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Investigating the Role of Shikonin in Enhancing Osteogenesis and Angiogenesis for the Treatment of Osteoporosis

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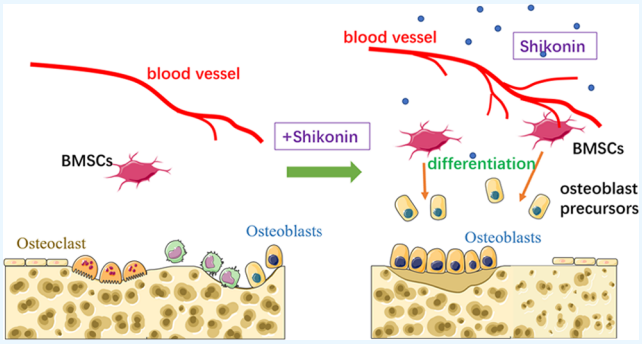
ABSTRACT: Osteoporosis, characterized by an increased risk of fractures, represents a significant global public health issue. Natural compounds have emerged as promising candidates for addressing this condition. Shikonin, derived from Lithospermum erythrorhizon as a purple-red naphthoquinone pigment, exhibits a diverse array of biological activities, including antibacterial, anti-inflammatory, and anticancer properties. Despite the well-documented bone-protective properties of shikonin, the precise molecular mechanisms underlying its role in the differentiation of bone marrow-derived mesenchymal stem cells (BMSCs) into osteoblasts, along with its implications on angiogenesis, are not fully elucidated. Our study showcases shikonin's ability to stimulate the differentiation of BMSCs into osteoblasts, leading to an upregulation of osteoblast-specific marker genes such as OC, Runx2, BMP2, and ALP. Furthermore, shikonin intervention triggers the upregulation of phosphorylation of p38, ERK, and JNK in the MAPK signaling pathway. Furthermore, shikonin has been shown to enhance the migration and angiogenic capabilities of human umbilical vein endothelial cells (HUVECs). Notably, the augmentation of HUVEC migration by shikonin can be counteracted by the addition of a JNK inhibitor. Furthermore, our findings indicate that shikonin effectively improves osteoporosis in aged mice by promoting osteoblast differentiation. In summary, our study elucidates the molecular mechanisms through which shikonin exerts its beneficial effects in the treatment of osteoporosis, highlighting its potential as a novel therapeutic option for both the prevention and management of this condition.



1. INTRODUCTION

Osteoporosis is a chronic condition characterized by reduced bone mass, disrupted bone microarchitecture, and an increased risk of fractures, often leading to long-term disability. Osteoporosis represents a substantial global public health issue, impacting over 200 million individuals worldwide and contributing to more than 9 million bone fractures annually.[1] Of particular concern is the age-related decline in bone density, which contributes to the development of osteoporosis.[2] Existing treatments for osteoporosis, including estrogen, bisphosphonates, and calcitonin, have limited overall efficacy and are associated with significant adverse effects.[3−5] While molecular targeted therapies like denosumab and romosozumab are available, their long-term follow-up data are insufficient, and they can be prohibitively expensive.[6,7]

Bone remodeling is a dynamic process regulated by the balance between bone formation mediated by osteoblasts and bone resorption conducted by osteoclasts.[8] In age-related osteoporosis, reduced differentiation of BMSCs into osteoblasts results in decreased bone formation.[9] Recent studies have highlighted the close relationship between bone regeneration/remodeling, bone formation, and angiogenesis.[10] Furthermore, research has revealed impaired differentiation of BMSCs into osteoblasts and compromised blood vessel formation as critical mechanisms underlying osteoporosis progression.[11]

The mitogen-activated protein kinase (MAPK) messenger family is a widely distributed group of protein kinases in eukaryotes, with crucial roles in cell proliferation, apoptosis, and differentiation.[12] This family encompasses subgroups such as ERK1/2, p38, and JNK, each exhibiting unique activity and substrate specificities that regulate cellular function.[12] During bone formation, the MAPK signaling pathway exerts a critical function in regulating osteoblast differentiation, maturation, and bone matrix synthesis. Specifically, the activation of the ERK signaling pathway promotes osteoblast differentiation and bone matrix synthesis, while the JNK and p38 signaling pathways participate in osteoblast differentiation and matura-

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Table 1. Sequences of Primers

gene	forward primer (5′-3′)	reversed primer (5′-3′)
OCN	GCAGCTTGGTGCACACCTAG	GGAGCTGCTGTGACATCCAT
ALP	AACCAGACACAAGCATTCC	CCAGCAAGAAGAAGCCTTTG
BMP2	AGCTGCAAGAGACACCCTTT	CATGCCTTAGGGATTTTGGA
Runx2	AGGGAATAGAGGGGATGCATTAG	AAGGGAGGACAGAGGGAAACA
β-Actin	GGCACCACACCTTCTACAATG	GGGGTGTTGAAGGTCTCAAAC

tion under stimuli-induced stress.[13−15] Additionally, research has indicated that the activation of the MAPK signaling pathway augments VEGF transcription, increases VEGF expression, and fosters blood vessel formation.[16]

Shikonin, a naphthoquinone compound derived from the roots of *Lithospermum erythrorhizon* Sieb. et Zucc. and *Arnebia guttata Bunge*, is a recognized constituent of traditional Chinese herbal medicine. Previous research has underscored the diverse biological activities of shikonin, including its anticancer, antibacterial, anti-inflammatory, and antiviral properties.[17−20] Additionally, shikonin has been demonstrated to enhance the differentiation of MC3T3-E1 cells via the BMP2/Smad5 signaling pathway[21] and mitigate cartilage destruction and joint swelling associated with osteoarthritis.[22,23] Although shikonin exhibits a certain bone-protective effect, current literature lacks reports on its impact on the osteogenic differentiation of BMSCs. Given shikonin's noteworthy bioactivity and bone-protective effects, alongside the pivotal role of the MAPK signaling pathway in the osteogenic differentiation and angiogenesis of BMSCs, our study seeks to investigate whether shikonin can enhance BMSCs' osteogenic differentiation and promote blood vessel formation through the MAPK signaling pathway. This exploration aims to potentially address osteoporosis resulting from diminished bone-forming capability.

2. MATERIALS AND METHODS

2.1. Animals and Reagents. The study utilized 18-month-old C57BL/6 mice obtained from Hunan Silaike Laboratory Animal Co., Ltd. These mice were carefully raised and maintained in the Animal Department of the Pediatric Research Institute at Hunan Children's Hospital. Shikonin, a compound of interest, was obtained from Sigma and prepared by diluting it in phosphate-buffered saline (PBS). The cell culture medium α-MEM was sourced from Hyclone, while fetal bovine serum, commonly used in cell culture, was purchased from Gibco. Bone marrow mesenchymal stem cells (CP-M131, Wuhan Procell Technology Co., Ltd., China). MC3T3-E1 cells (CL-0378, Wuhan Procell Technology Co., Ltd., China). Recombinant Human VEGF (P5590, Beyotime, China). To investigate specific proteins and markers, the study utilized antibodies against osteocalcin, p-P38, pERK, pJNK and GAPDH. These high-quality antibodies were procured from Cell Signaling Technology.

2.2. Cell Proliferation Assay. For the evaluation of cell viability, the Cell Counting Kit-8 (CCK-8) obtained from 7sea (China) was employed in accordance with the manufacturer's guidelines. BMSCs were seeded into 96-well plates at a density of 3×10^3 cells per well. Subsequently, BMSCs were exposed to various concentrations of shikonin (0, 0.1, 0.2, and 0.4 μM) for a duration of 1−4 days, as per previously published literature protocols.[19−21] Following the respective treatment periods, 10 μL of CCK-8 solution was dispensed into each well and incubated for 2 h in a protected environment shielded from light. Subsequently, the optical density (OD) was determined at 450 nm wavelength using a spectrophotometer.

2.3. Osteogenic Differentiation and Mineralization Assessment. BMSCs were inoculated into a 24-well plate at a density of 2×10^4 cells per well. Once the cell density surpassed 10%, the standard culture medium was substituted with a specialized osteogenic differentiation medium procured from Cyagen, China. Various concentrations of shikonin (0, 0.1, 0.2, and 0.4 μM) were then introduced for intervention. In addition to the above, we chose the preosteoblastic MC3T3-E1 cell line as a positive control group. These cells were cultured solely in osteogenic differentiation medium to promote their differentiation. After 7 days of differentiation, ALP activity was assessed with an ALP staining kit supplied by Beyotime, China. Subsequently, on day 14 of differentiation, bone mineralization nodules were visualized through staining with Alizarin Red S (ARS) solution, also obtained from Beyotime, China.

2.4. Real-Time PCR. BMSCs were seeded into 6-well plates at a density of 1×10^5 cells per well, and then exposed to shikonin at concentrations of 0.2 and 0.4 μM for 5 days. Total RNA was extracted from the treated cells with Trizol (Invitrogen), and cDNA was synthesized for Real-Time PCR analysis to assess gene expression. PCR reactions were carried out using primers specifically designed based on mouse gene sequences. The primer sequences are listed in Table 1.

2.5. Scratch Assay. In this investigation, HUVECs were plated onto 6-well plates and incubated until full confluence was achieved, in readiness for the scratch wound assay. When the cells achieved 100% confluence, a uniform scratch was made in each well using a 200 μL pipet tip. The scratch was made in a consistent manner to ensure uniformity in subsequent measurements. Following the creation of the scratch, an intervention was introduced by adding 0.2 μM of shikonin to the wells. Furthermore, the positive control group received an addition of 20 ng/mL human recombinant VEGF protein to the culture medium. Shikonin is a compound known for its potential therapeutic effects in various biological processes. By incorporating shikonin, our intent was to explore its effect on the healing dynamics of the scratch-induced wounds within the HUVECs. At specific time points, namely 12 and 24 h after the intervention, images of the scratch area were captured using appropriate imaging techniques. These images served as a visual representation of the scratch healing process and allowed for subsequent analysis. To quantify the degree of scratch closure, the scratch area was measured using reliable measurement tools or software. This provided objective data on the extent to which the HUVECs migrated and closed the scratch over the designated time intervals. This study was conducted to assess the impact of shikonin on the healing ability of HUVECs using the scratch assay.

2.6. Tube Formation Assay. In this experiment, HUVECs were seeded into 96-well plates which were precoated with Matrigel (Biocoat). A concentration of 5×10^3 cells per well

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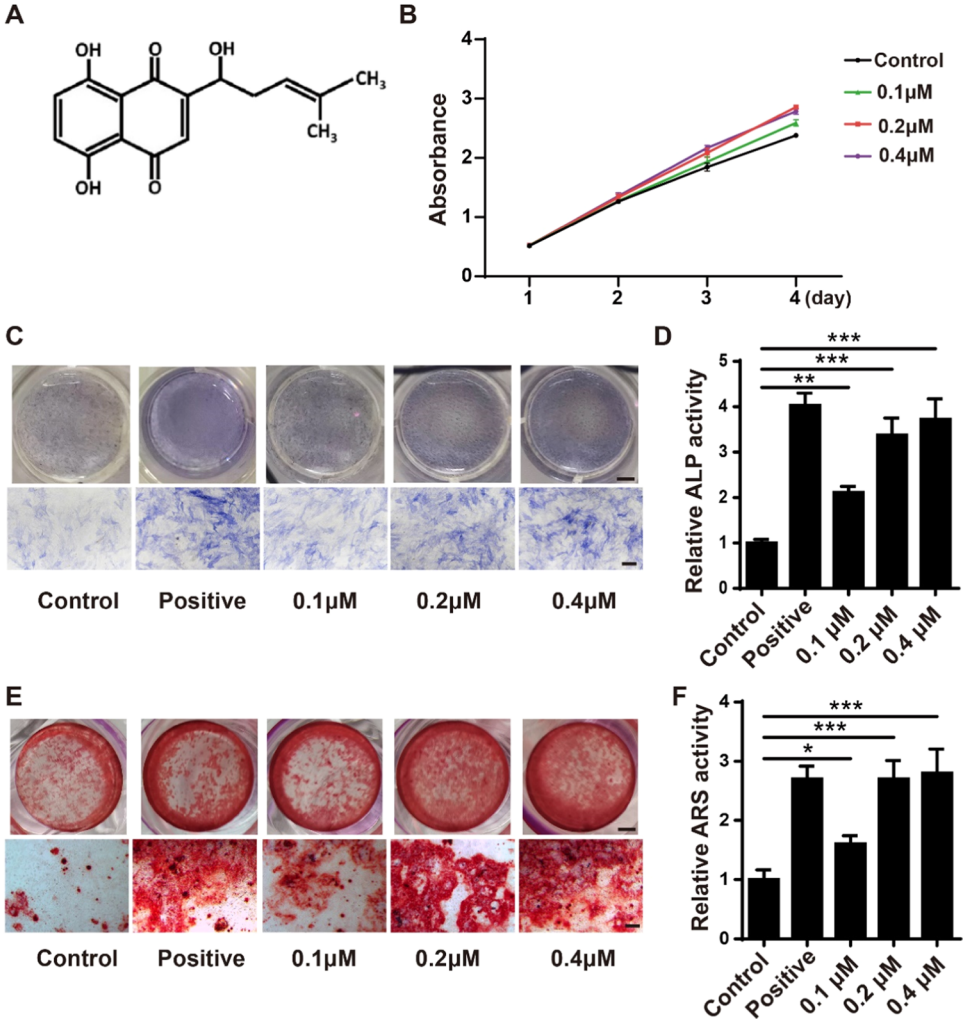

Figure 1. Shikonin promotes osteogenic differentiation of BMSCs. (A) Chemical structure of Shikonin; (B) The effect of different concentrations (0.1, 0.2, and 0.4 μM) of shikonin on BMSC proliferation. (n = 5). (C) ALP Staining Images of BMSCs Following 7 Days of Osteogenic Induction (top row scale bar, 0.5 cm; bottom row scale bar, 100 μm). (D) Quantitative Analysis of ALP Staining. (E) Images of Alizarin Red staining (top row scale bar, 0.5 cm; bottom row scale bar, 100 μm). (F) Quantitative Analysis of Alizarin Red staining. (*P < 0.05, **P < 0.01, ***P < 0.001 comparison to untreated control groups).

was used for seeding the plates. To evaluate the effect of shikonin on tube formation in HUVECs, various concentrations of shikonin (0, 0.1, 0.2, and 0.4 μM) were administered as interventions. Shikonin is a compound known for its potential therapeutic effects on cellular processes. The plates were incubated for 8 h to allow for tube formation by the HUVECs. Tube formation is a process by which these cells organize and create tubular structures, resembling those found in blood vessels. Following the incubation period, the tube formation of the HUVECs was observed and photographed using an inverted microscope. This provided a visual representation of the tubes formed by the cells. To quantify the extent of tube formation, the number of HUVEC tubes was measured using Image Pro Plus software or similar image analysis tools.

2.7. Western Blot Analysis. BMSCs were cultivated in 6-well plates and exposed to shikonin intervention (0.2 μM) for varying durations. After the intervention period, cellular proteins were isolated through lysis using RIPA buffer. The extracted protein samples were subsequently separated via 12% SDS-PAGE. This method facilitates the distinction of proteins according to their molecular weight. Following gel electrophoresis, the proteins were subsequently moved to a polyvinylidene fluoride (PVDF) membrane for further examination. This membrane provides a solid support for the proteins during subsequent steps. To minimize nonspecific binding, the membrane was incubated at room temperature for 1 h with a 5% solution of nonfat milk. This blocking step helps to create a barrier and reduce background noise during the antibody incubation. Subsequently, the membrane was incubated with the primary antibody at 4 °C overnight. The primary antibody specifically recognizes and binds to the target protein under investigation. This step aids in detecting the presence and/or levels of the target protein. Following incubation with the primary antibody, the membrane was washed three times to eliminate any unbound antibody. This step helps to remove any excess or nonspecifically bound antibodies. Subsequently, the membrane was incubated for 1 h with a secondary antibody conjugated to horseradish peroxidase (HRP). This secondary antibody specifically binds to the primary antibody and incorporates HRP, an enzyme that catalyzes a reaction to generate a detectable signal. After another round of washing to remove any unbound secondary antibody, the membrane was exposed and developed for

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visualization. The HRP enzyme catalyzes a reaction that produces a visible signal (e.g., chemiluminescence or colorimetric) to indicate the presence of the target protein.

2.8. Animal Studies. The study encompassed a total of 12 18-month-old C57BL/6 mice, which were divided into two groups: a control group and a shikonin treatment group. The mice in the treatment group received a safe dosage of 3 mg/kg of shikonin through tail vein injections, while the control group received an equivalent dosage of PBS. To evaluate the effects of shikonin treatment, the mice were injected twice a week for a duration of two months. After the treatment period, Micro-CT scanning and OC immunofluorescence staining were performed on the femurs of the same side to analyze and compare the results between the two groups. It must be noted that all animal experimentation procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals established by the National Institutes of Health (NIH). These guidelines are designed to ensure the ethical treatment and responsible use of animals in research studies.

2.9. Micro-CT Analysis. Upon collection, the mouse femur samples were fixed in 4% paraformaldehyde for 24 h. Subsequently, Micro-CT scanning was carried out using a vivCT80 model from Scanco Medical Inc., located in Brüttisellen, Switzerland. During the scanning process, the micro-CT system captured multiple cross-sectional images of the fixed femur samples. These images were then processed and reconstructed into three-dimensional (3D) representations of the bone structure using the built-in software provided by the micro-CT system. This software incorporated advanced algorithms to generate accurate 3D images from the digital data acquired during the scanning procedure. These 3D images served as valuable tools for further analysis and evaluation of the bone structure and potential effects of shikonin treatment on the femurs of the mice.

2.10. Statistical Analysis. In the experimental study, the raw data obtained from the sample analysis were recorded as mean values along with their corresponding standard deviations. To ascertain the statistical significance between the groups, the researchers utilized two widely accepted statistical tests: the t test and analysis of variance (ANOVA). The choice of test depended on the specific experimental design and the number of groups being compared. In both cases, the researchers set a significance level (α) of 0.05, which means that if the calculated p-value from the statistical test was less than 0.05, it was considered as statistically significant.

3. RESULTS

3.1. Shikonin Enhances the Osteogenic Differentiation of BMSCs. To investigate the effects of shikonin on BMSCs, we initially intervened with BMSCs using shikonin at varied concentrations. The CCK-8 assay measures the metabolic activity of live cells by reducing WST-8 to an orange-red formazan dye, thereby assessing cellular proliferation activity.[24] The CCK-8 assay results indicated that shikonin promotes the proliferation of BMSCs (Figure 1B). We subsequently observed the effects of shikonin on the osteogenic differentiation of BMSCs, intervening with BMSCs using shikonin at different concentrations (0.1, 0.2, 0.4 μM) combined with osteogenic induction medium. During the process of osteogenic differentiation, the expression of ALP significantly increases in the early stages, particularly as cells begin to differentiate toward an osteoblastic lineage.[25] In the later stages of osteogenic differentiation, alizarin red S staining is commonly used to visualize the deposition of calcium salts.[26] ALP staining demonstrated that the MC3T3-E1 cells in the positive control group exhibited strong ALP staining, while shikonin promoted the early osteogenic differentiation of BMSCs (Figure 1C,D). Alizarin Red staining revealed that the MC3T3-E1 cells in the positive control group exhibited distinct red mineralized nodules. Additionally, shikonin enhanced the mineralization capacity of BMSCs during the late differentiation stage following intervention (Figure 1E,F).

3.2. Shikonin Promotes Osteogenic Gene Expression in BMSCs. By quantifying the expression of osteogenic-related genes via PCR, such as ALP, OC, Runx2, BMP2, etc., we can assess the osteogenic differentiation process. ALP expression increases early in the osteogenic differentiation process, serving as an essential marker for osteoblast differentiation.[25] OC expression increases in the late stages of osteogenic differentiation and is a sign of mature osteoblasts; measuring its expression helps in evaluating bone formation and mineralization status.[27] BMP2 is a significant member of the bone morphogenetic protein family, involved in multiple stages of osteoblast differentiation, particularly in the initiation and regulation of the early stages.[28] Runx2 is a crucial transcription factor for osteogenic differentiation, regulating the entire process.[29] BMSCs were subjected to treatment with varying concentrations of shikonin (0.2 and 0.4 μM) for 48 h, followed by RNA extraction for real-time PCR analysis. The findings demonstrated that shikonin significantly upregulates the mRNA expression levels of ALP, OC, BMP2 and Runx2 (Figure 2A−D).

3.3. Shikonin Promotes Angiogenesis. To examine the impact of shikonin on angiogenesis, we performed scratch and tube formation assays using HUVECs. During angiogenesis, the migration ability of endothelial cells is a key step. The scratch assay can simulate the migration process of cells in vivo by observing and measuring the rate and manner of scratch closure, thereby evaluating the migratory capacity of cells.[30] It exposed to 0.2 μM shikonin demonstrated a significant enhancement in cell migration as observed in the scratch assay. Meanwhile, the positive control group, which received the addition of VEGF protein, exhibited the strongest cell migration ability (Figure 3A,B). Furthermore, in the tube formation assay, cotreating HUVECs with varying concentrations of shikonin (0.2, 0.4 μM) significantly promoted their ability to form tube-like structures. Meanwhile, the addition of VEGF protein significantly enhanced the ability of HUVECs to form tube-like structures compared to the control group (Figure 3C,D).

3.4. Shikonin Affects Phosphorylation in the MAPK Signaling Pathway. To elucidate the role of the MAPK signaling pathway in the osteogenic differentiation of BMSCs induced by shikonin, we treated the cells with 0.2 μM of shikonin for different time periods (30, 60, or 120 min) and assessed the expression of phosphorylated forms of p38, ERK, and JNK via protein blotting. 30, 60, and 120 min were selected as the time points for detection, as cellular signaling pathways are rapidly activated following stimulation. Within 30 min, early responses can be observed; at 60 min, activation may reach its peak; and at 120 min, the assay can reveal whether the signal remains continuously activated or begins to decline, thereby providing a comprehensive understanding of the dynamic changes in the signaling pathway.[31] Our findings revealed that shikonin treatment resulted in a significant upregulation of p-p38, pERK, and pJNK, with the most

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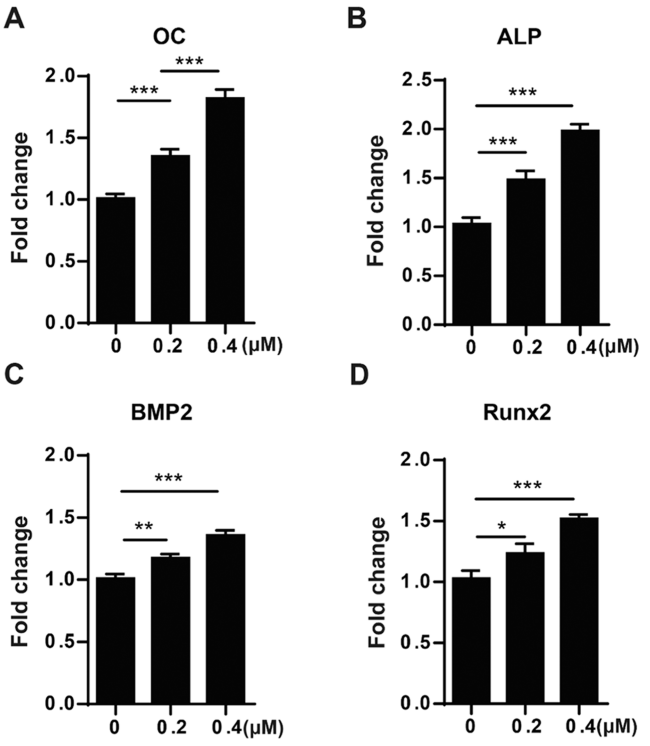

Figure 2. Shikonin promotes osteogenic gene expression in BMSCs. RT-PCR was used to measure the expression levels of osteogenic-related genes in BMSCs after intervention with 0.2 and 0.4 μM of shikonin. Gene expression was normalized to β-actin. (A) Osteocalcin (OC); (B) Alkaline Phosphatase (ALP); (C) Bone Morphogenetic Protein 2 (BMP2); (D) Runt-related Transcription Factor 2 (Runx2). (n = 3). (*P < 0.05, **P < 0.01, ***P < 0.001 comparison to untreated control groups).

pronounced effect observed after 60 min of treatment (Figure 4A−D). Furthermore, to explore the potential involvement of the JNK signaling pathway in the enhancement of cell migration induced by shikonin, we cotreated HUVECs with shikonin and the JNK signaling pathway inhibitor SP600125. Remarkably, the inhibitory effects of SP600125 on JNK signaling attenuated the shikonin-induced promotion of cell migration (Figure 4E,F). In summary, these findings suggest that shikonin promotes the activation of p38, ERK, and JNK within the MAPK signaling pathway. This, in turn, facilitates osteogenic differentiation in BMSCs and angiogenesis in HUVECs.

3.5. Shikonin Improves Bone Mass in Aged Mice. In the current investigation, the effects of shikonin on bone mass were investigated in 18-month-old aged mice. The mice were treated with shikonin for a duration of 2 months. Subsequently, after euthanasia, micro-CT scanning was conducted on the mice's femurs. Notably, our findings demonstrated that shikonin treatment led to a significant enhancement of bone mass in aged mice. Further analysis of the micro-CT data demonstrated that shikonin treatment increased key parameters associated with bone quality, including BV/TV (bone volume fraction), Tb.N (trabecular number) and Tb.Th (trabecular thickness). Conversely, shikonin treatment led to a decrease in Tb.Sp (trabecular separation), indicating a greater density and connectivity of trabecular bone in the shikonin-treated group as compared to the control group (Figure 5A−E). To elucidate the underlying mechanism, immunofluorescent staining of osteocalcin (OC) was performed on femur tissue sections. Remarkably, the shikonin-treated group demonstrated a significantly higher number of osteoblasts compared to the control group (Figure 5F,G). This suggests that shikonin may stimulate osteoblast differentiation and activity, contributing to the observed improvement in bone mass. Overall, our results indicate that shikonin administration holds the potential to enhance bone mass in aged mice by increasing bone volume, thickness, and number, while reducing trabecular separation. The upregulation of osteoblasts further supports the beneficial effects of shikonin on bone health.

4. DISCUSSION

Bone homeostasis denotes the equilibrium between bone resorption (breakdown) and bone formation within the body. It is a process that helps maintain stable bone tissue during normal growth, development, and repair.[8] However, age-related osteoporosis is a degenerative bone disorder frequently characterized by impaired bone quality, primarily resulting from reduced bone formation.[9] The imbalance in bone homeostasis can leads to fragile and brittle bones, increasing the risk of fractures. Recommended treatments for osteoporosis, including parathyroid hormone therapies and fluoride compounds, have been shown to enhance the proliferation and differentiation of osteoblasts. However, they may also be associated with risks of hypophosphatemia and gastrointestinal diseases.[32] In recent years, natural compounds have emerged as a new method for treating osteoporosis due to their relatively fewer side effects and potential effectiveness.[33] Studies have shown that certain active ingredients derived from Chinese herbal medicine can promote the proliferation and differentiation of osteoblasts, thereby improving osteoporosis.[34,35] Specifically, the compound shikonin has been identified to stimulate the differentiation of BMSCs into osteoblasts and enhance vascular regeneration, which in turn improves bone quality in aged mice. The mechanism behind this effect involves the activation of the MAPK signaling pathway, specifically the phosphorylation of proteins p38, ERK, and JNK. These findings suggest that shikonin may hold promise as a potential drug for preventing and treating osteoporosis.

Shikonin, a purple-red naphthoquinone compound, is primarily extracted from the roots of Lithospermum erythrorhizon. It has been extensively studied and found to have multiple beneficial effects.[36] A substantial body of research has demonstrated that shikonin inhibits the growth of various malignant tumors, including leukemia, colon cancer, breast cancer and gastric cancer.[37−40] Additionally, shikonin has shown promising anti-inflammatory, antibacterial, and analgesic effects.[18,19,41] In the context of the skeletal system, researchers have reported that shikonin can promote the proliferation and differentiation of MC3T3-E1 cells via the activation of the BMP2/Smad5 signaling pathway.[21] Moreover, shikonin has been found to promote the expression of pJNK and pp38 in the MAPK signaling pathway, leading to reduced chondrocyte inflammation and improved osteoarthritis.[42] The MAPK signaling pathway plays a vital role in bone formation and vascular regeneration processes.[43,44] Based on these findings, it is hypothesized that shikonin can enhance osteoblast differentiation and vascular regeneration, thereby improving bone formation and alleviating osteoporosis. This hypothesis is supported by the potential activation of pERK,

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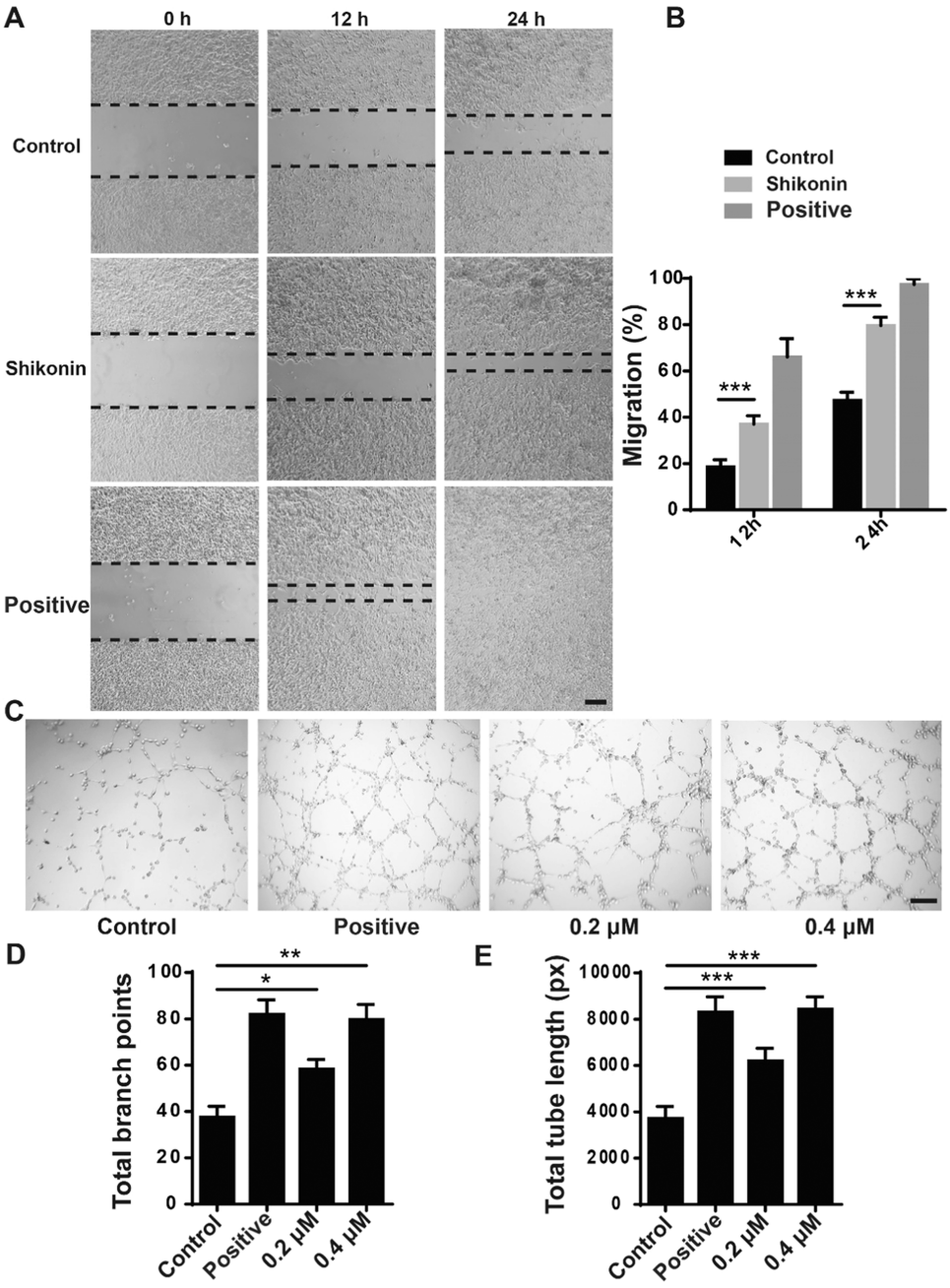

Figure 3. Shikonin promotes angiogenesis. (A) Scratch Assay Images of HUVECs (scale bar, 200 μm). (B) Quantitative Analysis of the Scratch Assay. (C) The tube formation assay was utilized to assess the impact of shikonin on the angiogenesis of HUVECs (scale bar, 100 μm). (D, E) Quantitative analysis was performed to determine the total number of branching points and the overall tube length in (C). ($n = 3$). (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ comparison to untreated control groups).

pJNK, and pp38 expressions in the MAPK signaling pathway by shikonin.

To elucidate the mechanism underlying shikonin's promotion of osteoblast differentiation, we first isolated BMSCs from mice. Our findings revealed that shikonin could enhance the proliferation of BMSCs and induce their differentiation into osteoblasts. It is well-established that the osteogenic potential of BMSCs is essential for bone repair and regeneration. Among these, the MAPK signaling pathway is considered a key regulatory factor in the osteogenic differentiation of BMSCs.[45,46] The MAPK signaling pathway comprises several family members, including ERK, JNK, and p38, which participate in regulating osteoblast differentiation, maturation, and synthesis of the bone matrix. ERK can regulate the proliferation, differentiation, and apoptosis of osteoblasts by regulating the activity of Runx2 and the expression of cell cycle regulatory factors.[47,48] Additionally, through the ERK signaling pathway, ALP activity can be enhanced, which participates in the generation of osteoblasts.[49] Some studies have indicated that the P38 signaling pathway regulates the activity of ALP and the expression of Runx2 and COL1A1, facilitating the differentiation of stem cells into osteoblasts.[50] Furthermore, it has been established that BMP2 requires activation of the p38 signaling pathway to facilitate osteogenic differentiation and maturation.[51] The JNK pathway has been found to participate in regulating the osteogenic differentiation of BMSCs or

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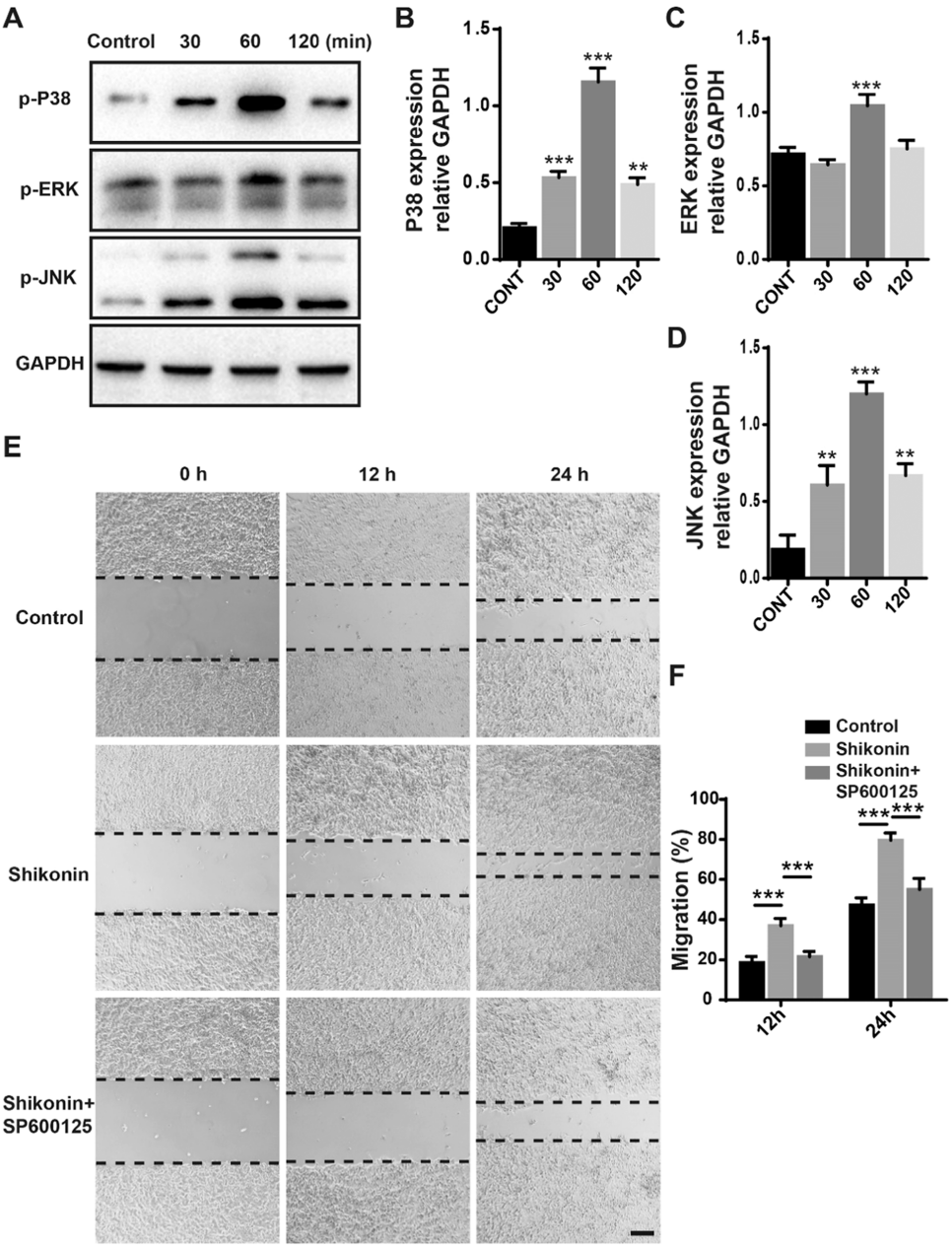

Figure 4. Shikonin affects phosphorylation in the MAPK signaling pathway. BMSCs were treated with 0.2 μM shikonin for 30, 60, or 120 min, and the expression of relevant proteins was detected by Western blot. Cell lysates were subjected to Western blot analysis using specific antibodies targeting phosphorylated p38, phosphorylated ERK, phosphorylated JNK, and GAPDH as the loading control. (A) Western Blot Band Results Image. The intensity of the bands was quantified utilizing ImageJ software. All experiments were performed at least three times to ensure reproducibility. (B–D) Quantitative Analysis of Western Blot Results. The migratory effect of shikonin on HUVECs is attenuated by the JNK signaling pathway inhibitor. (E) Scratch Assay Images of HUVECs (scale bar, 200 μm). (F) Quantitative Analysis of the Scratch Assay. (*P < 0.05, **P < 0.01, ***P < 0.001 comparison to untreated control groups).

periodontal ligament stem cells.[52,53] Previous studies have demonstrated that shikonin can promote the expression of pJNK and pp38 in the MAPK signaling pathway, thereby reducing the expression of pro-inflammatory mediators to improve osteoarthritis.[54] Additionally, shikonin has been shown to induce apoptosis in renal cancer cells by activating the Ras/MAPK and PI3K/AKT pathways.[55] Our study found that after treatment with shikonin, the phosphorylation of ERK, p38, and JNK in the MAPK signaling pathway of BMSCs was upregulated. Furthermore, the expression of osteogenic-related genes OC, ALP, BMP2, and Runx2 in BMSCs increased after shikonin intervention. These results indicate that shikonin can promote the osteoblastic differentiation of BMSCs through the MAPK signaling pathway.

The coupling of bone formation and vascular regeneration plays a crucial role in bone regeneration. It has been proven that activating the MAPK signaling pathway can enhance transcription of VEGF, increase the expression of VEGF, and promote the formation of blood vessels.[16,56] Liu et al. found that shikonin inhibited ROS production induced by ox-LDL, thereby protecting HUVECs.[57] Additionally, other studies have demonstrated that shikonin promotes wound healing in rat skin by stimulating proliferation and angiogenesis in fibroblasts and endothelial cells.[58] Our research demonstrated

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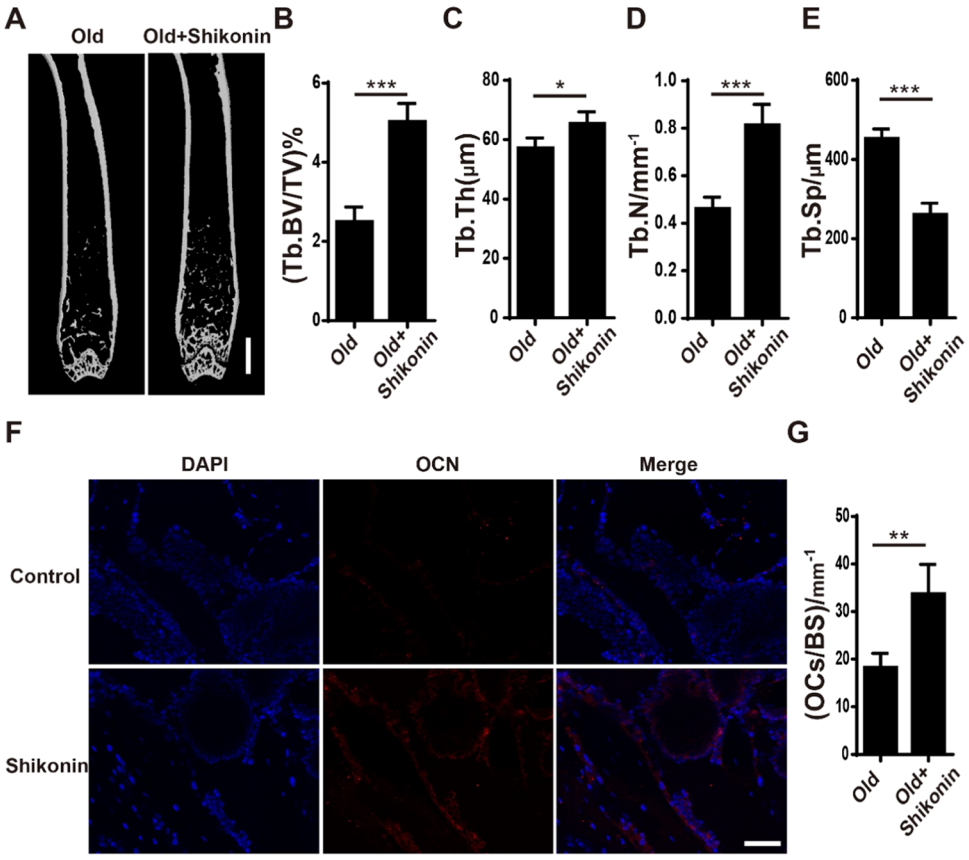

Figure 5. Shikonin improves bone mass in aged mice. (A) Representative μCT images of the femora are shown, with scale bars set at 1 mm. Quantification of Micro-CT data was conducted with a sample size of $n = 6$ per group: (B) Tb.BV/TV, (C) Tb.Th, (D) Tb.N, (E) Tb.Sp. (F) Immunofluorescence histochemistry staining (Scale bar: 25 μm). (G) Quantitative analysis of osteoblast numbers. $n = 6$. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ comparison to untreated control groups).

that shikonin can enhance the migration and angiogenesis of HUVECs. Furthermore, after the addition of the JNK signaling pathway inhibitor SP600125, the promotion effect of shikonin on HUVEC migration was suppressed. These results suggest that shikonin can promote angiogenesis of HUVECs through the MAPK signaling pathway.

5. CONCLUSIONS

Our study has demonstrated that shikonin can promote bone formation and angiogenesis, improve bone quality in aged mice, and that the ERK, p38, and JNK pathways in the MAPK signaling pathway are involved in this process. However, our injection of shikonin in vivo was mainly based on previous research literature without designing multiple concentration gradients, which has certain limitations. In future studies, we should further improve this aspect. Overall, this study provides scientific evidence for shikonin as a potential treatment for osteoporosis.

■ ASSOCIATED CONTENT

Data Availability Statement

All relevant data are within the manuscript.

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Author Contributions

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Notes

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■ ABBREVIATIONS

BMSCsbone marrow-derived mesenchymal stem cells
HUVECshuman umbilical vein endothelial cells
OCosteocalcin
ALPalkaline phosphates
BMPbone morphogenetic protein
Runx2runt-related transcription factor 2
ERKextracellular regulated protein kinases
JNKc-Jun N-terminal kinase
MAPKmitogen-activated protein kinase
OPosteoporosis
VEGFvascular endothelial growth factor
PVDFpolyvinylidene fluoride
HRPhorseradish peroxidase
PBSphosphate belanced solution

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