to measure the TLR4 mRNA levels of wild-type 143B cells and stable TLR4-overexpressing 143B cells. Data are represented as the mean ± SD (n=5). $^{**}P < 0.01$ compared with the wild-type 143B cells. (**B**) The stable TLR4-overexpressing 143B cells and wild-type 143B cells were treated with DMSO or SCU, then WB was used to analyze the indicated protein levels. (**C**) Quantification and normalization of p-p65 protein levels were performed relative to α-tubulin. (**D**) The stable TLR4-overexpressing 143B cells and wild-type 143B cells were treated with DMSO or SCU. Green fluorescence indicates NF-κB p65. Scale bar, 10 μm. (**E**) As described in (**D**), the nuclear staining dots per cell were quantified. Data are expressed as the mean ± SD (n=5). $^{**}P < 0.01$ compared with the control group.


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(Figure 4D and E). These findings strongly suggest that SCU suppression of NF-κB activation is dependent on TLR4 inhibition.

## SCU Inhibits OS Cell Proliferation Through TLR4 in vitro

To explore whether TLR4 is required for SCU to inhibit the proliferation of OS cells in vitro, we performed colony formation assay, CCK8 assay, EdU assay and cell counting experiments. As shown in Figure 5A and B, the colony number of stable TLR4-overexpressing 143B cells was higher than that of wild-type 143B cells, suggesting TLR4 overexpression dramatically increased 143B cell colony formation, and TLR4 overexpression reversed the decrease of colony number observed under treatment with SCU. TLR4 overexpression also attenuated the decrease in the viability of 143B cells induced by SCU treatment (Figure 5C). In agreement with this, SCU treatment inhibited 143B cell proliferation when assessed by EdU assay, and this phenomenon was also abolished by TLR4 overexpression (Figure 5D and E). Cell counting assay revealed that TLR4 overexpression blocked the cell number decrease induced by SCU (Figure 5F). All these results confirmed that TLR4 is required for the inhibition of OS cell proliferation regulated by SCU.

## SCU Inhibits OS Tumor Growth Through TLR4 in vivo

To investigate whether SCU inhibition of OS tumor growth is dependent on TLR4 in vivo, we performed xenograft experiments with SCU treatment. A diagram of the xenograft design is illustrated in Figure 6A. Nude mice were injected with TLR4 overexpressing stable 143B cells or wild-type 143B cells, and then injected with SCU or DMSO intraperitoneally every two days. As shown in Figure 6B–D, SCU treatment resulted in significant inhibition of OS tumor growth compared with the control group ($P < 0.01$), whereas TLR4 overexpression attenuated the suppression of OS growth induced by SCU injection, moreover, the tumor volume and weight in the TLR4 OE group were dramatically increased

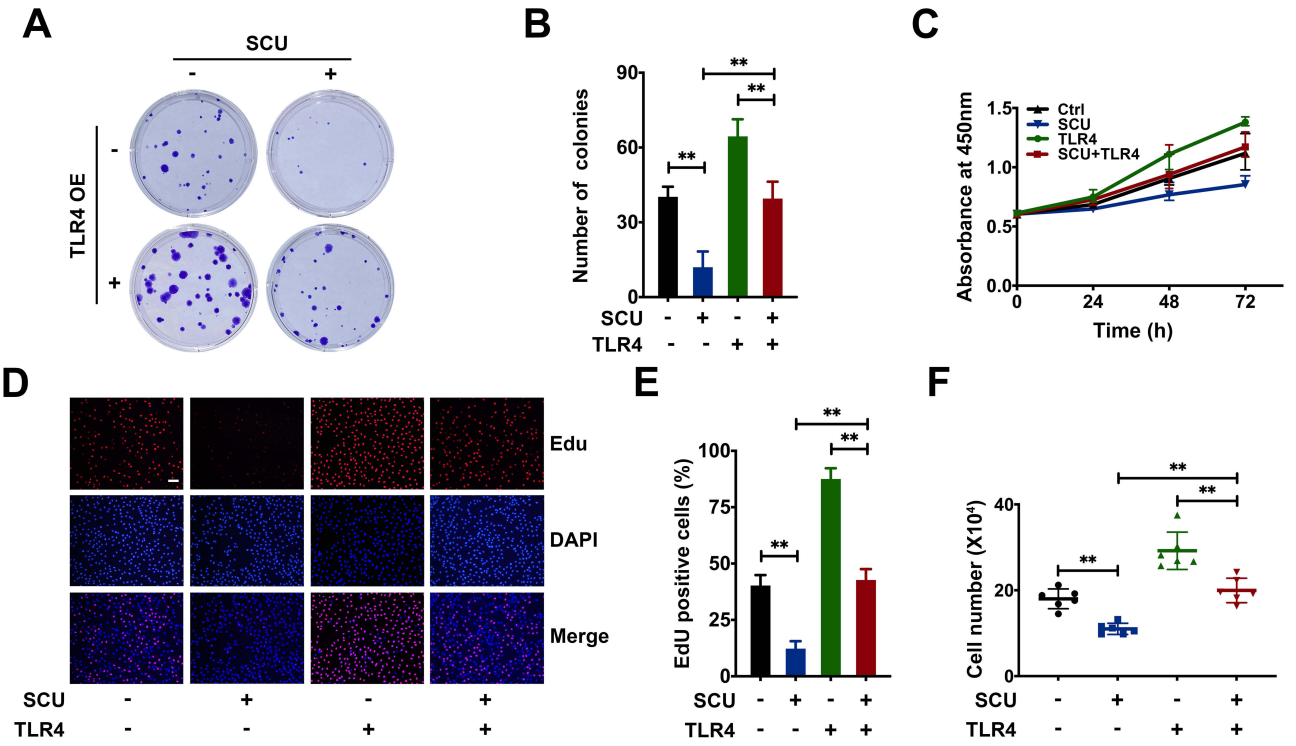


**Figure 5** SCU inhibits OS cell proliferation through TLR4 in vitro. (**A**) Stable TLR4-overexpressing 143B cells and wild-type 143B cells were treated with DMSO or SCU, and colony formation assay was performed to investigate the effect of TLR4 overexpression and SCU treatment on 143B cell proliferation. (**B**) Quantification of 143B cell proliferation shown in (**A**). (**C**) The effect of TLR4 overexpression and SCU treatment on the cell viability of 143B cells was determined by CCK-8 assay. (**D**) The stable TLR4-overexpressing 143B cells and wild-type 143B cells were treated with DMSO or SCU (100 μM), and the EdU incorporation assay was used to analyze cell proliferation. Scale bar, 100 μm. (**E**) Quantification of 143B cell proliferation shown in (**D**). (**F**) The cell number of indicated cells was evaluated to reveal the influence of TLR4 overexpression and SCU treatment on OS cell proliferation. Data are shown as mean ± SD (n=5). $^{**}P < 0.01$ compared with the control group.


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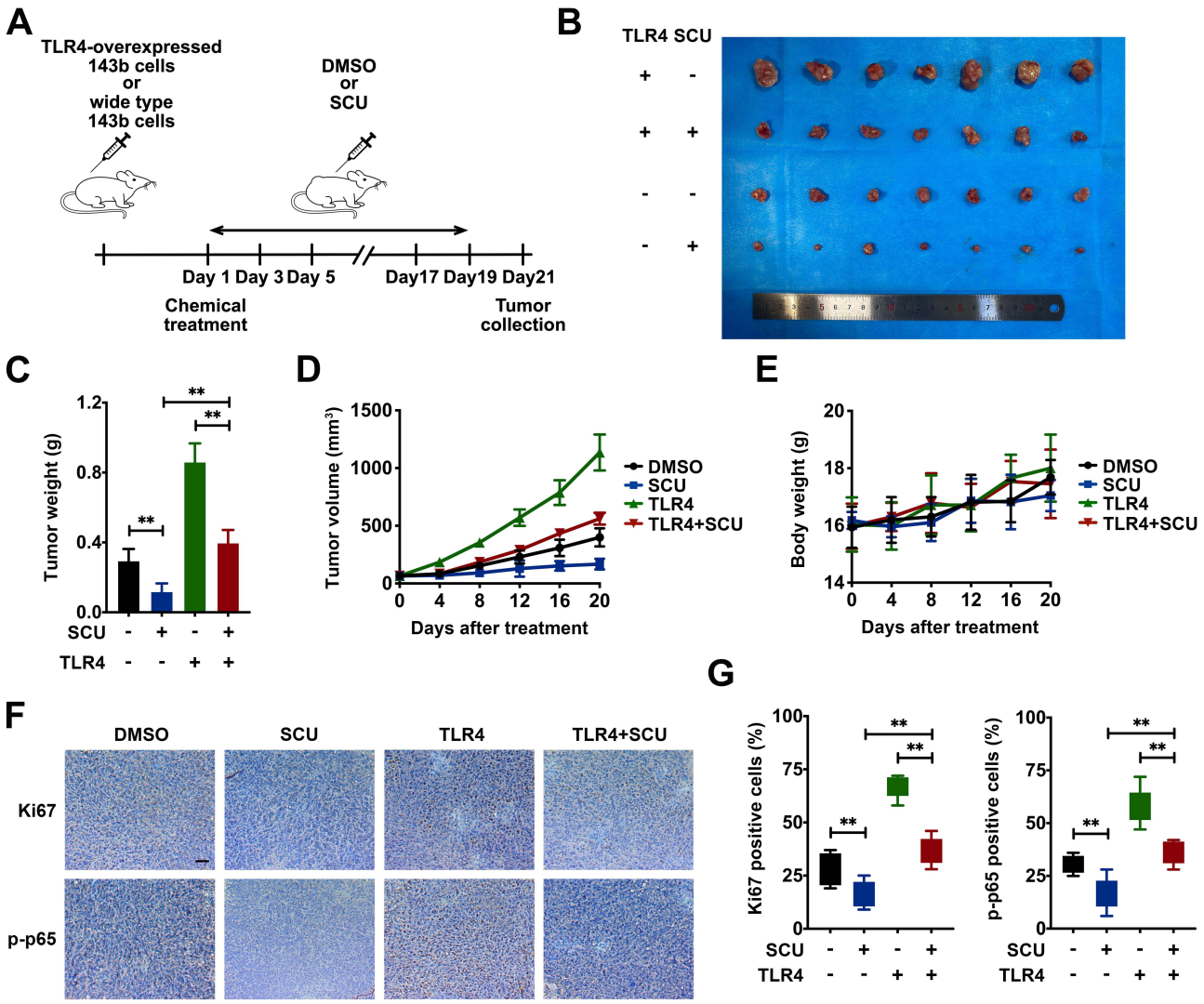


**Figure 6** SCU inhibits OS tumor growth through TLR4 in vivo. (**A**) Illustration of the in vivo mouse xenograft experiment. (**B**) Macroscopic image showing the collected tumor specimens after the mice were sacrificed. (**C**) Effects of SCU and TLR4 overexpression on the tumor weight of tumor-bearing mice. (**D**) Tumor volume was calculated at the indicated times. (**E**) Mouse body weights were monitored every 4 days. (**F**) Ki67 and p-p65 immunohistochemical staining of representative tumors in each group. Scale bar, 50 μm. (**G**) The numbers of Ki67- and p-p65-positive cells in tumors were calculated and quantified following SCU and TLR4 overexpression in vivo. n=7 mice for each group. $^{**}P < 0.01$ compared with the control group.

compared with the control group ($P < 0.01$), indicating that TLR4 overexpression promotes OS growth and that TLR4 acted as an oncogene for OS. Figure 6E shows that SCU treatment and TLR4 overexpression had no impact on mouse body weight. Ki67 is a cellular marker of proliferation, and as expected, IHC staining showed that the number of Ki67-positive cells in tumor tissue decreased following SCU treatment, and TLR4 overexpression abolished this phenomenon ($P < 0.01$). Similar results were obtained with p-p65-positive cells, which were consistent with the results of in vitro experiments ($P < 0.01$) (Figure 6F and G). Collectively, these results indicate that SCU inhibition of OS tumor growth is dependent on TLR4 in vivo.

## Discussion

The lack of effective chemotherapeutic drugs has a deleterious effect on the prognosis of OS patients, and consequently, novel chemotherapeutic agents and strategies are urgently required to enhance the survival rate.[3,29] In the present study, we found that SCU strongly inhibits OS progression. Mechanistically, SCU inhibits the canonical NF-κB pathway by downregulating TLR4 expression and disrupting the interaction of TLR4 and its downstream mediator TRAF6.


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Moreover, TLR4 overexpression facilitates OS growth in vitro and in vivo, indicating the positive regulatory effect of TLR4 on OS progression and showing that TLR4 is a promising OS therapeutic biomarker and target.

SCU has gained widespread attention for its regulatory role in both inflammation and carcinogenesis.[8,10,11] For anti-tumor activity, previous studies mostly focused on the function of SCU in facilitating tumor cell apoptosis,[10,11] while the effect of SCU on OS cells remained completely unknown. Our study revealed that SCU inhibits OS growth without obvious toxicity (Figures 5 and 6). Mechanistically, SCU suppresses the TLR4/TRAF6/NF-κB pathway, and since this pathway is closely associated with regulation of inflammation, our work further unravels the importance of immunotherapy in OS treatment. The molecular docking result revealed that SCU directly interacts with TLR4 protein, with binding energy of −7.5 kcal/mol, which is similar to the binding energy between TLR4 and its inhibitor TAK242,[30–32] suggesting that SCU binds firmly to TLR4. A 3D ribbon model of the SCU–TLR4 complex showed that hydrogen bonds formed at T232 and E154 of TLR4, and hydrophobic interactions were also found at N156, D181, L180, H179 and L155 (Figure 3F), which probably impaired the interaction of TLR4 and TRAF6 and consequently contributed to the inactivation of NF-κB.

TLR4 positively modulates NF-κB signaling through two major signaling cascades, the TRIF pathway (also called the MyD88-independent pathway) and the MyD88-dependent pathway.[21] Among the molecules associated with these two pathways, TRAF6 is a very important effector of TLR4. In previous studies, some compounds were found to significantly regulate both TLR4 and TRAF6 expression, resulting in changes of NF-κB activation,[27,33] but in the present work, we found that the expression of TRAF6 was not consistent with the expression of TLR4 under SCU treatment (Figure 3A and B). Consequently we speculated that there must be an under-emphasized but crucial mechanism mediating the inactivation of NF-κB. Lin et al reported that LPS affects the interaction of TLR4 and TRAF6, which is a scaffold and bridge participating in the TLR4/NF-κB signal transduction pathway.[28] Considering that SCU binds directly to TLR4, we deduced that the interaction of SCU and TLR4 disrupts the interaction of TLR4 and TRAF6, and the results verified our hypothesis (Figure 3G-J). Our work demonstrates that the interaction of TLR4 and TRAF6 is also important for NF-κB activation. Furthermore, auto-K63 ubiquitination of TRAF6 is essential for the signal transduction of TLR4/NF-κB,[34,35] but whether the interaction of SCU and TLR4 affects TRAF6 K63 ubiquitination needs to be further explored.

TLR4 signals participate in the regulation of several cancers, so cancer treatment targeting TLR4 is of considerable and increasing interest.[20,22,36] However, whether TLR4 can function as the drug target of OS therapy is controversial. Richert et al reported that activation of TLR4 prevents the occurrence of OS metastasis according to the GSE33382 dataset, indicating the negative role of TLR4 on OS progression,[23] whereas Wang et al found that knockdown of TLR4 expression inhibits migration and invasion of MG63 and Saos-2 OS cells, suggesting the positive role of TLR4 on OS oncogenesis.[24] In line with the findings of this study, our data showed that TLR4 overexpression promotes OS growth in vivo and in vitro (Figures 5 and 6), thus TLR4 is an appealing target for OS therapy, but additional studies need to be carried out to fully elucidate the advantages and side effects of anti-TLR4-based strategies in OS therapy.

The present study has several limitations. Here, the inhibitory role of SCU on OS has only been measured using a cell line-derived xenograft model. Future research should explore the inhibitory effect of SCU on human osteosarcoma through patient-derived xenograft models or clinical trials. Additionally, more in-depth and comprehensive toxicological studies are required to understand the in vivo side effects of SCU before its clinical application. With advancements in molecular profiling, such as single-cell RNA sequencing, different cell types and cellular populations of OS are being identified.[37–39] However, further research is needed to determine which cell type is affected by SCU, resulting in the inhibition of OS growth. Furthermore, the application of SCU in the precision therapy of OS warrants continued investigation.

## Conclusion

In conclusion, SCU exhibited a dual impact by inhibiting TLR4 expression and disrupting TLR4–TRAF6 interaction, resulting in NF-κB inactivation, thereby blocking OS growth. This work contributes to identification of a novel chemotherapeutic drug and reveals a crucial mechanism for OS treatment.

## Abbreviations

EdU, 5-ethynyl-20-deoxyuridine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IκB, inhibitor of NF-κB; IKK, IκB kinase; IRAK4, interleukin 1 receptor-associated kinase 1; KEGG, Kyoto encyclopedia of genes and genomes; LPS; lipopolysaccharide; MyD88, myeloid differentiation factor-88 adaptor protein; NF-κB, nuclear factor-κB; OS, osteosarcoma; qRT-PCR, quantitative real-time PCR; RNA-seq, RNA sequencing; SCU, scutellarein; TAK1, transforming growth factor-activated kinase 1; TLR4, toll-like receptor 4; TRAF6, TNF receptor-associated factor 6; TRIF, toll/interleukin 1 receptor domain-containing adaptor inducing the interferon-β.

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## Disclosure

The authors report no conflicts of interest in this work.

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