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Original Article

Morinda officinalis polysaccharide enhances osteogenic differentiation and migration of bone marrow mesenchymal cells by activating P38MAPK signal transduction

Manxiang Lai[1], Xia Chen[2], Zhiyan Ruan[1], Hong Ren[1], Zhe Xu[1], Jiwei Lin[3]

[1]Department of Pharmacy, Guangdong Food and Drug Vocational College, Guangzhou 510520, Guangdong, China; [2]Department of Nursing, Guangdong Food and Drug Vocational College, Guangzhou 510520, Guangdong, China; [3]Prevention and Treatment Center, Shenzhen Hospital of Traditional Chinese Medicine, Shenzhen 518000, Guangdong, China

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Abstract: *Morinda officinalis* polysaccharide (MOP), a derivative extracted from the traditional Chinese medicine *Morinda officinalis*, exhibits immunomodulatory and anti-osteoporosis (OP) effects. However, the mechanism underlying MOP's promotion of bone mesenchymal stem cell (BMSC) osteogenic differentiation remains unclear. Flow cytometry was employed to characterize BMSCs isolated from SD rats. The biological characteristics of BMSCs were detected using methyl thiazolyl tetrazolium (MTT), scratch healing, and colony formation assays. To assess the effect of MOP on BMSC osteogenic differentiation, Alizarin Red S staining and alkaline phosphatase (ALP) activity assays were performed. Osteogenic-associated proteins and gene expression were measured by western blot and RT-qPCR. MOP (40 μg/mL) promoted BMSC proliferation, enhanced colony formation and migration, promoted the formation of mineralized nodules of BMSCs, and increased ALP activity. Moreover, MOP treatment upregulated osteoblast-related protein and gene levels. In addition, MOP activated the P38 MAPK signaling pathway, and the addition of p38 pathway inhibitor SB203580 inhibited cell viability, colony formation, migration, and osteogenic differentiation of BMSCs. These findings suggest that MOP enhances BMSC proliferation and migration through P38 MAPK pathway activation, thereby promoting osteogenic differentiation.

Keywords: *Morinda officinalis* polysaccharides, bone mesenchymal stem cell, osteoporosis, P38 MAPK signaling, osteogenic differentiation

Introduction

Osteoporosis (OP) is a chronic bone disorder characterized by reduced bone strength, altered bone structure, and a heightened risk of fracture. It is a common manifestation of physiological aging in bone [1-3]. With the aging population and extended life expectancy, the incidence of OP is increasing, posing a significant threat to a global health [4, 5]. Therefore, it is particularly important to define its pathogenesis and safe and effective prevention and treatment drugs.

Bone mesenchymal stem cells (BMSCs) are multipotent adult stem cells derived from bone marrow [6, 7]. The diminished ability of BMSCs to differentiate into osteoblasts (OB) is the primary cause of insufficient OB production, decreased bone formation, and the onset of OP [8, 9]. Therefore, promoting BMSC proliferation and inducing osteogenic differentiation represents a potential therapeutic approach for OP.

Morinda citrifolia is a warm tonic Chinese medicine, widely distributed in nature, and is used to promote the health of the kidneys, bones and muscles, as well as combat wind-dampness [10, 11]. Morinda *officinalis* polysaccharide (MOP), the main bioactive component of *Morinda officinalis*, exhibits various functions such as antioxidation, antidepression, anti-fatigue, anti-inflammation, immunomodulation, and the promotion of angiogenesis and bone

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formation [12, 13]. Research has shown that MOP can reduce oxidative stress in rats through impeding the PGC-1α/PPARγ pathway, increase serum trace elements, and alleviate osteoporosis caused by ovariectomy in rats [14]. Nevertheless, the effect of MOP on BMSC osteogenic differentiation and its precise mechanism remain unclear.

As an important member of the MAPK family, p38 MAPK is widely expressed in various cells and plays a critical role in regulating cell proliferation and apoptosis [15, 16]. Recent studies have highlighted the close relationship between the p38 MAPK pathway and osteogenic differentiation [17, 18]. However, whether MOP also affects BMSC osteogenic differentiation through regulating the p38 MAPK pathway has yet to be explored.

This study aims to investigate the effects of MOP on the biological characteristics of BMSCs, elucidate its impact on osteogenesis-related proteins and genes in BMSCs, and further explore the mechanism by which MOP promotes osteogenic differentiation of BMSC through the p38 MAPK signaling pathway.

Materials and methods

Cell acquisition and culture

BMSCs were isolated from the femurs of 4-week-old healthy male SD rats (Vitalriver Co., LTD., Beijing, China). The SD rats were euthanized by intraperitoneal injection of sodium pentobarbital (100 mg/kg), followed by sterilization using 75% ethanol. Using sterilized surgical scissors and forceps, the bilateral femurs and tibias of rats were carefully dissected, and the surrounding connective tissues were removed. The bones were then rinsed with PBS. The femurs and tibias were cut at the center to expose the marrow cavity. DMEM medium (11965092, Gibco, Grand Island, NY, USA) containing fetal bovine serum (FBS, 10%, A5256701, Gibco) and a penicillin-streptomycin mixture (100 U/mL, 15140122, Gibco) was aspirated using a sterile syringe and injected into one end of the bone. The marrow cavity was repeatedly flushed until the bone was whitish and translucent. The flushed bone marrow fluid was collected into a centrifuge tube and then pipetted repeatedly to make a single-cell suspension. The single-cell suspension was cultured in sterile culture flasks containing DMEM medium (containing FBS and antibiotics) at 37°C in a 5% CO_2 incubator, with the medium replaced every 3 days. When the cells reached 80%-90% confluence, they were digested with 0.25% trypsin (25200114, Gibco) and sub-cultured. The cells were cultured up to the third generation for subsequent experiments.

BMSC isolation and identification

BMSCs at the third passage in the logarithmic phase were digested with trypsin and centrifuged for 5 min. BMSCs were resuspended in PBS at a density of 1×10^6/mL. Surface markers were assessed by incubating the cells with the following antibodies: PE anti-rat CD90 (12-0902-82) and PE anti-rat CD44 (12-0441-82) from Invitrogen (Carlsbad, CA, USA), PE anti-rat CD29 (102207), FITC anti-rat CD45 (202205), and FITC anti-rat CD11b (201805) from BioLegend (San Diego, CA, USA). The mixture was incubated for 30 min, protected from light. Flow cytometry was then employed to assess the expression of BMSC surface marker molecules.

Methyl thiazolyl tetrazolium (MTT) assay

BMSCs at the third passage were digested with trypsin, centrifuged, and seeded in 96-well plates at a density of 1×10^4 cells/well. Once the monolayer of cells covered 90% of the bottom of the wells, the medium was replaced with fresh medium containing MOP (Shifengbio, Shanghai, China) or MOP combined with the p38 MAPK pathway inhibitor SB203580 (2 μg/mL, HY-10256, MedChemExpress, Monmouth Junction, NJ, USA), and incubated for 24 h [19]. After 24 hours, 50 μL/well of MTT (C0009S, Beyotime, Shanghai, China) solution was added, and the plates were incubated for 2 h. Dimethyl sulfoxide (34869, Sigma-Aldrich, St. Louis, MO, USA) was then added, and the mixture was incubated for an additional 10 min to dissolve the formazan crystals. The microplate reader was employed to determine the OD_{570} value of each well, and cell viability was then calculated.

Clone formation assay

A total of 1000 cells were seeded into each well of a 6-well plate and incubated for two weeks

(37°C, 5% CO_2). Once clonal cell clusters were observed, the medium was discarded, and the cells were rinsed with PBS twice. The cells were then fixed with 4% paraformaldehyde (P885233, Macklin Inc., Shanghai, China) for half an hour. After removing the fixative, the cells were stained with 0.1% crystal violet (C776041, Macklin Inc.) for 15 min. Following staining, the cells were washed three times with deionized water, and images were captured for colony counting.

Scratch-wound assay

BMSCs were inoculated in 6-well plates, with horizontal lines drawn in advance. After 24 hours, a 20 μL sterile pipette tip was used to scratch a vertical line intersecting the horizontal line at the bottom of the 6-well plate. The cells were subsequently washed twice with PBS, followed by culture in serum-free DMEM medium supplemented with MOP (40 μg/mL). The plates were incubated at 37°C, and wound healing was observed at 0 and 24 hours.

Alkaline phosphatase (ALP) staining

BMSCs were cultured in DMEM medium for 2 days, followed by osteogenic differentiation in RASMX-90021 osteogenic differentiation medium. After one week, the cells were fixed with 4% paraformaldehyde for 20 min and incubated with BCIP/NBT ALP reagent (C3206, Beyotime) for 20 min. The cells were then rinsed twice with PBS, photographed and observed under a microscope. The OD_{410} value was examined using a microplate reader for quantitative analysis of ALP activity.

Alizarin red staining

BMSCs were cultured in DMEM medium for two days, followed by osteogenic differentiation in RASMX-90021 osteogenic differentiation medium (Cyagen Biosciences, Suzhou, China), with medium changed every two days. After two weeks, the cells were fixed with 4% paraformaldehyde for 20 min. The cells were then stained with alizarin red (A765602, Macklin Inc.) for 30 min, washed with PBS, and observed under a microscope. Calcium nodules were dissolved using sodium dodecyl sulfate (L6026, Sigma-Aldrich), the optical density (OD) at 546 nm was measured to quantify calcium deposition.

Western blot

Proteins were extracted from BMSCs using RIPA lysate (P0013C, Beyotime), and the protein concentration was assessed using a BCA kit (P0011, Beyotime). After gel electrophoresis, the protein samples were transferred to a polyvinylidene fluoride (PVDF) membrane (LC2005, Invitrogen) and blocked for 1 hour. The membrane was then washed and incubated overnight with primary antibodies: osteocalcin (OCN, 33-5400, dilution 1:1000, Invitrogen), runt-related transcription factor 2 (RUNX2, PA5-82787, dilution 1:1000, Invitrogen), ALP (PA5-63148, dilution 1:1000, Invitrogen), collagen I (Col I, MA1-26771, dilution 1:1000, Invitrogen), p-ERK1/2 (44-680G, dilution 1:1000, Invitrogen), ERK1/2 (61-7400, dilution 1:1000, Invitrogen), p-p38 (44-684G, dilution 1:1000, Invitrogen), p38 (33-8700, dilution 1:1000, Invitrogen), p-JNK (sc-6254, dilution 1:1000, Santa Cruz, CA, USA), or JNK (sc-7345, dilution 1:1000, Santa Cruz). Subsequently, the membrane was incubated with goat anti-rabbit secondary IgG (31460, dilution ratio 1:10,000, Invitrogen) for 1.5 h. GAPDH (MA1-16757, dilution 1:1000, Invitrogen) was used as the internal reference. The grey value of the bands was assessed using Image J software.

RT-qPCR

Log-phase BMSCs were seeded in 6-well plates, and after cell adhesion, 1 mL of TRIzol reagent (15596018CN, Invitrogen) was added to each well for RNA extraction. cDNA was synthesized using AMV reverse transcriptase (10109118001, Sigma-Aldrich) for reverse transcription. PCR amplification was accomplished using the SYBR Green qPCR Mix kit (TaKaRa, Tokyo, Japan). Relative quantification of target genes was determined by the $2^{-\Delta\Delta Ct}$ method, with GAPDH serving as the endogenous control. The primer sequences used are listed below: OCN: F: 5'-GGAGTGGCCTCTCTGGGTTG-3', R: 5'-CAGCCAACTCGTCACAGTCC-3'; RUNX2: F: 5'-AGGAGTCCTGCCTCTTGTCT-3', R: 5'-AGGGGAGTACTTACTTCTGGGT-3'; ALP: F: 5'-ACCCTCCTACCGGAACTGAA-3', R: 5'-TTGGTGTCATGGGCTCAGTC-3'; Col I: F: 5'-CAGGAGATTACCTCGACGCC-3', R: 5'-AAAGGTGGTTAAGCTGGGGG-3'; GAPDH: F: 5'-GTGGCTGGCTCAGAAAAAGG-3', R: 5'-GGGGAGATTCAGTGTGGTGG-3'.

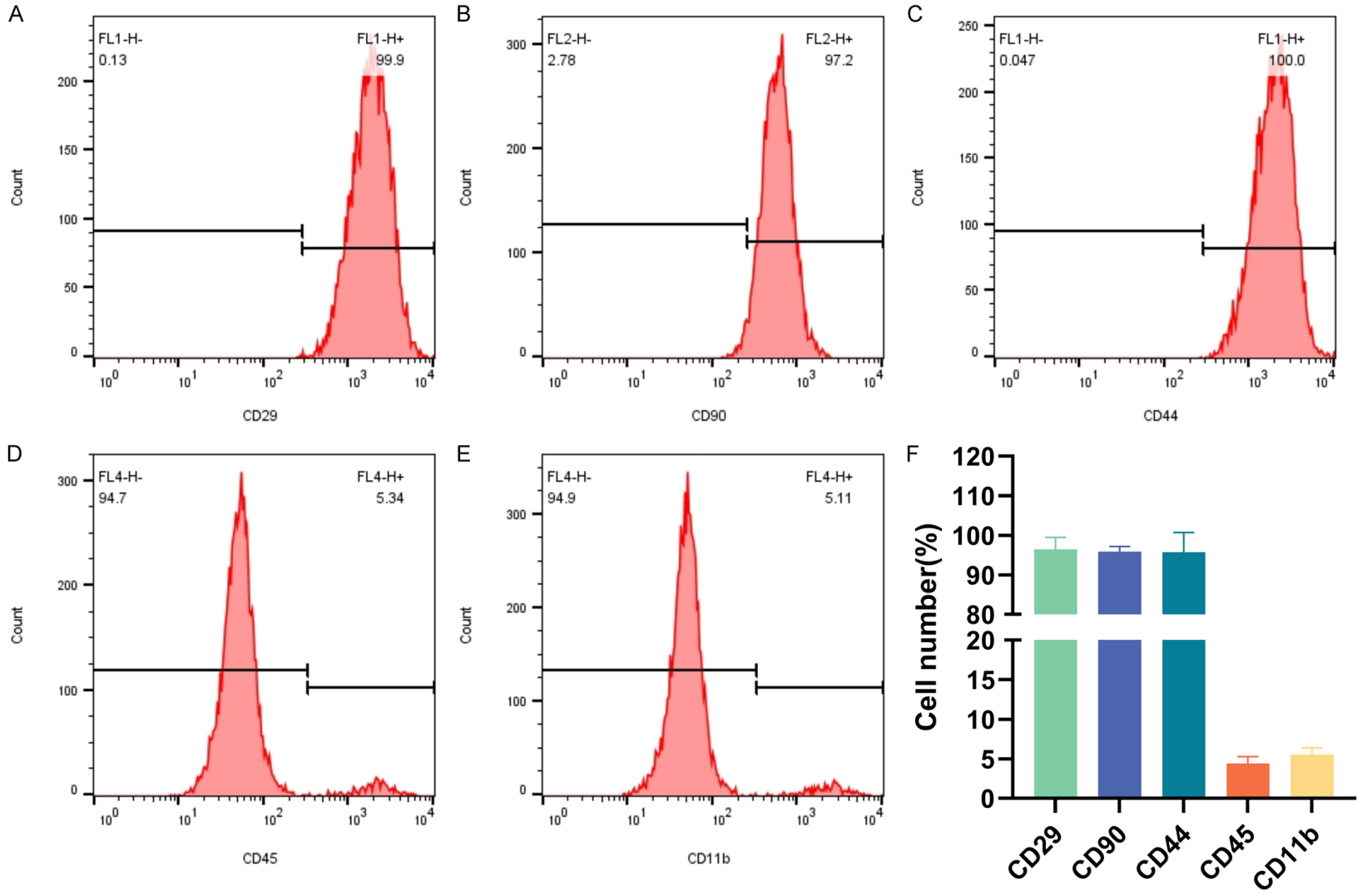

Figure 1. BMSC isolation and characterization. A-E. CD29, CD90, CD44, CD45 and CD11b levels were assessed by flow cytometry. F. The quantitative results of CD29, CD90, CD44, CD45, and CD11b contents.

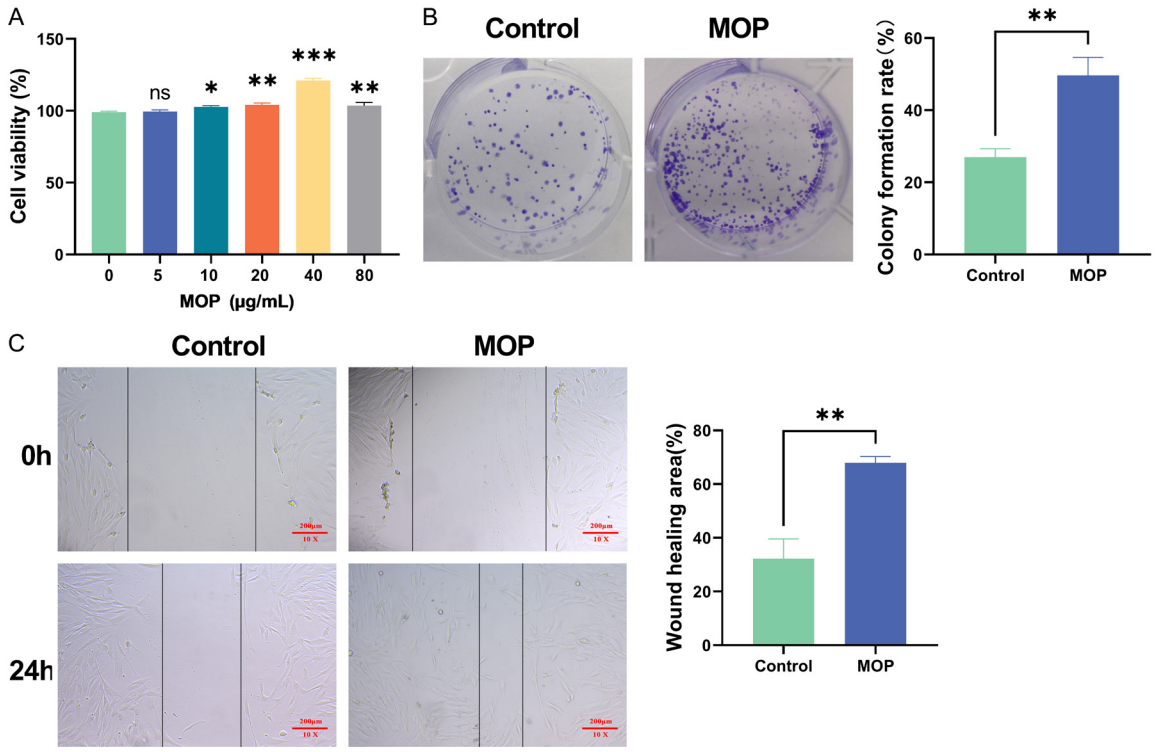

Figure 2. Effects of MOP on BMSC proliferation and migration. A. BMSC viability evaluated using Methyl thiazolyl tetrazolium (MTT) assay. B. BMSC clone formation detected using the colony formation assay. C. BMSC migration evaluated using Scratch-wound assay (10×, 200 μm). *P<0.05, **P<0.01, ***P<0.001.

Statistical analysis

Each experiment was run at least three times, and the outcomes were reported as mean ± standard deviation. Statistical analyses were performed using the SPSS 26.0 (IBM, Armonk, NY, USA). The Student's *t*-test was utilized for normally distributed data with equal variances. For normally distributed data with unequal variances, the Welch's corrected t-test was applied. Nonparametric Wilcoxon rank-sum test was performed when data did not meet the normality assumption. A *p*-value of <0.05 was considered significant. Graphs were generated using Prism software (Graphpad 9.0, La Jolla, CA, USA).

Results

BMSC isolation and characterization

BMSCs were characterized by flow cytometry, which showed positive expression of CD44, CD90, and CD29, and negative expression of CD11b and CD45 (**Figure 1A-E**). In addition, the expression rates of CD29, CD90, CD44, CD45, and CD11b were 96.50%, 95.90%, 95.72%, 4.42%, and 5.54%, respectively (**Figure 1F**). These results confirm that the isolated BMSCs exhibited typical mesenchymal stem cell immunophenotypes, with high expression of mesenchymal stem cell markers and low expression of hematopoietic cell markers.

MOP promoted the proliferation of BMSCs

MTT assay was used to ascertain the impacts of MOP (5, 10, 20, 40 and 80 μg/mL) on BMSC cell viability. The results showed that 40 μg/mL of MOP significantly enhanced BMSC viability (**Figure 2A**), which was selected as the optimal concentration for subsequent experiments. Clone formation assay demonstrated a marked increase in the clone formation rate of BMSCs after MOP treatment (**Figure 2B**). Additionally, the scratch-wound assay revealed that MOP treatment significantly accelerated the scratch healing rate of BMSCs, indicating an enhancement in BMSC migration (**Figure 2C**). These findings indicate enhanced proliferation and migration of BMSCs following MOP intervention.

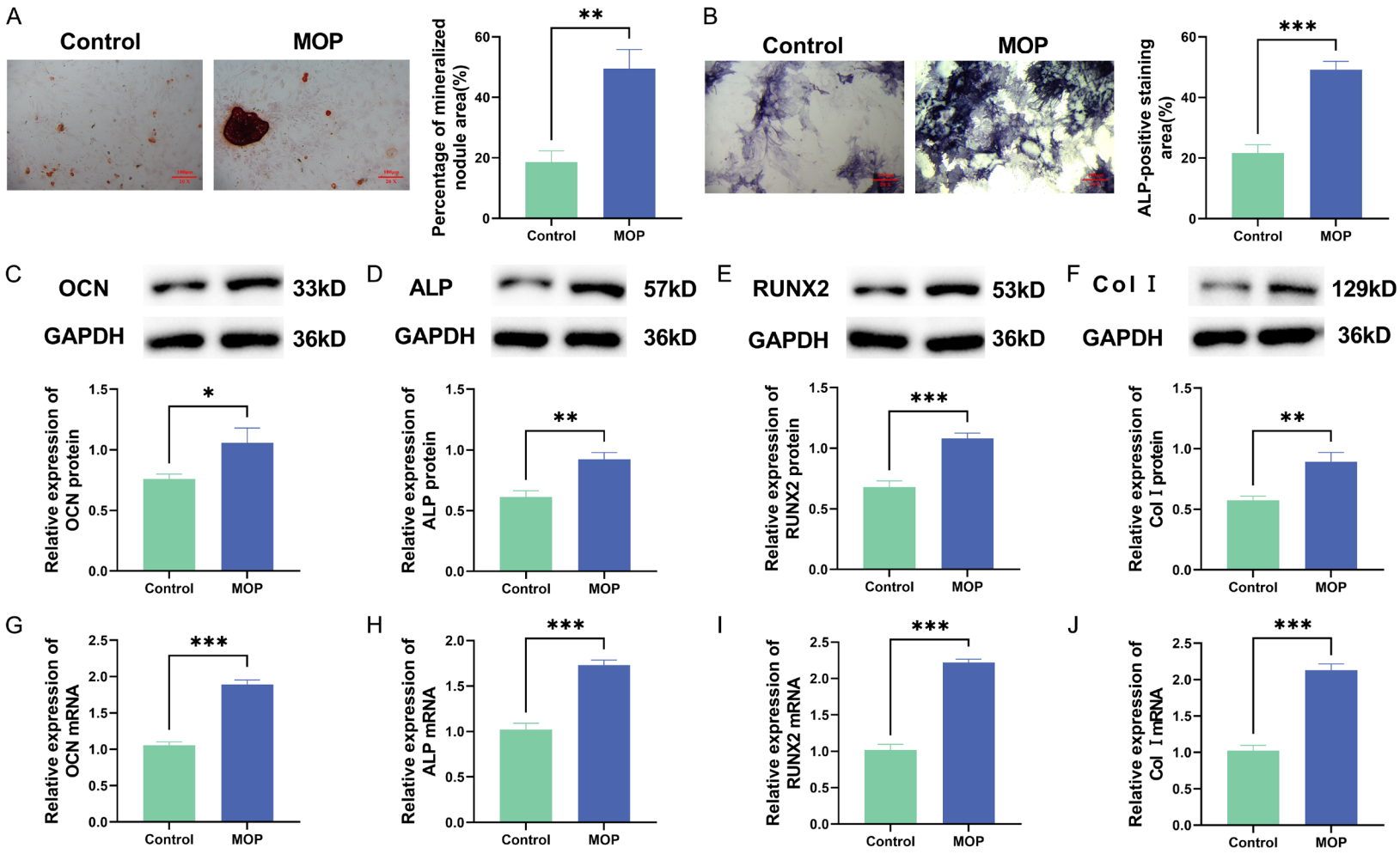

Figure 3. Effects of MOP on the osteogenic differentiation of BMSCs. A. Alizarin red staining for detection of calcium deposits (20×, 100 μm). B. Alkaline phosphatase (ALP) staining for detection of enzyme activity (20×, 100 μm). C-F. The protein expression levels of osteocalcin (OCN), runt-related transcription factor 2 (RUNX2), ALP, and collagen I (Col I) in BMSCs were examined using western blot. G-J. The mRNA expression levels of OCN, ALP, RUNX2 and Col I in BMSCs assessed using RT-qPCR. **P*<0.05, ***P*<0.01, ****P*<0.001.

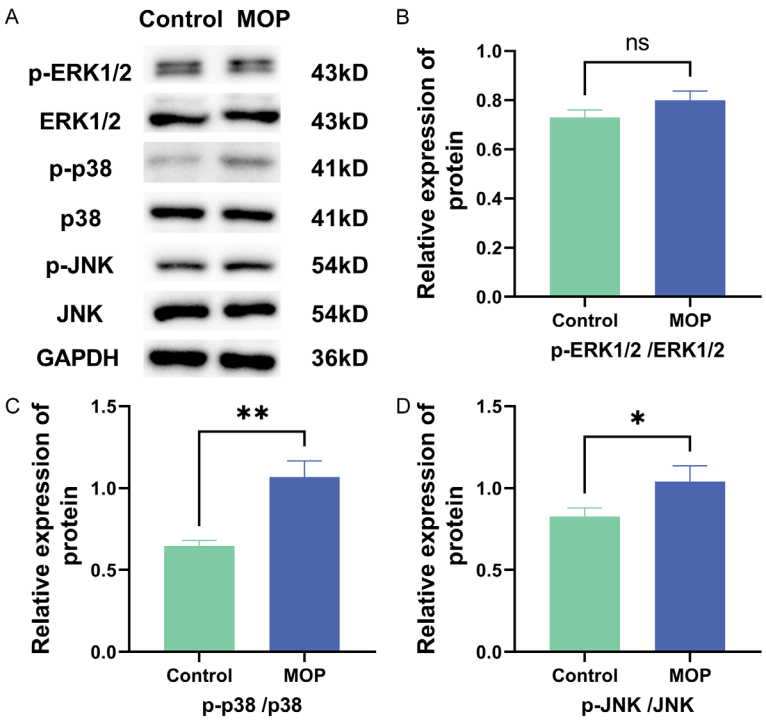

Figure 4. Effects of MOP on MAPK signaling pathway. A. Phosphorylation of p38, ERK1/2, and JNK in BMSCs analyzed using western blotting. B. Quantitative results of ERK1/2 phosphorylation. C. Quantitative results of p38 phosphorylation. D. Quantitative results of JNK phosphorylation. *P<0.05, **P<0.01.

MOP promoted osteogenic differentiation of BMSCs

Alizarin red staining and subsequent quantitative analysis showed a significant increase in the number and volume of mineralized nodules after MOP treatment compared to the control group, indicating enhanced osteogenic differentiation of BMSCs (**Figure 3A**). Similarly, the ALP positivity rate of BMSC was significantly increased after MOP treatment, indicating that MOP enhanced the osteogenic differentiation of BMSC (**Figure 3B**). Western blot analysis revealed a significant upregulation of osteogenesis-related proteins, including OCN, RUNX2, Col I and ALP (**Figure 3C-F**). Additionally, mRNA expression levels of OCN, ALP, RUNX2 and Col I were also markedly elevated (**Figure 3G-J**). Collectively, these findings indicate that MOP promotes the osteogenic differentiation of BMSCs.

Effects of MOP on MAPK signaling pathway

Western blot results showed that MOP treatment increased the phosphorylation levels of ERK1/2, p38 and JNK in BMSCs (**Figure 4A**). Quantitative analysis revealed that the p38 phosphorylation level in BMSC was significantly heightened after MOP treatment, indicating that MOP treatment activates the P38 MAPK signaling pathway (**Figure 4B-D**).

MOP promoted BMSC proliferation through activating the P38 MAPK pathway

MTT assay results revealed that the addition of p38 pathway inhibitor SB203580 significantly inhibited BMSC viability (**Figure 5A**). In the presence of SB203580, the clone formation rate of BMSCs was markedly reduced compared to the MOP-treated group (**Figure 5B**, **5C**). Additionally, the scratch healing rate of BMSCs following SB203580 treatment was significantly reduced, suggesting that the inhibitor impaired BMSC migration (**Figure 5D**, **5E**). In addition, SB203580 treatment alone significantly reduced BMSC cell viability and inhibited their proliferation and migration. These findings imply that the p38 MAPK pathway plays a crucial role in MOP-mediated promotion of BMSC viability, proliferation, and migration.

MOP enhanced BMSC osteogenic differentiation by activating the P38 MAPK pathway

Alizarin red staining and subsequent quantitative analysis indicated a decrease in the number and volume of mineralized nodules after MOP+B203580 treatment compared with the MOP- only group, indicating weakened osteogenic differentiation of BMSCs (**Figure 6A**, **6B**). Similarly, the ALP positivity rate was significantly reduced in BMSCs after MOP+SB203580 treatment, further suggesting that the inhibitor impaired osteogenic differentiation (**Figure 6C**, **6D**). Expression levels of osteogenesis-related proteins, including OCN, ALP, RUNX2, and Col I, were significantly reduced after MOP+SB203580 treatment (**Figure 6E-I**). Furthermore, mRNA expression levels of OCN, ALP,

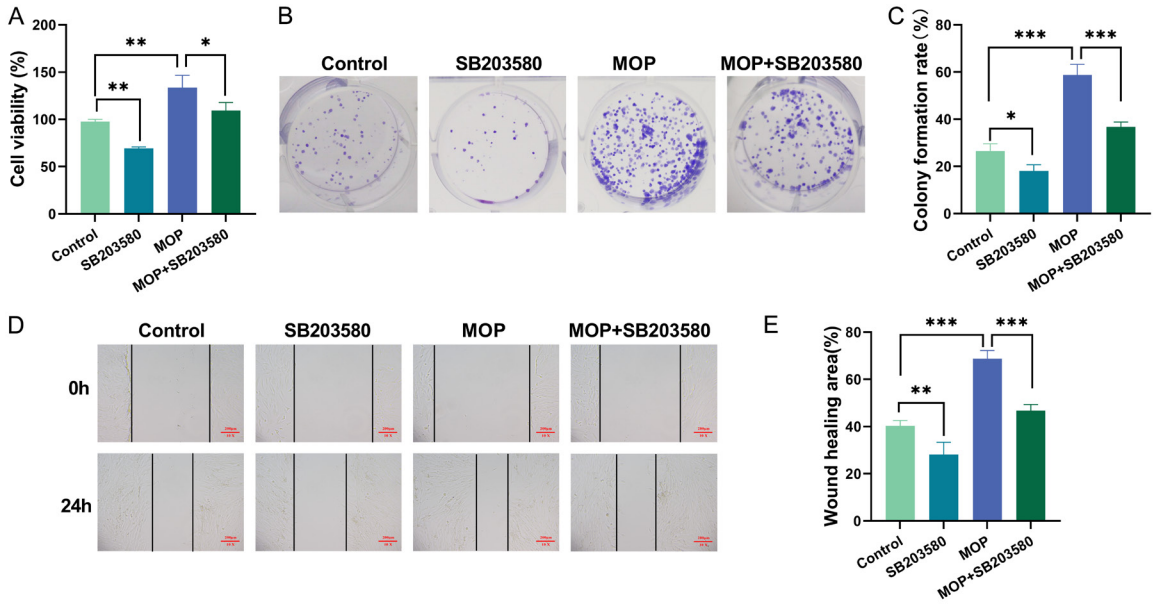

Figure 5. Effects of P38 MAPK signaling pathway inhibitor and MOP on MSC proliferation. A. BMSC viability evaluated using MTT assay. B, C. BMSC clone formation rate evaluated using colony formation experiment (10×, 200 μm). D, E. BMSC migration assessed using wound-healing assay. *P<0.05, **P<0.01, ***P<0.001.

RUNX2, and Col I were also noticeably decreased after MOP+SB203580 treatment (**Figure 6J-M**). In addition, SB203580 treatment alone also markedly reduced BMSC cell osteogenic differentiation. Collectively, these findings confirm that MOP stimulates osteogenic differentiation of BMSC by activating the P38 MAPK pathway.

Discussion

Bone marrow stem cells (BMSCs) are precursor cells of osteoblasts (OBs) in the bone marrow, and reduction in their osteogenic differentiation leads to reduced bone formation, which is a key contributor to the pathogenesis of OP [20-22]. In recent years, the study of the osteogenic differentiation capacity of BMSC has become a hotspot in OP pathogenesis research. The isolation and culture of BMSCs with high purity is crucial for further study. However, because BMSCs do not have unique surface antigens, no specific molecules for BMSCs have been definitively identified to date [23, 24]. It is generally accepted that CD29, CD44, CD166, and CD105 are important markers of BMSCs [25]. In contrast, BMSCs lack surface antigens present on hematopoietic stem cells, such as CD34 (a precursor cell marker), CD38 (a marker for mature hematopoietic cells), and CD45 (a leukocyte marker). In this study, flow cytometry was utilized to identify BMSC surface antigens, yielding a positive expression of CD29, CD90 and CD44, with a positive rate of more than 95%; conversely, CD11b and CD45 were negative for expression, confirming that the cells cultured in this experiment were BMSCs, not hematopoietic stem cells, consistent with the report of Zhao *et al*. [20].

Bone metabolism is a dynamic process where osteoblasts and osteoclasts play critical roles in bone formation and bone resorption, respectively, maintaining a balance essential for bone health. Disruption of this balance can lead to bone damage and even bone disease [26, 27]. Previous studies have shown that MOP can regulate the osteogenic-lipogenic differentiation of rat BMSCs by upregulating miR-21 and activating the PI3K/AKT pathway, demonstrating its potential for preventing osteoporosis induced by ovariectomization in rats [28]. Additionally, MOP can stimulate the growth and division of osteoblasts while suppressing osteoclast formation, thus regulating bone resorption and bone formation, contributing to its anti-osteoporosis effect [29]. Lai *et al.* reported that 40 μg/mL MOP promoted osteogenic differentiation in BMSCs [30]. In contrast, 10 μg/mL MOP exerted weaker regulatory effects,

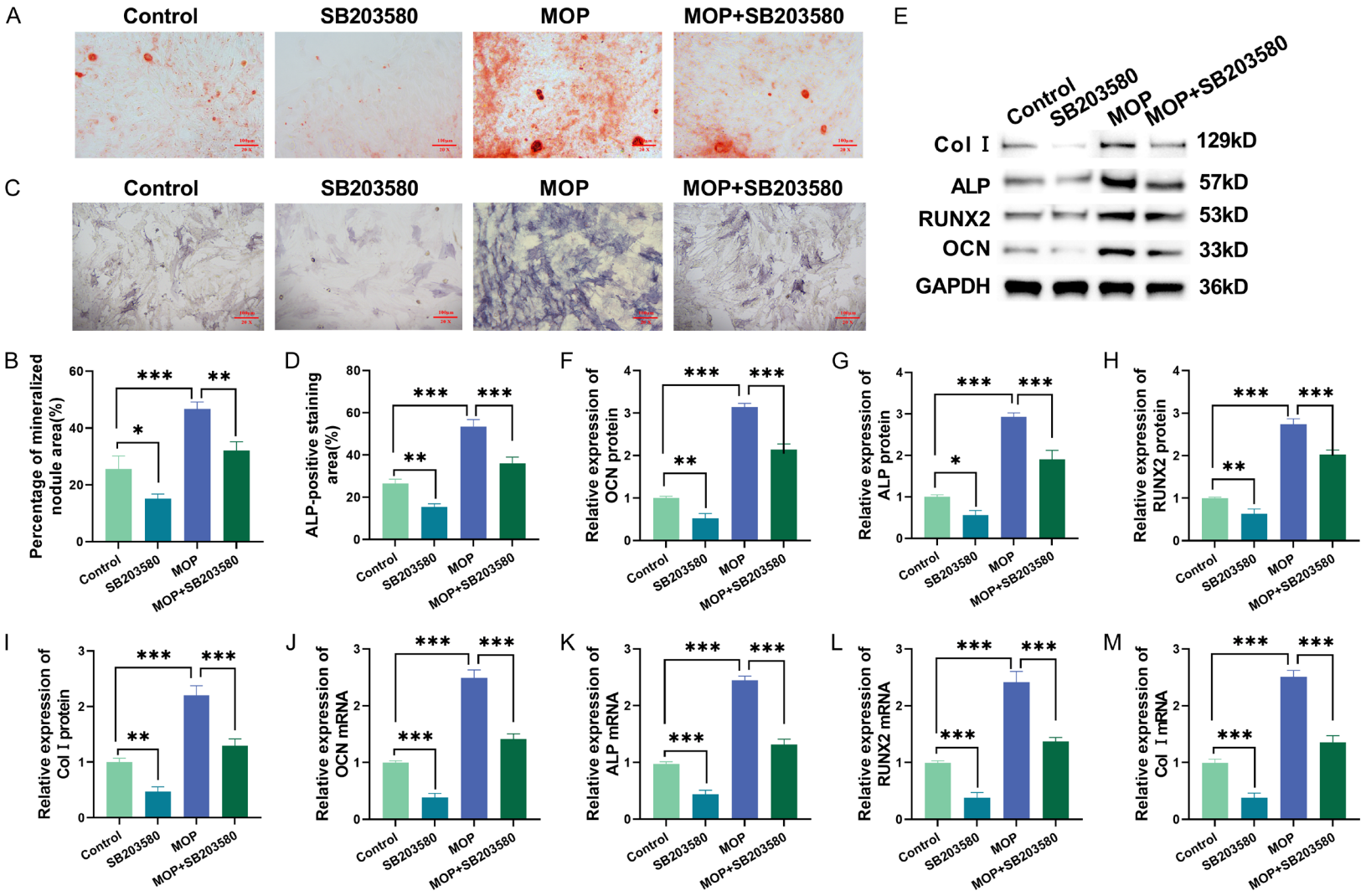

Figure 6. Effect of P38 MAPK signaling pathway inhibitor and MOP on BMSC osteogenic differentiation. A, B. Alizarin red staining for detection of calcium deposits (20×, 100 μm). C, D. Alkaline phosphatase (ALP) staining for detection of enzyme activity (20×, 100 μm). E-I. The protein expression levels of collagen I (Col I), osteocalcin (OCN), runt-related transcription factor 2 (RUNX2) and ALP levels examined using western blot. J-M. The mRNA expression levels of OCN, ALP, RUNX2, and Col I in BMSCs assessed using RT-qPCR. **P*<0.05, ***P*<0.01, ****P*<0.001.

with less pronounced promotion of osteogenesis compared to the 50 μg/mL concentration. In our study, we examined the effects of MOP at 5-80 μg/mL on BMSC cell viability and found that 40 μg/mL was the optimal concentration for promoting BMSC proliferation.

The osteogenic differentiation of BMSCs is critically dependent on the ability of these cells to synthesize and secrete osteogenic-specific proteins and extracellular matrix components [31, 32]. Key proteins involved in early osteogenic differentiation include ALP, Col I, and osteogenic proteins, while OCN and bone salivary proteins appear later during the process, among which ALP and OCN are two critical markers used to assess osteogenesis [33, 34]. ALP is an essential enzyme for OB differentiation and maturation, with its expression increasing as osteogenic differentiation progresses [35]. OCN, also known as bone gla protein, is the only non-collagenous protein synthesized and secreted by OBs and is the most abundant protein in bone tissue [36]. Activation of RUNX2 is a hallmark of the initiation of osteoblast differentiation and is one of the primary members of the RUNX family. High RUNX2 levels stimulate the secretion and maturation of osteoblasts, regulating the expression of a variety of markers specific to osteoblast proliferation and differentiation [37]. Our research uncovered a notable increase in the expression of osteogenesis-related proteins and genes, including OCN, ALP, RUNX2, and Col I, following MOP treatment. This suggests that MOP effectively promoted BMSC osteogenic differentiation.

Osteogenic differentiation of BMSCs is a complex process regulated by multiple signaling pathways, among which MAPK pathway plays a critical role [20, 38]. Research has shown that the ERK, p38, and JNK pathways within the MAPK family significantly influence BMSC differentiation and proliferation [39]. Lin *et al.* found that shikonin activates the p38 MAPK pathway, promoting BMSC osteogenic differentiation and potentially improving periodontal bone defects in rats [40]. However, whether MOP can regulate the P38 MAPK signaling pathway remains unclear. In this study, MOP treatment caused a notable increase in p38 phosphorylation levels in BMSCs, indicating activation of the P38 MAPK pathway. This suggests that P38 MAPK may play a role in regulating BMSC osteogenic differentiation by MOP. To further investigate the contribution of the p38 MAPK signaling pathway to BMSC osteogenic differentiation, the p38 pathway inhibitor SB203580 was added in this study for verification. The findings illustrated that p38 pathway inhibitors inhibited the promotion of MOP on BMSC cell proliferation, migration, and osteogenic differentiation.

Conclusion

MOP enhanced BMSC proliferation, clone formation, and migration by activating the P38 MAPK signaling pathway. While the findings suggest that MOP promotes osteogenic differentiation and migration of BMSCs through the p38 MAPK pathway *in vitro*, the complex *in vivo* physiologic milieu and systemic regulatory networks, including cellular crosstalk, immune responses, and metabolic pathways, were not investigated in this study. Future studies should focus on investigating the role of MOP in enhancing osteogenic differentiation in animal models.

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Disclosure of conflict of interest

None.

Address correspondence to: Jiwei Lin, Prevention and Treatment Center, Shenzhen Hospital of Traditional Chinese Medicine, No. 1, Fuhua Road, Futian District, Shenzhen 518000, Guangdong, China. Tel: +86-0755-23484542; E-mail: linjiwei2023@126.com

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