Article

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# Baicalin Plays an Anti-Osteosarcoma Role *in Vitro* and Promotes Osteogenic Differentiation by Inhibiting NF-$\kappa$B Signaling

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**Background: Osteosarcoma (OS) is commonly recognized as a malignant cancer originating from bone-forming mesenchymal stem cells, comprising approximately 20% of sarcomas. Baicalin, a bioactive flavonoid glycoside isolated from *Scutellaria baicalensis*, has been demonstrated to possess potent anti-inflammatory and neuroprotective properties.**

**Objective: To explore the potential mechanisms through which baicalin exerts anti-osteosarcoma effects and facilitates osteogenesis *in vitro*.**

**Methods: Cell Counting Kit-8 (CCK-8), scratch assay, and transwell assay were employed to assess the effects of baicalin at varying concentrations (20, 40, and 80 µM) on U2OS cell proliferation, invasion, and migration, respectively. Western blot and qRT-PCR analyses were conducted to evaluate the influence of baicalin on the osteogenic potential of OS cells by examining osteoblast markers such as osteocalcin (*OCN*), osteopontin (*OPN*), and runt-related transcription factor 2 (*RUNX2*), as well as the osteoclast marker—receptor activator of nuclear factor kappa B ligand (*RANKL*). Additionally, the impact of baicalin on epithelial-mesenchymal transition (EMT) markers (N-cadherin, E-cadherin, Vimentin) and proteins related to the Nuclear factor $\kappa$B (NF-$\kappa$B) signaling pathway (p-p65, p-I$\kappa$B$\alpha$, p65, I$\kappa$B$\alpha$) in OS cells was evaluated via western blot analysis. The activity and mineralization capacity of Alkaline Phosphatase (ALP) in baicalin-treated cells were examined through ALP staining and Alizarin Red S (ARS) staining.**

**Results: Baicalin exhibited significant suppression of OS cell U2OS invasion ($p < 0.01$), migration ($p < 0.01$), and proliferation ($p < 0.05$) at various concentrations. Additionally, baicalin treatment notably increased the E-cadherin protein level, while decreasing the expression levels of Vimentin and N-cadherin proteins ($p < 0.01$), thus promoting EMT. Following baicalin treatment, there was a marked elevation in the protein and mRNA expression levels of RUNX2, OPN, and OCN, while the expression level of RANKL protein was reduced ($p < 0.05$), indicating enhanced osteogenic differentiation. The groups treated with baicalin exhibited higher ALP activity and mineralization ability ($p < 0.01$). Moreover, baicalin treatment significantly reduced the expression levels of p-I$\kappa$B$\alpha$ and p-p65 proteins, as well as the ratios of p-I$\kappa$B$\alpha$/I$\kappa$B$\alpha$ and p-p65/p65 ($p < 0.01$). These effects of baicalin were concentration-dependent, with higher concentrations yielding stronger effects.**

**Conclusion: *In vitro*, baicalin demonstrates anti-OS effects and facilitates osteogenic differentiation, potentially by inhibiting NF-$\kappa$B pathway activity.**

**Keywords:** baicalin; osteosarcoma; osteogenic differentiation; NF-$\kappa$B pathway

## Introduction

Osteosarcoma (OS) stands as a prevalent solid malignant bone-forming cancer, characterized by high recurrence and metastasis rates. Its incidence peaks among adolescents and children, with the median age of patients being 18 years old, coinciding with the period of adolescent growth spurt [1]. Regrettably, OS has emerged as a leading cause of tumor-related deaths in kids and teenagers [2,3], with approximately 5 new cases per 1 million reported annually. Patients diagnosed with non-metastatic OS and treated solely with surgery face a grim prognosis, with a 5-year survival rate below 20% [4]. However, significant strides have been made in both chemotherapy and surgical interventions, with the 5-year survival rate of OS patients reaching 66–82%, particularly with the implementation of limb salvage surgery and neoadjuvant chemotherapy [5]. Despite these advancements, cancer progression remains a formidable challenge in OS treatment, resulting in a meager 5-year survival rate of around 20% in patients with metastatic or recurrent disease [6]. Hence, there is an

urgent need to explore practical, effective, and safe therapeutic agents for the treatment of OS.

Baicalin, a flavonoid-derived compound extracted from the *Scutellaria baicalensis* root [7], has garnered attention for its reported safety and lack of toxicity, coupled with its antioxidant and anti-inflammatory properties [8,9]. Notably, studies have highlighted baicalin's ability to significantly impede cancer metastasis and growth across various tumor types, including breast cancer, human glioblastoma, and OS [10–13]. This inhibitory effect is primarily attributed to the reactive oxygen species-mediated inhibition of mitochondrial and protein kinase B (AKT) pathways [14,15]. However, the precise mechanism underlying baicalin's role in OS remains incompletely understood and warrants further investigation.

Nuclear factor $\kappa$B (NF-$\kappa$B), a transcription factor involved in numerous biological processes such as cell survival, apoptosis, proliferation, immune response, and the regulation of genes related to intracellular redox homeostasis [16], plays a crucial role in osteogenic differentiation. Recent studies have revealed that activation of the NF-$\kappa$B signaling pathway in inflammatory dental pulp stem cells leads to significant downregulation of the expression of osteogenic differentiation markers (Alkaline Phosphatase (*ALP*)/runt-related transcription factor 2 (*RUNX2*)/osteocalcin (*OCN*)/Collagen-I) and inhibition of osteogenic differentiation. Baicalin has been shown to reduce the release of tumor necrosis-associated factors and promote osteogenic differentiation by inhibiting the NF-$\kappa$B signaling pathway [17].

Furthermore, NF-$\kappa$B activation in OS promotes the differentiation of tumor cells into osteoid cells and enhances their invasive ability into bone tissue [18]. However, no study has yet reported on baicalin's ability to exert anti-OS effects and osteogenic differentiation through suppression of the NF-$\kappa$B signaling pathway. Building upon this gap, the current research posits the hypothesis that baicalin may indeed exert anti-OS functions and encourage osteogenic differentiation by suppressing the NF-$\kappa$B signaling pathway. Thus, this paper aims to explore this hypothesis at the *in vitro* level, thereby presenting novel insights and strategies for the treatment of OS.

## Materials and Procedures

### *Drugs*

Baicalin was procured from Sichuan Xieli Pharmaceutical Co., Ltd. (No. 20053191, Sichuan, China). Physiological saline was employed for the preparation of baicalin culture solutions at concentrations of 20 µM, 40 µM, and 80 µM.

### *Cell Culture and Grouping Intervention*

The OS cells (U2OS) were obtained from the National Collection of Authenticated Cell Cultures (SCSP-5030, Shanghai, China). These cell samples underwent proper verification via short tandem repeat (STR) analysis, and testing confirmed the absence of mycoplasma contamination. U2OS cells were cultured in RPMI-1640 medium (11875093, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 1% penicillin-streptomycin (P1400, Solarbio, Beijing, China) and 10% fetal bovine serum (FBS, 10099158, Gibco, Carlsbad, CA, USA). Cell cultures were maintained in an incubator with 5% $CO_2$ and 95% humidity at 37 °C. Prior to experimentation, cells were cultured until reaching the logarithmic growth phase and then harvested for passaging.

Cells were inoculated and cultured to the desired density based on the specific experimental requirements. Following this, they were subjected to the following treatments:

Control group: U2OS cells received no treatment; Treatment groups: U2OS cells were divided into groups treated with 20 µM, 40 µM, and 80 µM concentrations of baicalin for 24 hours.

### *Cell Counting Kit-8 (CCK-8) Assay*

After seeding at a density of $1 \times 10^4$ cells/well in 96-well plates, U2OS cells were cultured until they adhered to the plate surface. Subsequently, they were treated with different concentrations of baicalin. Following a 24-hour incubation period, the culture medium was aspirated, and the cells were supplemented with a medium containing 10% CCK-8 solution (CA1210, Solarbio, Beijing, China) for an additional 2 hours of incubation. The optical density (OD) of the cells at 450 nm was then measured using a microplate reader (Varioskan™ LUX, VLBLATGD2, Thermo Fisher Scientific, Waltham, MA, USA). Finally, cell viability was calculated.

### *Scratch Assay for Cell Migration Ability*

U2OS cells were seeded into 6-well plates at a density of $5 \times 10^6$ cells/well and cultured until they reached confluence. Subsequently, a pipette tip was used to create a straight scratch line perpendicular to the cell monolayer in each well. The PBS was then used to rinse away the scratched cells and debris, followed by replacing the old medium with a fresh medium.

At 0 hours and 24 hours post-scratching, images of the scratched area were captured and recorded. The width of the scratch was measured using Image J software (V1.8.0, National Institutes of Health, Bethesda, MD, USA). The cell migration rate was calculated using the following equation:

$$\text{24-hour cell migration rate} = [(\text{0-hour scratched width} - \text{24-hour scratched width})/\text{0-hour scratched width}] \times 100\%$$

### *Transwell Detection of Cell Invasion Ability*

The upper chamber of the transwell (3422, Corning Incorporated, NY, USA) was coated with pre-solubilized matrix gel and allowed to solidify at 37 °C. Subsequently, 200 µL of serum-free cell suspension containing $5 \times 10^4$ baicalin-treated U2OS cells was added to the upper chamber, while 600 µL of RPMI-1640 medium supplemented with 20% FBS was added to the lower chamber. The cell samples were then incubated at 37 °C for 24 hours.

Following incubation, the transwell chambers were fixed with 4% paraformaldehyde (P0099, Beyotime, Shanghai, China) for 15 minutes, and the fixed solution was rinsed with pure water several times. Any cells that had not migrated through the membrane were gently wiped off from the upper chamber using a cotton ball. Subsequently, a 30-minute staining with 0.1% crystal violet (C0121, Beyotime, Shanghai, China) was carried out. After staining, excess dye was removed by several rinses.

Finally, five randomly selected fields of view were photographed, and the migrated cells were counted under an IX71 inverted microscope (Olympus, Tokyo, Japan) [19].

### *qRT-PCR Detection of Gene Expression Levels*

Total RNA was extracted from the cells using Trizol reagent (15596026, Thermo Fisher Scientific, Waltham, MA, USA). The absorbance values at 260 nm were measured using a Nanodrop spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, Waltham, MA, USA) to determine the RNA concentration. Subsequently, the extracted RNA was reversely transcribed into cDNA using a reverse transcription kit (RR037A, Takara, Tokyo, Japan).

Quantification of *RUNX2*, osteopontin (*OPN*), *OCN*, receptor activator of nuclear factor kappa B ligand (*RANKL*), and Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) levels was performed using fluorescent quantitative PCR kits (RR820L, TaKaRa, Tokyo, Japan). The primer sequences used are shown in Table 1. The reaction conditions were as follows: 48 °C for 30 minutes, 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. GAPDH was utilized as an internal control, and the quantitative results were calculated using the $2^{-\Delta\Delta Ct}$ method.

### *Alkaline Phosphatase (ALP) Staining and Alizarin Red S (ARS) Staining for Assessing Osteogenic Differentiation and Mineralization Capacity*

U2OS cells at the logarithmic growth phase were maintained in culture until they reached approximately 60% confluence. Subsequently, the old medium was replaced with an osteogenic differentiation medium supplemented with various concentrations of baicalin (0, 20, 40, and 80 µM) to induce osteogenic differentiation of U2OS cells. The medium was refreshed every three days with a complete medium containing 50 mg/mL ascorbic acid phosphate, 10 mM $\beta$-glycerophosphate, and 10 nM dexamethasone.

**Table 1. RT-qPCR primer sequences.**

| Genes | Primer sequences |
|---|---|
| *RUNX2* | F: 5′-TGTTCCAAAGACTCCGGCAA-3′<br>R: 5′-CCCATCTGGTACCTCTCCGA-3′ |
| *OPN* | F: 5′-AATCTCCTAGCCCCACAGACC-3′<br>R: 5′-CCACACTATCACCTCGGCCA-3′ |
| *OCN* | F: 5′-GTGCAGCCTTTGTGTCCAAG-3′<br>R: 5′-TCCGGATTGAGCTCACACAC-3′ |
| *RANKL* | F: 5′-CCATCGGGTTCCCATAAAG-3′<br>R: 5′-TGAAGCAAATGTTGGCGTA-3′ |
| *GAPDH* | F: 5′-GGAGCGAGATCCCTCCAAAAT-3′<br>R: 5′-GGCTGTTGTCATACTTCTCATGG-3′ |

*RUNX2*, runt-related transcription factor 2; *OPN*, osteopontin; *OCN*, osteocalcin; *RANKL*, receptor activator of nuclear factor kappa B ligand; *GAPDH*, Glyceraldehyde-3-phosphate dehydrogenase.

After 5 days of culture, the cells were fixed with paraformaldehyde for 20 minutes and then washed three times with PBS. Subsequently, the samples were stained in the dark following the instructions provided in the ALP kit (P0321S, Beyotime, Shanghai, China). The stained samples were observed and photographed in each group.

Ten days later, the cells were stained using an osteoblast-mineralized nodule staining kit (C0148S, Beyotime, Shanghai, China) based on the ARS method. After staining, the samples were observed with an optical microscope and photographed. Finally, the relative ALP activity and mineralized nodule levels were evaluated using Image J software (National Institutes of Health, Bethesda, MD, USA).

### *Western Blotting*

After 30 minutes of cell lysis using protein lysis solution (Beyotime, Shanghai, China), the cells were centrifuged to collect the supernatant for protein concentration detection. Subsequently, the protein was denatured, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis, and transferred to a Polyvinylidene fluoride (PVDF) membrane.

Next, the membrane was sealed with 5% skimmed milk powder for 2 hours, followed by overnight incubation with primary antibodies including E-cadherin (ab40772, 1/50,000, Abcam, Cambridge, UK), N-cadherin (ab76011, 1/20,000, Abcam, Cambridge, UK), Vimentin (ab92547, 1/5000, Abcam, Cambridge, UK), RUNX2 (ab236639, 1/1000, Abcam, Cambridge, UK), OPN (ab283669, 1/1000, Abcam, Cambridge, UK), OCN (ab133612, 1/10,000, Abcam, Cambridge, UK), RANKL (ab239607, 1/1000, Abcam, Cambridge, UK), p-p65 (ab31624, 1/500, Abcam, Cambridge, UK), p65 (ab32536, 1/100,000, Abcam, Cambridge, UK), p-I$\kappa$B$\alpha$ (FS-K0390, 1/1000, Shanghai Fusheng Industrial Co., Ltd., Shanghai, China), I$\kappa$B$\alpha$ (yb-

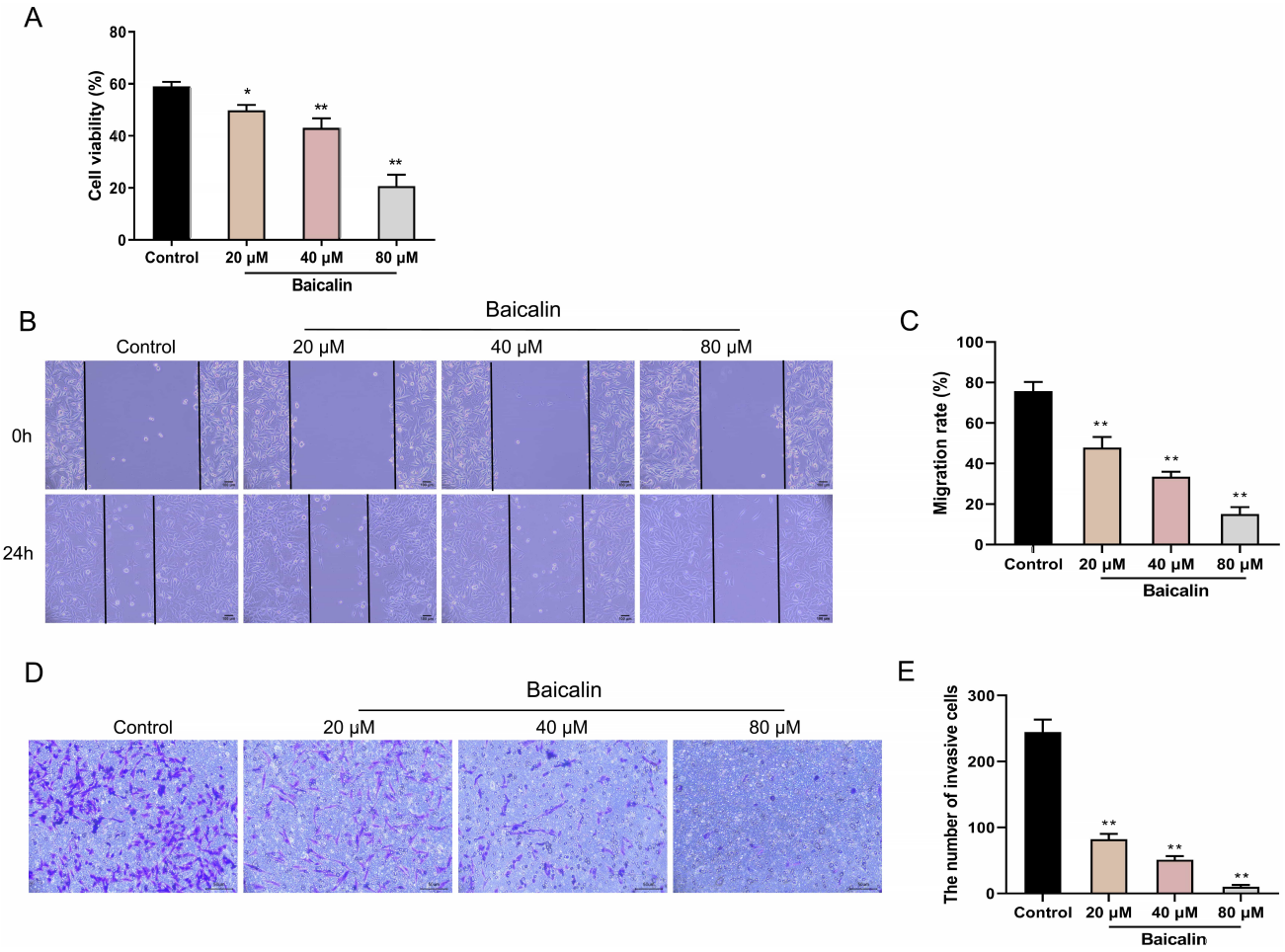


**Fig. 1. Baicalin treatment inhibits osteosarcoma cells' migration, proliferation and invasion.** (A) Cell Counting Kit-8 (CCK-8) detected the viability of U2OS cells in Control, 20 μM, 40 μM, and 80 μM groups (n = 3). (B,C) Scratch assay detected the migratory ability of U2OS cells in each group (n = 3); Scale bar = 100 μm. (D,E) Transwell assay detected the invasion ability of U2OS cells (n = 3). Scale bar = 50 μm. *$p < 0.05$ and **$p < 0.01$ *vs.* Control.

848Hu01, 1/1000, Shanghai Yubo Biotechnology Co., Ltd., Shanghai, China), and GAPDH (ab181602, 1/1000, Abcam, Cambridge, UK) at 4 °C.

Subsequently, the membrane was incubated with horseradish peroxidase (HRP)-coupled secondary antibodies, goat anti-rabbit (ab6721, 1/2000, Abcam, Cambridge, UK) and goat anti-mouse (ab205719, 1/5000, Abcam, Cambridge, UK) for 2 hours at room temperature. The ECL luminescent solution (P0018M, Beyotime, Shanghai, China) was added for development and exposure before photography. GAPDH was used as an internal reference, and the blot images were semi-quantified through Image J software (National Institutes of Health, Bethesda, MD, USA).

### *Statistical Treatment*

The experimental data were statistically analyzed using SPSS 24.0 software (IBM, Armonk, NY, USA). All assays were performed independently in triplicate, and the results were presented as mean ± standard error of the mean (SEM). Additionally, the data were visualized using GraphPad Prism software (7.0, GraphPad Software, San Diego, CA, USA). Comparison among multiple groups was conducted using one-way ANOVA, followed by the Bonferroni post hoc test for comparing differences between two groups. A *p*-value of less than 0.05 was considered statistically significant.

## Results

### *Baicalin Treatment Inhibits Proliferation, Invasion and Migration of Osteosarcoma Cells*

In this study, we initially observed the effects of baicalin on the proliferation, migration, and invasion of OS cells. We found that different concentrations of baicalin significantly reduced the viability of U2OS cells in a concentration-dependent manner ($p < 0.05$, Fig. 1A). Furthermore, compared to the 0 μM group, the invasion ($p < 0.01$), proliferation ($p < 0.05$), and migration ($p < 0.01$) abilities of cells in the 20 μM, 40 μM, and 80 μM groups

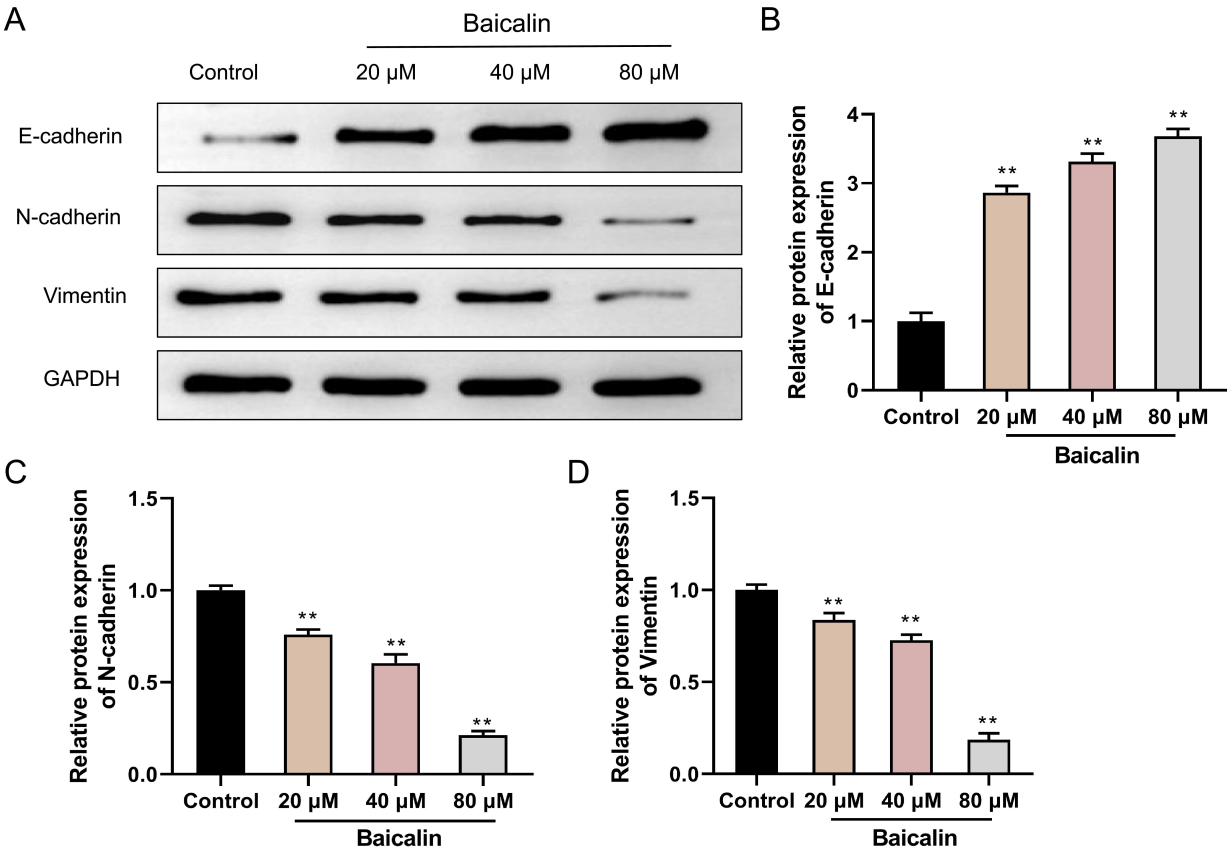


**Fig. 2. Baicalin regulates the epithelial-mesenchymal transition (EMT) in osteosarcoma cells.** (A–D) Western blot detection for E-cadherin, N-cadherin and Vimentin proteins' relative expression levels in U2OS cells in Control, 20 μM, 40 μM and 80 μM groups (n = 3), $^{**}p < 0.01$ *vs*. Control.

were notably reduced (Fig. 1B–E). Importantly, this reduction was concentration-dependent, indicating that higher concentrations of baicalin corresponded to lower migration and invasion abilities of U2OS cells. Therefore, baicalin effectively inhibits the proliferation, invasion, and migration of OS cells, with 80 μM baicalin exhibiting the most significant effects.

### *Baicalin Treatment Inhibits the Epithelial-Mesenchymal Transition (EMT) of Osteosarcoma Cells*

Furthermore, we investigated the impact of baicalin on the EMT of OS cells. Compared to the Control group, the treatment groups showed significantly increased levels of E-cadherin protein and decreased levels of N-cadherin and Vimentin proteins in U2OS cells, following a concentration-dependent trend ($p < 0.01$) (Fig. 2A–D). These results clearly indicate that baicalin inhibits the EMT process in OS cells.

### *Baicalin Regulates Osteogenic and Osteoblastic Differentiation of Osteosarcoma Cells*

Subsequently, we examined the influence of baicalin on the osteogenic and osteoblastic differentiation of OS cells. Compared to the Control group, the treatment groups displayed significantly elevated mRNA and protein expression levels of osteogenesis-associated markers (RUNX2, OPN, and OCN), while the mRNA and protein expression levels of the osteoblastogenic marker RANKL in U2OS cells were markedly decreased. These changes were concentration-dependent, with higher concentrations of baicalin leading to higher expression levels of RUNX2, OPN, and OCN proteins, and lower expression levels of RANKL protein ($p < 0.05$) (Fig. 3A–E). In summary, baicalin has the ability to promote osteogenesis in OS cells.

### *Baicalin Promotes Osteosarcoma Cells' Osteogenic Differentiation*

Subsequently, we assessed the effect of baicalin on the osteogenic differentiation of OS cells using ALP staining and ARS staining. The results revealed a significant enhancement in ALP activity in U2OS cells after baicalin treatment in the 20 μM, 40 μM, and 80 μM groups compared to the Control group. Importantly, the ALP activity increased in a concentration-dependent manner, with higher concentrations of baicalin leading to higher ALP activity ($p < 0.01$, Fig. 4A,B). Similarly, the ARS staining results showed a considerable enhancement in the miner-

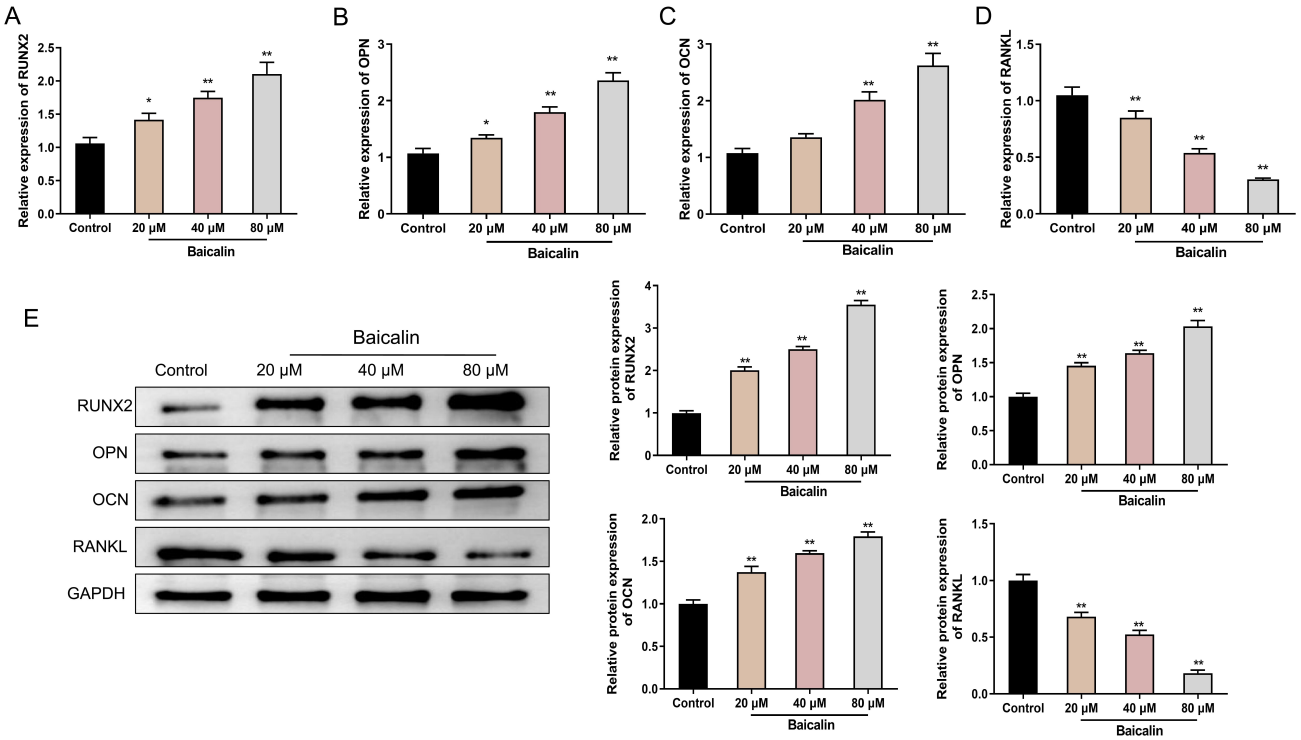


**Fig. 3. Baicalin regulates the expression of osteogenic markers in osteosarcoma cells.** (A–D) qRT-PCR for testing the *RUNX2*, *OPN*, *OCN*, and *RANKL* expression levels in U2OS cells of Control, 20 μM, 40 μM, and 80 μM groups (n = 3). (E) Western blot for determining the relative protein expression levels of RUNX2, OPN, OCN, and RANKL in cells of Control, 20 μM, 40 μM, and 80 μM groups (n = 3), *$p < 0.05$ and **$p < 0.01$ *vs.* Control.

alization capacity of U2OS cells in the 20 μM, 40 μM, and 80 μM groups compared to the control cells, also in a concentration-dependent pattern ($p < 0.01$, Fig. 4C,D). Overall, baicalin effectively promotes the osteogenic differentiation of OS cells.

### *Baicalin Treatment Inhibits the NF-κB Signaling Pathway Activity in Osteosarcoma Cells*

Finally, we investigated changes in the NF-κB signaling pathway activity in OS cells following baicalin treatment. The results showed that baicalin treatment significantly decreased the protein phosphorylation levels of IκBα and p65, as well as the ratios of p-p65/p65 and p-IκBα/IκBα, in a concentration-dependent manner compared to the controls ($p < 0.01$, Fig. 5). Thus, baicalin effectively inhibits the NF-κB signaling pathway activity in OS U2OS cells.

## Discussion

OS has emerged as a major health concern among children and adolescents, often originating from the terminal differentiation of osteoid cells or the differentiation of bone marrow stromal cells into osteoid cells. Patients with OS typically succumb to increased early migration and local invasion [20]. Therefore, the identification of effective drugs and the elucidation of their potential roles and mechanisms in halting the progression of OS are imperative for advancing clinical diagnosis and therapy for this disease.

In this study, we found that baicalin at concentrations of 20 μM, 40 μM, and 80 μM significantly suppressed the proliferation, migration, and invasion abilities of human OS cells. Furthermore, the anti-tumor effect of baicalin exhibited a concentration-dependent increase within a certain range. These findings indicate that baicalin indeed exerts an anti-tumor effect, consistent with the observations of Pang *et al.* [13], who reported that baicalin notably inhibited the proliferation, migration, and invasion abilities of OS cells.

EMT plays a crucial role in guiding OS cells through invasion and metastasis [21]. It is a process wherein polarized epithelial cells lose their adhesive properties and acquire the functional phenotypes of mesenchymal cells. This transition is accompanied by various changes in molecular markers, among which the "cadherin switch" is the most prominent molecular alteration. The gradual down-regulation of E-cadherin (E-cad) and up-regulation of N-cadherin (N-cad) on the surface of tumor cells promote their metastasis and invasion [22].

In this study, treatment with 20 μM, 40 μM, and 80 μM of baicalin significantly increased the E-cadherin protein level while decreasing the levels of N-cadherin and Vimentin proteins in OS cells. This suggests that baicalin may exert an anti-OS effect by inhibiting EMT. Previous

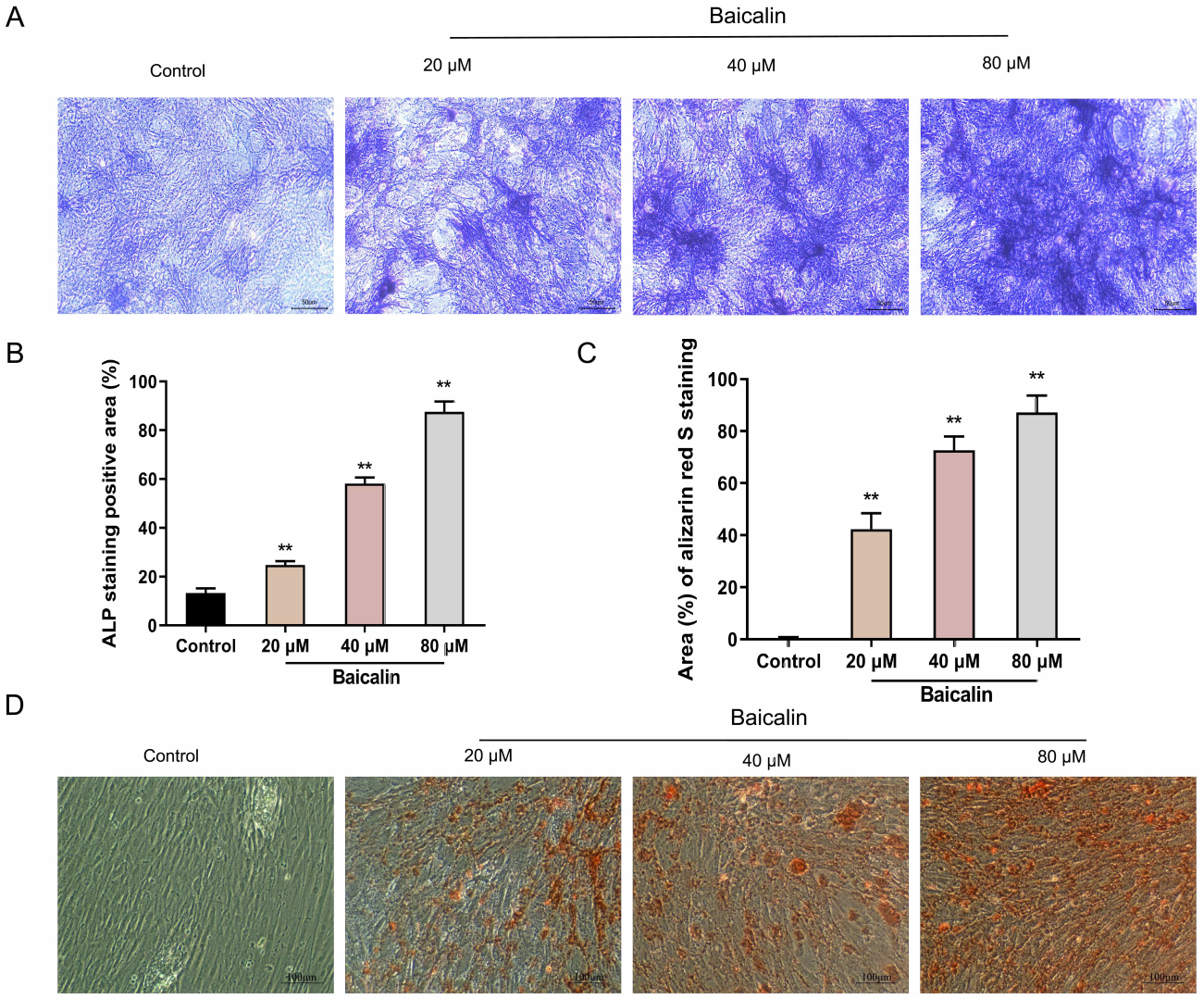


**Fig. 4. Baicalin promotes osteogenic differentiation of osteosarcoma cells.** (A–D) Alkaline Phosphatase (ALP) staining (Scale bar = 50 μm) and Alizarin Red S (ARS) (Scale bar = 100 μm) to detect ALP activity (A,B) and mineralization capacity (C,D) in U2OS cells of Control, 20 μM, 40 μM and 80 μM groups, respectively (n = 3), $^{**}p < 0.01$ *vs.* Control.

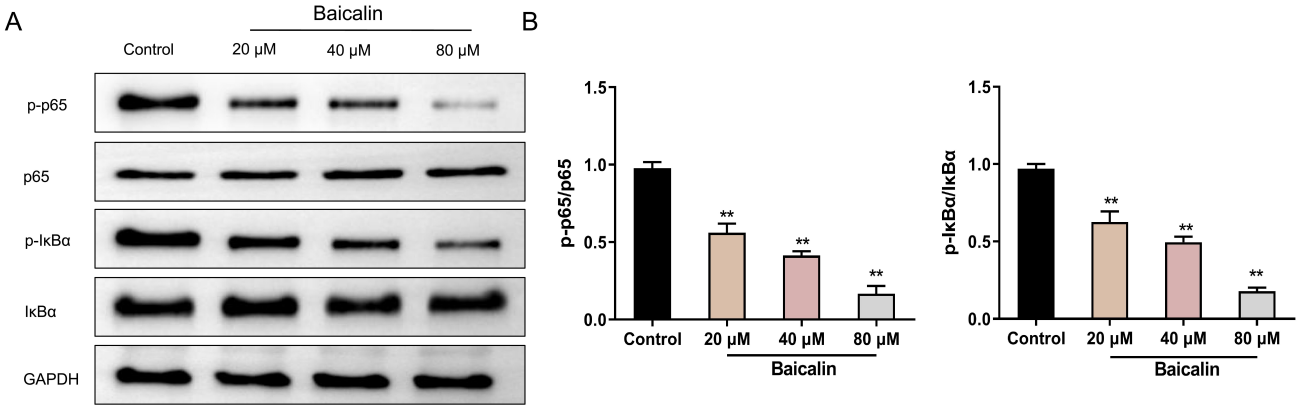


**Fig. 5. Baicalin treatment inhibits Nuclear factor κB (NF-κB) signaling pathway activity in osteosarcoma cells.** (A,B) Western blot detection of relative protein expression levels of IκBα, p-IκBα, p65 and p-p65 in U2OS cells of Control, 20 μM, 40 μM, and 80 μM groups (n = 3), $^{**}p < 0.01$ *vs.* Control.

research has indicated that OS is a differentiation-related disease. Specifically, it arises when multipotent skeletal stem cells (MSSCs) undergoing differentiation into mature osteoblasts are affected, leading to disruptions in normal osteogenic differentiation. The degree of malignancy is higher when the interruption of normal differentiation occurs at an earlier stage [23].

The process of differentiation is governed by numerous factors, among which RUNX2 plays a pivotal role in bone formation and osteogenic differentiation. *RUNX2* determines the lineage commitment of osteoblasts and regulates the expression of key proteins such as OPN and OCN by binding to osteoblast-specific cis-acting elements. Additionally, osteoblasts facilitate osteogenesis and differentiation by secreting ALP and suppressing the secretion of RANKL, a crucial factor that triggers the differentiation of osteoblast precursor cells into osteoclasts [24,25].

In this study, we observed that different concentrations of baicalin significantly increased the expression of RUNX2, OPN, and OCN, while reducing the expression of RANKL in OS cells. This suggests that baicalin effectively promotes osteogenesis and differentiation in OS cells.

Baicalin has demonstrated anti-OS and osteogenesis-promoting effects [13,26], yet its mechanism of action remains unclear. Nuclear factor $\kappa$B (NF-$\kappa$B) proteins are a family of pleiotropic transcription factors that remain inactive under normal conditions but become activated and encode numerous proteins in conditions such as tumors, inflammation, or immune dysfunction [27]. Briefly, stimulation by immune receptors and cytokine receptors triggers a cascade of proximal membrane events leading to the activation of the IKK (I$\kappa$B kinase), which phosphorylates I$\kappa$B, resulting in its proteasome degradation and the release of the NF-$\kappa$B release for nuclear translocation and gene transcriptional activation [28].

The IKK complex consists of IKKb and IKKa, which phosphorylate the downstream IKBa protein kinase. Once phosphorylated, IKBa enhances the phosphorylation of the downstream p65 protein and its translocation into the nucleus; where it binds to specific gene sequence promoters and regulates tumor development [29]. Research has shown a positive correlation between OS cell proliferation and the NF-$\kappa$B protein expression. Additionally, NF-$\kappa$B promotes the metastasis and invasion of OS cells [18]. Li *et al.* [17] found that baicalin promoted osteogenic differentiation of inflammatory dental pulp stem cells by inhibiting the NF-$\kappa$B signaling pathway. In this study, baicalin significantly reduced protein expression levels of p-p65 and p-I$\kappa$B$\alpha$ as well as the ratios of p-p65/p65 and p-I$\kappa$B$\alpha$/I$\kappa$B$\alpha$ in OS cells. Overall, baicalin may exert its anti-tumor effects and promote osteogenesis by inhibiting the NF-$\kappa$B signaling pathway.

However, there are some limitations to our study. For instance, we only utilized one OS cell line for phenotypic and mechanistic studies, and relevant *in vivo* experiments were not conducted. Additionally, functional validation experiments of activators or inhibitors of the NF-$\kappa$B pathway were lacking in the validation experiments of signaling pathways. Therefore, future studies should include multiple cell lines, conduct *in vivo* experiments, and incorporate pathway validation investigations.

## Conclusion

In summary, our research demonstrates that baicalin exerts its anti-OS effect and promotes osteogenesis by blocking the NF-$\kappa$B pathway, providing a theoretical basis for its potential use as a clinical drug for OS.

## Availability of Data and Materials

Data involved in the present work are available from the corresponding author upon request.

## Author Contributions

ZFZ and TZ designed the research study. ZFZ and TZ performed the research. XFC, MY and WFS provided help and advice on experiments. XFC, MY and WFS analyzed the data. All authors were involved in drafting and critical revision of the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

## Ethics Approval and Consent to Participate

Not applicable.

## Acknowledgment

Not applicable.

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## Conflict of Interest

The authors declare no conflict of interest.

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