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Original Article

Original Article

miR-126 mitigates the osteogenic differentiation of human bone marrow-derived mesenchymal stem cells by targeting the ERK1/2 and Bcl-2 pathways

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Abstract

Human bone marrow mesenchymal stem cells (hBMMSCs) are a promising cell source for bone engineering owing to their high potential to differentiate into osteoblasts. The objective of the present study is to assess microRNA-126 (miR-126) and examine its effects on the osteogenic differentiation of hBMMSCs. In this study, we investigate the role of miR-126 in the progression of osteogenic differentiation (OD) as well as the apoptosis and inflammation of hBMMSCs during OD induction. OD is induced in hBMMSCs, and matrix mineralization along with other OD-associated markers are evaluated by Alizarin Red S (AR) staining and quantitative PCR (qPCR). Gain- and loss-of-function studies are performed to demonstrate the role of miR-126 in the OD of hBMMSCs. Flow cytometry and qPCR-based cytokine expression studies are performed to investigate the effect of miR-126 on the apoptosis and inflammation of hBMMSCs. The results indicate that miR-126 expression is downregulated during the OD of hBMMSCs. Gain- and loss-of function assays reveal that miR-126 upregulation inhibits the differentiation of hBMMSCs into osteoblasts, whereas the downregulation of miR-126 promotes hBMMSC differentiation, as assessed by the determination of osteogenic genes and alkaline phosphatase activity. Furthermore, the miR-126 level is positively correlated with the production of inflammatory cytokines and apoptotic cell death. Additionally, our results suggest that miR-126 negatively regulates not only B-cell lymphoma 2 (Bcl-2) expression but also the phosphorylation of extracellular signal-regulated protein kinase (ERK) 1/2. Moreover, restoring ERK1/2 activity and upregulating Bcl-2 expression counteract the miR-126-mediated suppression of OD in hBMMSCs by promoting inflammation and apoptosis, respectively. Overall, our findings suggest a novel molecular mechanism relevant to the differentiation of hBMMSCs into osteoblasts, which can potentially facilitate bone formation by counteracting miR-126-mediated suppression of ERK1/2 activity and Bcl-2 expression.

Key words Bcl-2, ERK1/2, hBMMSCs, miR-126, osteogenic differentiation

Introduction

Osteoporosis is characterized by degenerating bone structure and reduced bone mass, which ultimately leads to severe bone fragility and elevated risks of fracture [1,2]. It is classified into primary osteoporosis, which comprises type 1 and type 2 (also termed postmenopausal and senile osteoporosis, respectively), and secondary osteoporosis (*e.g.*, glucocorticoid- or steroid-caused osteoporosis). Osteoporosis results from the imbalance of bone

generation and the degeneration process in the microenvironment of bone marrow, which is modulated by osteoblasts [3,4]. Bone marrow-derived mesenchymal stem cells (BMMSCs) belong to a class of multipotent stem cells that can differentiate into osteoblasts via osteogenesis—a series of complicated reactions that regulate the phenomenon of differentiation [5,6]. In humans, the generation of different phenotypes of BMMSCs (hBMSCs) is modulated by different kinds of growth factors and signaling pathways that are often associated with a common precursor existing in fat cells, bone marrow, etc. [7]. Therefore, delineating the mechanism underlying BMMSC osteogenic differentiation (OD) will be beneficial for the development of new BMMSC-based treatment modalities for patients with fractures or bone loss.

MicroRNAs belong to the conservative class of small RNAs consisting of 20-22 nucleotides, which are capable of regulating gene expression posttranscriptionally by interacting with the 3 - UTR of its target gene mRNA. The interaction then leads to mRNA degradation and translational suppression [8]. Emerging facts confirmed that miRNAs evidently influence diverse biological processes, including osteoporosis [9]. For instance, miR-214 expression is significantly reduced during the activation of osteogenesis. miR-214 overexpression has been reported to suppress the expression of bone morphogenetic protein 2 (BMP2), while KCNQ1 opposite strand/antisense transcript 1 (KCNQ1OT1) enhances BMP2 expression by sponging miR-214 to promote the OD of hBMMSCs [10]. Moreover, miR-21 promotes the migration and OD of hBMMSCs by increasing p-Akt and activating hypoxia-inducible factor (HIF)-1α. In addition, a study demonstrated that human mesenchymal stem cells modified with the miR-21/β-tricalcium phosphate complex exhibit osteogenesis in the repair of major size defects [11]. Interestingly, miR-126 is highly enriched in cardiac tissues [12] and implicated in various cardiovascular diseases [13,14]. However, its function in osteoporosis remains unclear. Previous studies have demonstrated that inhibiting miR-126 causes the activation of extracellular signal-regulated protein kinase (ERK) 1/2 and transcriptional upregulation of B-cell lymphoma 2 (Bcl-2) [15,16], which acts as a key modulator of OD [17,18]. Therefore, we hypothesized that miR-126 might participate in the progression of OD by regulating the functions of BMMSCs via ERK1/2- and Bcl-2-associated inflammation and apoptosis. Hence, investigating the effect of miR-126 on the OD, inflammation, and apoptosis of hBMMSCs might provide additional information about the potential of miR-126 as a promising target in the development of innovative therapeutics for osteoporosis. Kong *et al.* [19] have already shown that miR-126 promotes the proliferation, migration, invasion, and endothelial differentiation, while it inhibits the apoptosis and OD of bone marrow-derived mesenchymal stem cells. However, detailed mechanism for how miR-126 regulates hBMMSCs requires further investigation and confirmation.

In this study, induction of OD in hBMMSCs was used as the experimental model for investigating the role of miR-126 in the OD of hBMMSCs and its potential molecular mechanism. We found that miR-126 was decreased in hBMMSCs during OD induction. Upregulation of miR-126 led to reduced OD progression and induced inflammation and apoptosis of hBMMSCs, while down-regulated miR-126 showed the opposite effect. Bcl-2 and ERK1/2, two key targets of miR-126, accounted for the facilitative role of miR-126 in apoptosis and inflammation, respectively.

Materials and Methods

hBMMSC culture and induction of OD

Telomerase-immortalized hBMMSCs were obtained from primary hBMMSCs as described previously [20] and cultivated in DMEM (Invitrogen, Waltham, USA) containing 20% FBS (Gibco, Waltham, USA), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen). The OD of hBMMSCs was induced according to the procedures reported in a previous study [21]. Briefly, mineralization-inducing media (Invitrogen) containing 100 μM ascorbic acid, 2 mM β-glycerophosphate, and 10 nM dexamethasone was used for osteogenic induction in BMMSCs. Osteoblasts were characterized by examining alkaline phosphatase (ALP) activity after 14 days of osteogenic induction. Matrix mineralization was evaluated by Alizarin red S (AR) staining.

AR staining

The culture medium was removed, and the cells were rinsed with PBS twice prior to being fixed in paraformaldehyde (PFA, 4%) for 25 min. After fixation with PFA, the cells were rinsed twice with distilled water and air dried. The cells were then incubated with AR stain solution (1%, pH 6.3; Sigma-Aldrich, St Louis, USA) at 37°C for 15 min for the detection of calcium. The cells were subsequently rinsed with distilled water twice and incubated with HCl (1 mL, 1%) in EtOH (70%) at 4°C for 60 min [22]. The resultant supernatant solutions (10 μL) were subsequently added into 96-well assay plates (Sumilon, Utsunomiya, Japan), and the absorbance was determined at 450 nm using a Microplate Reader Model 680 for quantitative analysis (Bio-Rad Laboratories, Hercules, USA).

Cell transfection

For miR-126 up- or downregulation, a total of 2.0×10^5 hBMMSCs in a 6-well plate were transfected with agomiR-126 (5′-CAUUAUUACU UUUGGUACGCG-3′)/NC (5′-UCACAACCUCCUAGAAAGAGUAGA-3′) (50 nM; Ribobio, Guangzhou, China) or antagomir-126 (5′-CAUUAUUACUUUUGGUACGCG-3′)/NC (5′-UUGUACUACACAAA AGUACUG-3′) (50 nM; Ribobio) at 60%–80% confluency and incubated for 36–48 h. qPCR was used to verify the transfection efficiency. Additionally, the overexpression vectors pcDNA3-ERK and pcDNA3-Bcl-2a (Genscript, Suzhou, China) were constructed and used for further experiments. The pcDNA3-empty vector (Genscript) served as the negative control. BMMSCs were transfected with these constructs using Lipofectamine 2000 (Invitrogen) at day 3 post OD stimulation.

Western blot analysis

The hBMMSC lysates were extracted using radioimmunoprecipitation assay buffer (Thermo Fisher Scientific, Waltham, USA), and protein quantification was performed using a BCA protein estimation kit (Thermo Fisher Scientific). Subsequently, the proteins were resolved by 10% SDS-PAGE and transferred to PVDF membranes (Thermo Fisher Scientific). The membranes were blocked with BSA (5%; Sigma) dissolved in PBS containing 0.02% Tween 20 for 60 min. The membrane was initially incubated with primary antibodies at room temperature for 1 h, followed by incubation with secondary antibodies at 4°C overnight. The antibodies used are listed as follows: anti-OCN (ab93876, 1:2000; Abcam, Cambridge, UK), anti-OPN (ab8448 1:2000; Abcam), anti-RUNX2 (ab192256, 1:500, Abcam), anti-ALP (ab67228, 1:2000; Abcam), anti-BSP (ab52128, 1:2000; Abcam), anti-Bcl-2 (ab59348, 1:5000;

Abcam), anti-p-ERK1/2 (ab214362, 1:500; Abcam), anti-ERK1/2 (ab54230, 1:1000; Abcam), anti-β-actin (ab8227, 1:5000; Abcam), and HRP-conjugated goat anti-mouse/rabbit (ab6789/ab6721, 1:5000; Abcam). Protein bands were detected using Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific), and gray images and values were recorded using a Blot Scanner (LiCor, Lincoln, USA).

RNA extraction and qPCR

Total RNA was extracted from the pelleted cells using TRIzol reagent (Thermo Fisher Scientific), followed by DNase treatment. The RNA was subsequently quantified using a NanoDrop™ 2000 (Thermo-Fisher Scientific). To analyse the expression of miR-126, the TaqMan MicroRNA assay kit (Thermo Fisher Scientific) was used following the protocol supplied by the manufacturer. The expression level of miR-126 was normalized to that of *U6* snRNA as the internal control. To analyse the expression of mRNAs, cDNA was synthesized using the PrimerScript™ RT Reagent kit (Takara, Shiga, Japan), and qPCR was performed using the FastStart Universal SYBR Green Master kit (Roche Diagnostics GmbH, Mannheim, Germany) following the protocol provided by the manufacturer. The expression level of target mRNAs was normalized to that of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) as the internal control. Briefly, qPCR was conducted in a total reaction mix volume of 20 μL. The thermal amplification program was set as follows: initial denaturation for 10 min at 95°C and 35 cycles of denaturation for 15 s at 95°C, annealing for 30 s at 60°C, and extension for 30 s at 72°C. The sequences of primers were shown in Table 1. The expression levels were calculated using the $2^{-\Delta\Delta Ct}$ analysis method.

Determination of ALP activity

hBMMSCs were lysed with 0.05% Triton X-100, and total protein extracts were obtained through centrifugation at 10,000 *g* for 15 min. ALP activity was evaluated as per the relevant instructions. Briefly, the protein extract was mixed with ALP chromogenic substrate (50 μL) and its reaction solution (ab83369; Abcam). After incubation, the absorbance of the reaction product was measured at 405 nm with a SpectraMax M5 microplate reader (Molecular Devices, San Jose, USA), and the ALP enzyme activity was determined.

Flow cytometry

The apoptosis of hBMMSCs was assessed by flow cytometry using an Annexin V-FITC/PI apoptosis detection kit (BD Pharmingen™, San Jose, USA). Briefly, the cells were resuspended in binding buffer (20 μL) and then incubated with Annexin V-FITC (10 μL) and PI (5 μL). The rate of apoptosis was then determined with a flow cytometer (ZE5 Cell Analyzer; Bio-Rad).

Statistical analysis

Data are presented as the mean ± SD. All experiments were repeated at least three times with triplicate samples in each assay. Two-tailed *t*-test or ANOVA was used to evaluate the differences between the different experimental groups. $P < 0.05$ was considered statistically significant.

Results

miR-126 expression is decreased during the OD of hBMMSCs

To investigate the expression of various genes associated with OD, hBMMSCs were isolated from human bone marrow tissues and cultivated. Subsequently, the cells were subject to the induction of OD by incubating them in a medium that could induce osteoblast generation. At 0, 7, 14, and 28 days post induction, the expressions of OD-related genes, including osteocalcin (*OCN*), osteopontin (*OPN*), RUNX family transcription factor 2 (*RUNX2*), ALP, and bone sialoprotein (*BSP*) were evaluated and found to be dramatically increased at both the transcription and protein levels during the OD of hBMMSCs (Figure 1A–F). ALP activity in the OD-induced cells, which is a typical characteristic of the osteoblastic phenotype, was also increased relative to that in the control group (Figure 1G). AR staining was also utilized to confirm the OD of hBMMSCs (Figure 1H). In addition, qPCR results indicated a significant downregulation of miR-126 expression at days 14 and 28 after OD induction (Figure 1I). These data indicated that miR-126 expression is decreased during the OD of hBMMSCs.

Table 1. The sequences of the primers used in this study

Target	Primer sequence
GAPDH	F, 5′-GTCTCCTCTGACTTCAACAGCG-3′
	R, 5′-ACCACCCTGTTGCTGTAGCCAA-3′
OCN	F, 5′-TCATGTCCAAGCAGGAGGGCAGTAA-3′
	R, 5′-TTGTAGGCGTCCTGGAAGCCAATGT-3′
OPN	F, 5′-CCCGATGCCACAGATGAG-3′
	R, 5′-TCCCGTTGCTGTCCTGAT-3′
RUNX2	F, 5′-CCCAGTATGAGAGTAGGTGTCC-3′
	R, 5′-GGGTAAGACTGGTCATAGGACC-3′
ALP	F, 5′-CCTGATGGAGATGACAGAGGCT-3′
	R, 5′-TCAGTGAGTGCCTGGTAAGCCA-3′
BSP	F, 5′-AGAAAGAGCAGCACGGTTGAGT-3′
	R, 5′-GACCCTCGTAGCCTTCATAGCC-3′
miR-126	F, 5′-GGCTCGTACCGTGAGTAAT-3′
	R, 5′-GTGCAGGGTCCGAGGT-3′
IL-1β	F, 5′-CCACAGACCTTCCAGGAGAATG-3′
	R, 5′-GTGCAGTTCAGTGATCGTACAGG-3′
IL-6	F, 5′-AGACAGCCACTCACCTCTTCAG-3′
	R, 5′-TTCTGCCAGTGCCTCTTTGCTG-3′
TNF-α	F, 5′-CTCTTCTGCCTGCTGCACTTTG-3′
	R, 5′-ATGGGCTACAGGCTTGTCACTC-3′
Bcl-2	F, 5′-CATTTCCACGTCAACAGAATTG-3′
	R, 5′-AGCACAGGATTGGATATTCCAT-3′
ERK	F, 5′-ACAGGCTGTTCCCAAATGCT-3′
	R, 5′-CGAACTTGAATGGTGCTTCG-3′

miR-126 suppresses the OD of hBMMSCs

To investigate the role of miR-126 in the OD of hBMMSCs, cells were transfected with agomiR-126 to increase the intracellular miR-126 level. qPCR results demonstrated that miR-126 expression was considerably upregulated after transfection with agomiR-126 (Figure 2A). Subsequent assessment of OD-related gene expression by qPCR revealed that the levels were significantly downregulated in the OD-induced cells concomitant with miR-126 upregulation (Figure 2B–G). Additionally, the ALP activity in the hBMMSCs was significantly decreased with miR-126 upregulation (Figure 2H).

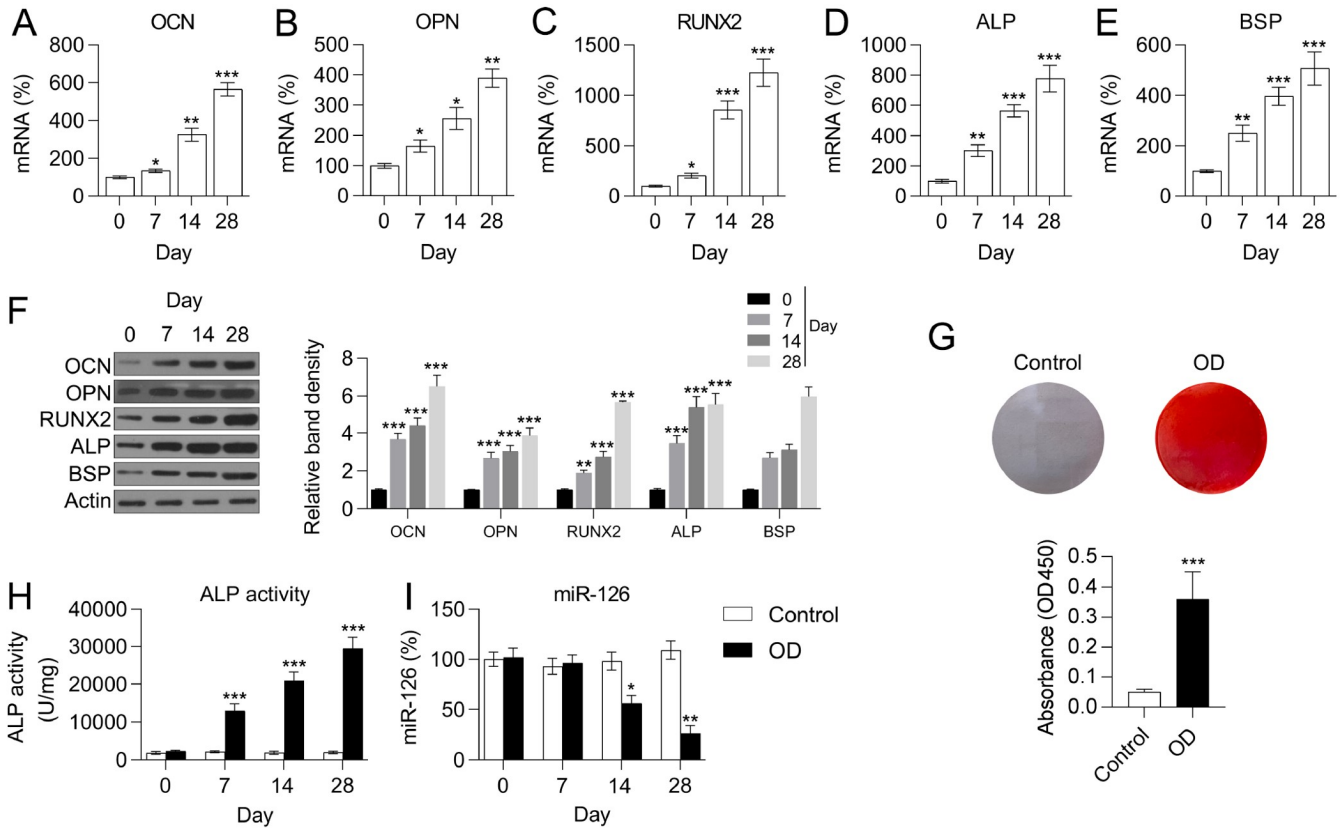

Figure 1. Expression of miR-126 during OD of hBMMSCs hBMMSCs were treated with 100 μM ascorbic acid, 2 mM β-glycerophosphate, and 10 nM dexamethasone for the induction of osteogenic differentiation. (A–E) qPCR was performed at days 0, 7, 14, and 28 to evaluate the mRNA levels of OD-relevant genes, including *OCN*, *OPN*, *RUNX2*, *ALP*, and *BSP*, while *GAPDH* served as the reference gene. The data in each group were normalized to that on day 0. (F) Western blot analysis was performed to detect the protein levels of OCN, OPN, RUNX2, ALP, and BSP at the indicated time points. (G) ALP activity was determined at day 14 during the OD. (H) Representative AR staining images showing extracellular matrix mineralization at day 14 post OD induction. (I) qPCR was performed at days 0, 7, 14, and 28 to determine the miR-126 expression level with *U6* as the reference gene. The data in each group were normalized to that on day 0. $n=4$. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs day 0 groups.

Furthermore, AR staining of hBMMSCs demonstrated that miR-126 attenuated matrix mineralization of hBMMSCs during OD induction (Figure 2I).

To demonstrate the inhibitory role of miR-126 in the OD of hBMMSCs, the expression of miR-126 was downregulated in hBMMSCs and then cells were subject to OD induction. The qPCR data revealed that miR-126 level was noticeably downregulated after transfection with antagomiR-126 (Figure 3A). Moreover, the expressions of OD-related genes were further increased at both the mRNA and protein levels in the hBMMSCs subject to OD induction post miR-126 downregulation compared with that in NC control hBMMSCs subject to OD induction (Figure 3B–G). Furthermore, ALP activity in hBMMSCs subject to miR-126 downregulation was further increased compared with that in cells transfected with antagomiR-NC (Figure 3H). Additionally, AR staining demonstrated that miR-126 inhibition promoted the matrix mineralization of hBMMSCs during OD induction (Figure 3I). Collectively, these findings suggest that miR-126 suppresses the OD of hBMMSCs.

miR-126 induces inflammation and apoptosis of hBMMSCs

To investigate the underlying mechanism by which miR-126 suppresses the OD of hBMMSCs, we analysed the effect of miR-126 on the inflammation and apoptosis of hBMMSCs post OD induction. The results revealed that the levels of three well-known proinflammatory cytokines, interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α, were elevated in the hBMMSCs subject to miR-126 upregulation compared with those in the agomiR-NC-transfected cells (Figure 4A). In contrast, the downregulation of miR-126 enhanced the production of IL-1β and IL-6 (Figure 4B); however, the change in TNF-α levels was not statistically significant compared with that in antagomiR-NC-transfected hBMMSCs. These data suggest that miR-126 induces inflammation during the OD of hBMMSCs.

Furthermore, assessment of apoptotic cells using flow cytometry with Annexin V and PI staining for hBMMSCs revealed a higher percentage of apoptotic cells in the agomiR-126-transfected group than in the agomiR-NC group (Figure 4C). In contrast, miR-126 downregulation apparently led to a lower percentage of apoptotic cells, although the difference was not statistically significant (Figure 4D). Overall, these data suggest that miR-126 induces apoptosis in hBMMSCs undergoing OD.

miR-126 expression is inversely associated with Bcl-2 expression and ERK1/2 phosphorylation in hBMMSCs

Previous studies have reported that miR-126 inhibition leads to

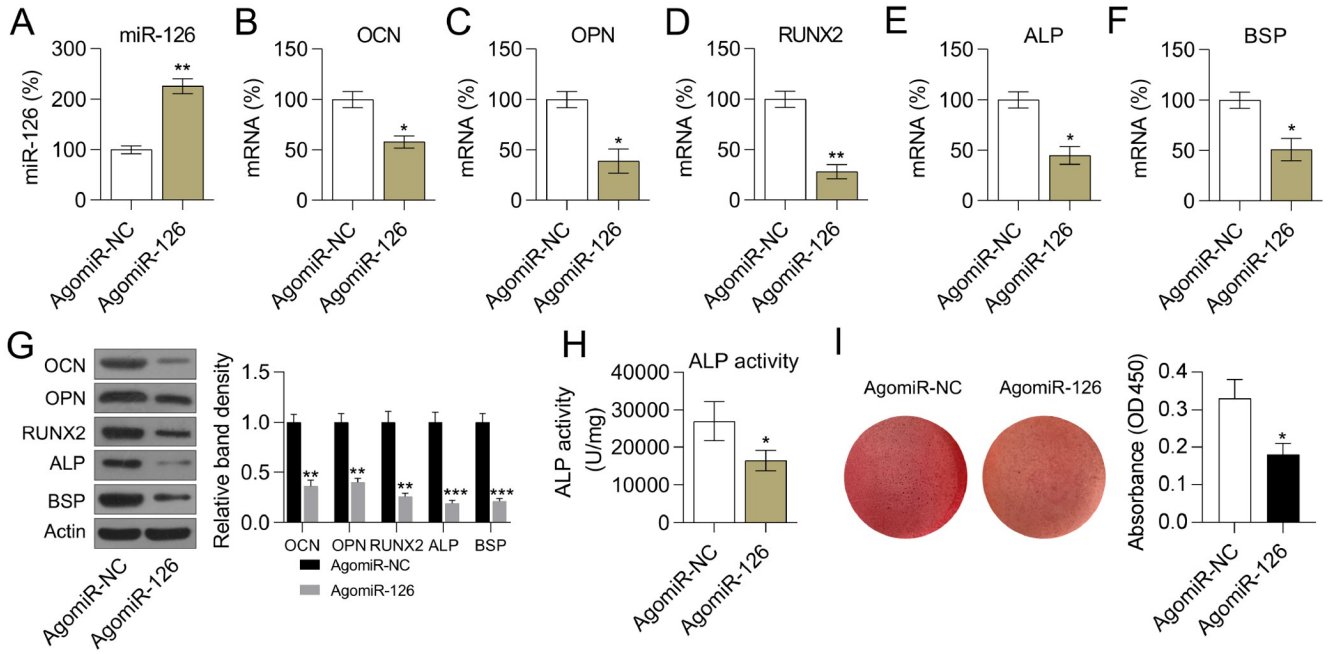

Figure 2. miR-126 suppresses the OD of hBMMSCs hBMMSCs were treated with 100 mM ascorbic acid, 2 mM β-glycerophosphate, and 10 μM dexamethasone for the induction of osteogenic differentiation. At day 3 after induction, the cells were transfected with agomiR-126/NC. (A) qPCR was performed to determine the miR-126 level in hBMMSCs at day 14 post induction. The data in each group were normalized to that in the agomiR-NC group. (B–F) qPCR was performed to examine the mRNA expressions of OCN, OPN, RUNX2, ALP, and BSP at day 14 post induction. The data in each group were normalized to that in the agomiR-NC group. (G) Western blot analysis was performed to detect the protein levels of OCN, OPN, RUNX2, ALP, and BSP at day 14 post induction. (H) ALP activity was determined at day 14 during the OD. (I) Representative AR staining images showing extracellular matrix mineralization at day 14 post induction. $n=4$. $^{*}P<0.05$, $^{**}P<0.01$ vs agomiR-NC groups.

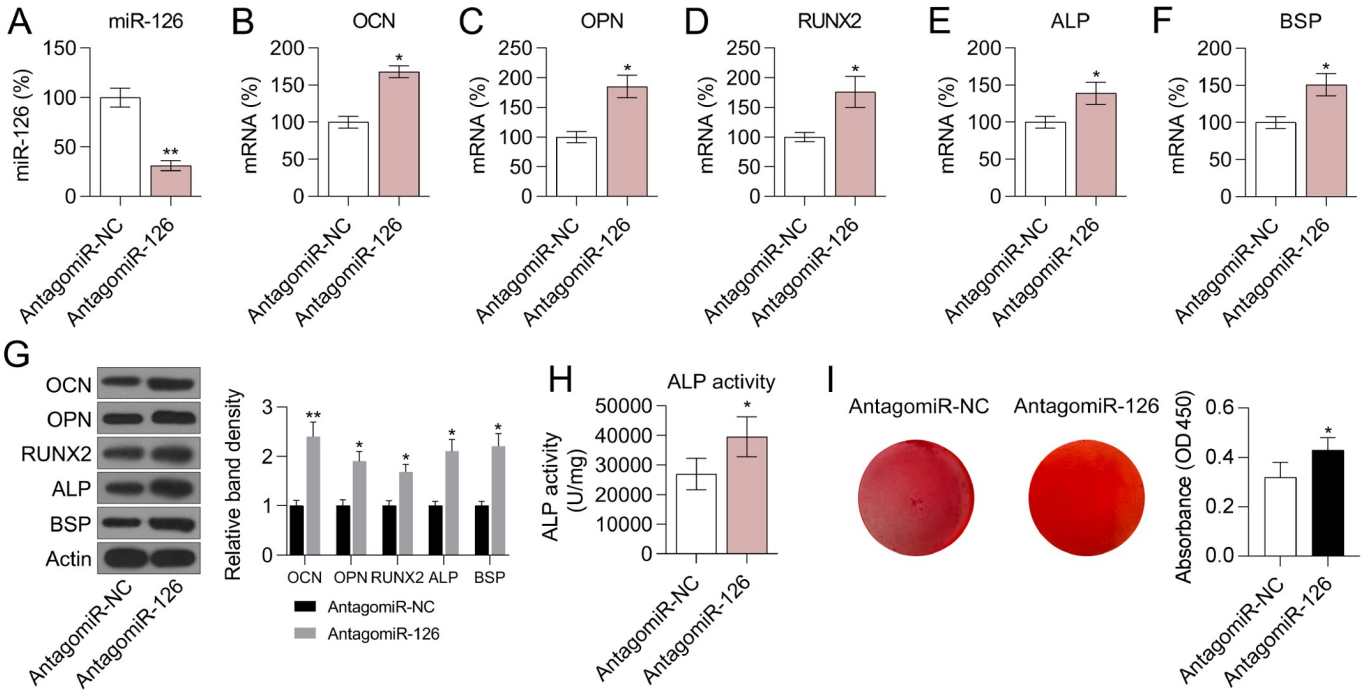

Figure 3. Inhibition of miR-126 promotes the OD of hBMMSCs hBMMSCs were treated with 100 μM ascorbic acid, 2 mM β-glycerophosphate, and 10 nM dexamethasone for the induction of osteogenic differentiation. At day 3 after induction, the cells were transfected with antagomiR-126/NC. (A) qPCR was performed to determine the miR-126 level in hBMMSCs at day 14 post induction. The data in each group were normalized to that in the antagomiR-NC group. (B–F) qPCR was performed to examine the mRNA expressions of OCN, OPN, RUNX2, ALP, and BSP at day 14 post induction. The data in each group were normalized to that in the antagomiR-NC group. (G) Western blot analysis was performed to detect the protein levels of OCN, OPN, RUNX2, ALP, and BSP at day 14 post induction. (H) ALP activity was determined at day 14 during the OD. (I) Representative AR staining images showing extracellular matrix mineralization at day 14 post induction. $n=4$. $^{*}P<0.05$, $^{**}P<0.01$ vs antagomiR-NC groups.

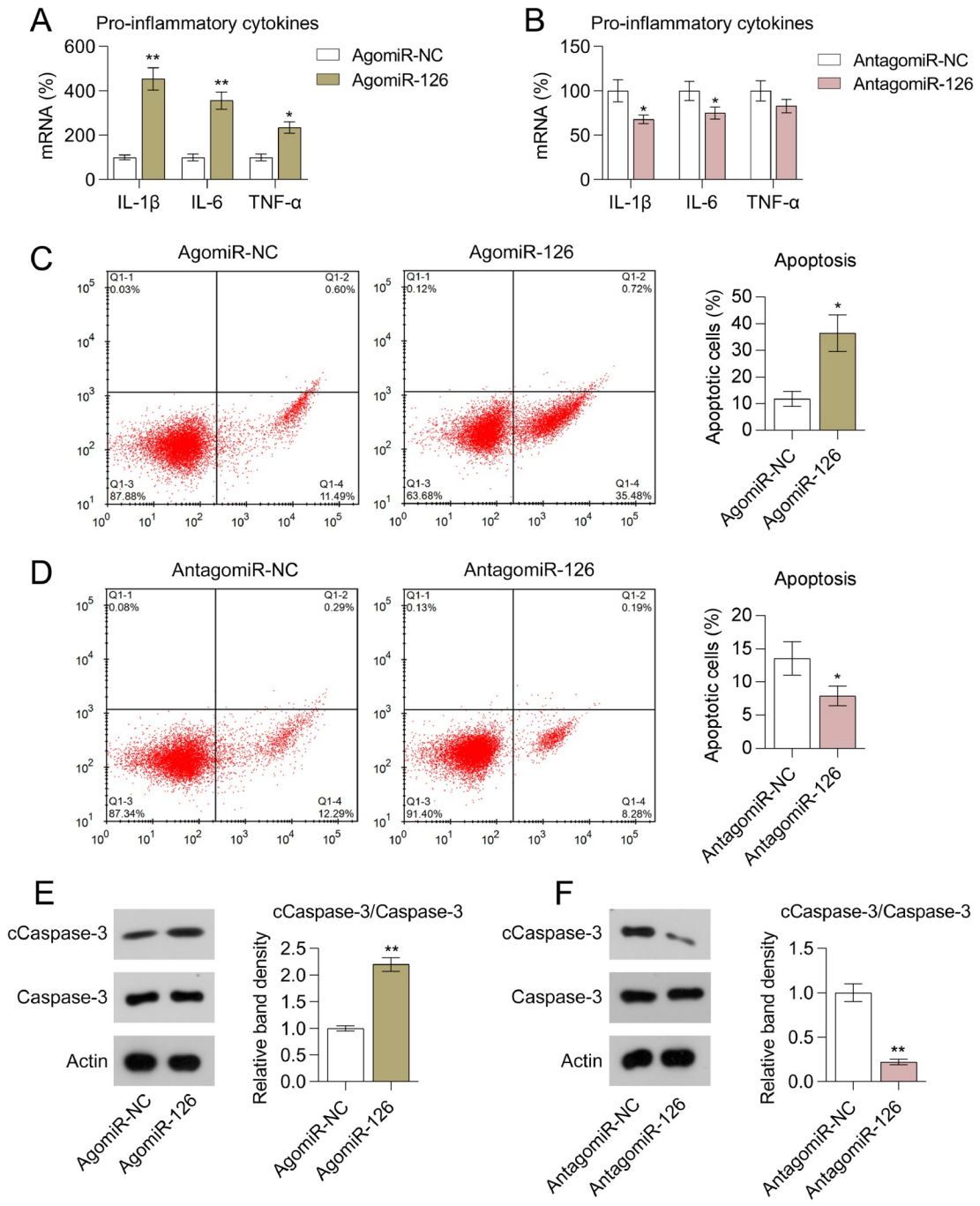

Figure 4. miR-126 induces inflammation and apoptosis of hBMMSCs hBMMSCs were treated with 100 mM ascorbic acid, 2 mM β-glycerophosphate, and 10 μM dexamethasone for the induction of osteogenic differentiation. At day 3 post induction, the cells were transfected with agomiR-126/NC or antagomiR-126/NC for 36 h. (A, B) qPCR was performed to examine the expressions of proinflammatory cytokines (IL-1β, IL-6, and TNF-α) at day 14 post induction. The data in each group were normalized to that in the agomiR/antagomiR-NC group. (C,D) Flow cytometry was performed to determine the percentage of Annexin V-positive and PI-negative cells in each group. The percentage of early apoptotic cells is displayed in the right panel. (E,F) Caspase cleavage was examined by western blot analysis. $n=4$. $^{*}P<0.05$, $^{**}P<0.01$ vs agomir/antagomiR-NC groups.

activation of ERK1/2 and transcriptional upregulation of Bcl-2 [23,24]. Hence, we hypothesized that miR-126 may suppress the OD of hBMMSCs through the inhibition of ERK1/2 and Bcl-2. Accordingly, we examined the expression of Bcl-2 and ERK1/2 phosphorylation in OD-induced hBMMSCs with/without miR-126 dysregulation. Our results revealed that at the transcriptional level, the upregulation of miR-126 did not affect the mRNA level of ERK but decreased the expression of Bcl-2 compared with those noted in agomiR-NC-transfected control cells. In contrast, the mRNA level of Bcl-2 was elevated in hBMMSCs subject to miR-126 downregulation, but no difference was found in ERK transcript level compared with those noted in the antagomiR-NC transfected control cells

(Figure 5A–D). However, our western blot analyses demonstrated that ERK1/2 phosphorylation, and not ERK1/2 expression, was decreased in the OD-induced hBMMSCs subject to miR-126 upregulation, while ERK1/2 phosphorylation was enhanced with reduced miR-126 level compared to those in the NC control cells (Figure 5E,F). These results demonstrate that miR-126 expression is negatively associated with ERK1/2 phosphorylation and Bcl-2 expression in hBMMSCs.

ERK1/2 and Bcl-2 participate in miR-126-mediated inflammation and apoptosis of hBMMSCs

To further investigate the role of ERK1/2 and Bcl-2 in the OD of hBMMSCs, cells were cotransfected with agomiR-126 and an ERK/Bcl-2 overexpression vector to compensate for the miR-126-mediated suppression of Bcl-2 expression and ERK1/2 phosphorylation. Our results showed that the ERK1/2 protein and transcript levels were markedly increased in the ERK overexpression group (Figure 6A,B). Notably, phosphorylated ERK1/2 was also increased in the ERK overexpression group compared with the pCDNA3-empty vector group (Figure 6B). Bcl-2 overexpression was confirmed by both qPCR and western blot analysis in the OD-induced hBMMSCs subject to miR-126 upregulation (Figure 6C,D). Subsequently, qPCR and flow cytometry were used to examine the inflammation and apoptosis of hBMMSCs during OD. The data showed that three proinflammatory cytokines, IL-1β, IL-6, and TNF-α, were enhanced due to the upregulated phosphorylation of ERK1/2 but not Bcl-2 (Figure 6E). Additionally, the percentage of miR-126-induced apoptotic cells was not influenced by ERK overexpression but was reduced after restoration of Bcl-2 expression (Figure 6F). These data suggest that miR-126 induces inflammation and apoptosis in hBMMSCs by regulating ERK1/2 activity and Bcl-2 expression, respectively.

miR-126 mitigates the OD of hBMMSCs by regulating ERK1/2 and Bcl-2

To determine whether miR-126-repressed OD of hBMMSCs is mediated by regulating ERK1/2 and Bcl-2, we first analysed the mRNA and protein expressions of genes associated with OD. The results revealed that the expression levels of these genes were partially increased in hBMMSCs subject to ERK1/2 and Bcl-2 overexpression compared with those in the single/miR-126 agomiR-transfected group (Figure 7A–F). Furthermore, partially increased ALP activity and matrix mineralization were detected during the OD of hBMMSCs post ERK1/2 and Bcl-2 overexpression, as assessed by

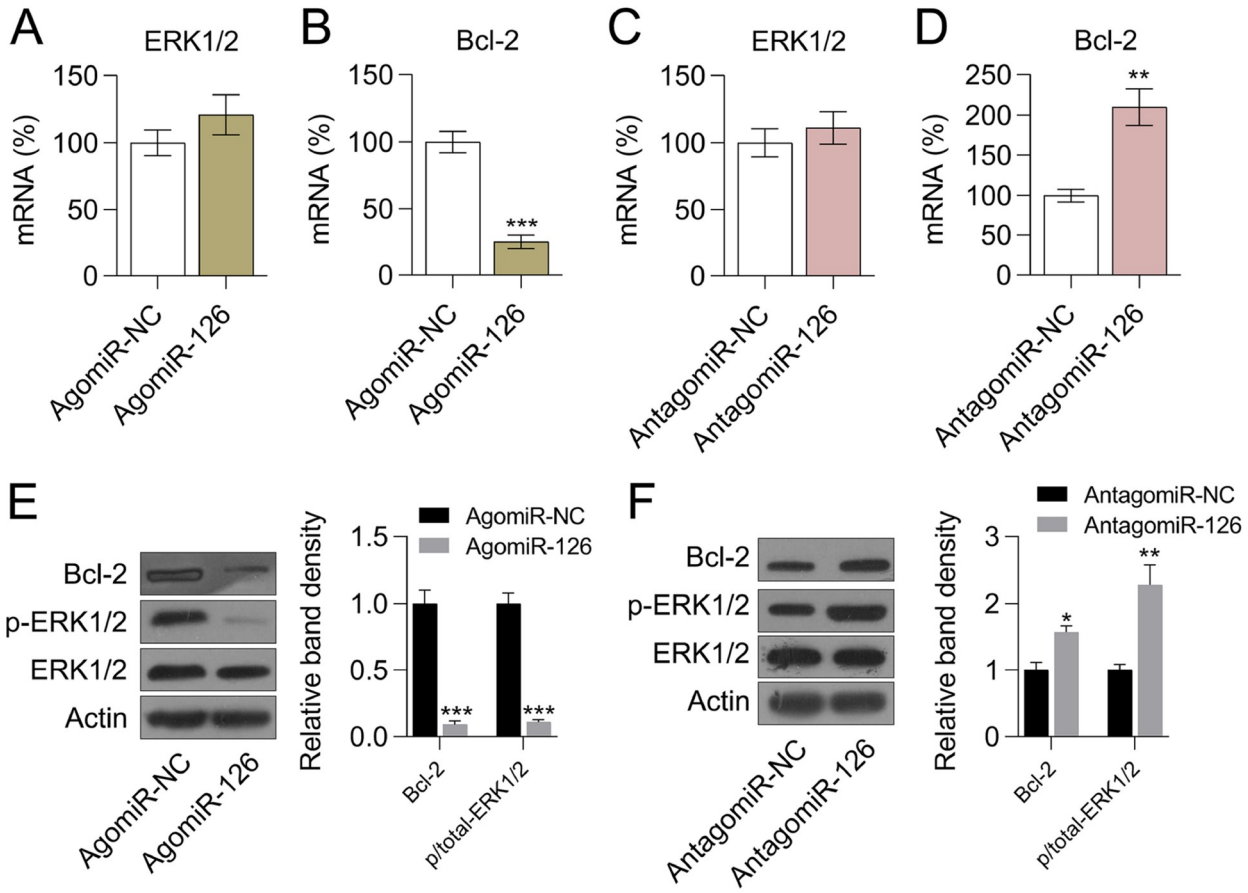

Figure 5. miR-126 suppresses ERK1/2 phosphorylation and Bcl-2 expression during OD of hBMMSCs hBMMSCs were treated with 100 μM ascorbic acid, 2 mM β-glycerophosphate, and 10 nM dexamethasone for the induction of osteogenic differentiation. At day 3 post induction, the cells were transfected with agomiR-126/NC or antagomiR-126/NC for 36 h. (A–D) qPCR was performed to examine the mRNA expressions of ERK and Bcl-2 at day 14 post induction. The data in each group were normalized to that in the agomiR/antagomiR-NC group. (E,F) Western blot analysis was performed to detect the protein expressions of ERK and Bcl-2, as well as ERK1/2 phosphorylation at day 14 post induction. $n = 4$. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs agomir/antagomiR-NC groups.

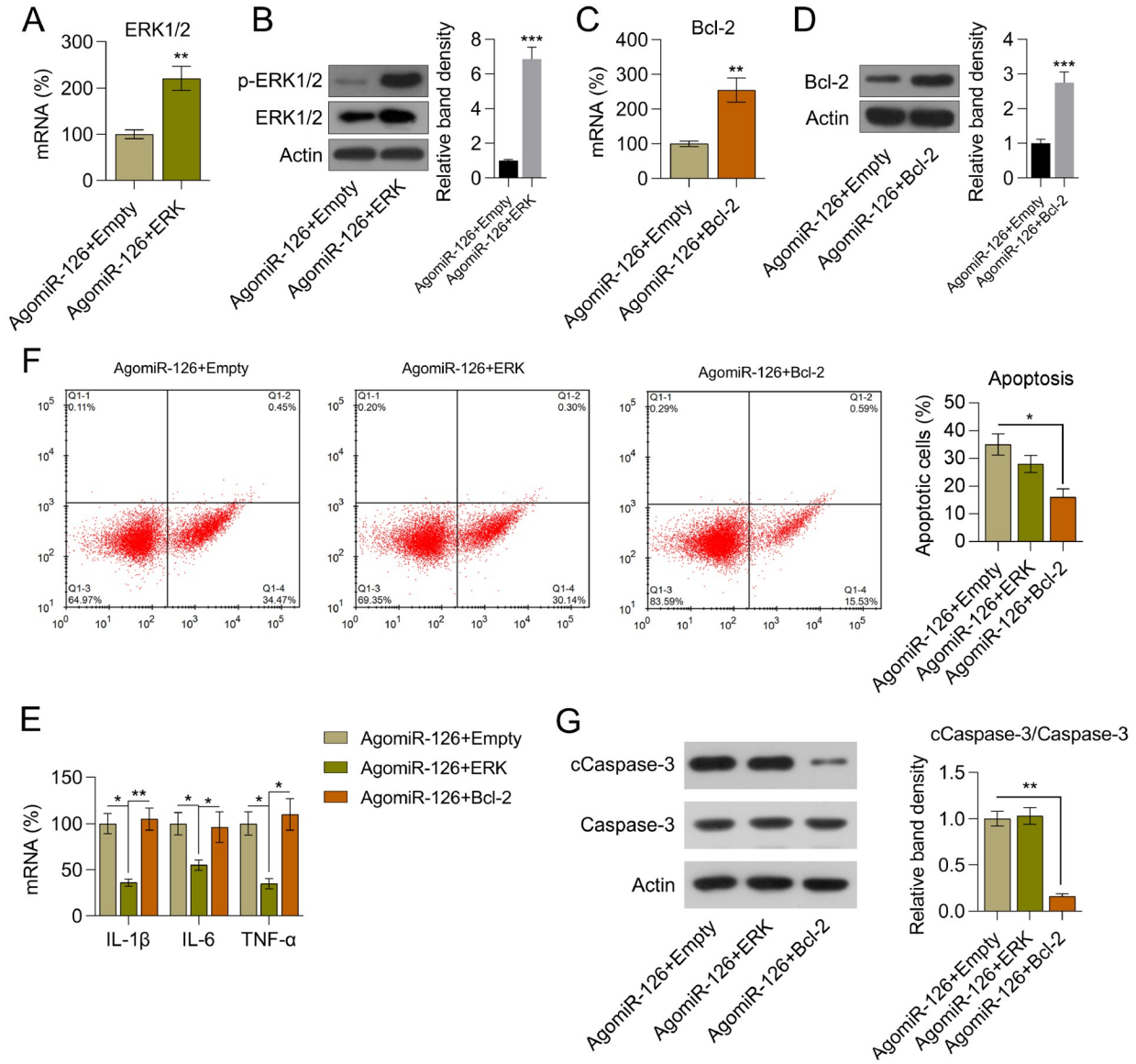

Figure 6. Role of Bcl-2 expression and ERK1/2 phosphorylation in inflammation and apoptosis during miR-126-mitigated OD of hBMMSCs
hBMMSCs were treated with 100 mM ascorbic acid, 2 mM β-glycerophosphate, and 10 μM dexamethasone for the induction of osteogenic differentiation. At day 3 post induction, the cells were transfected with agomiR-126/NC and pCDNA3-ERK1/2 or pCDNA3-Bcl-2 for 36 h. (A,B) qPCR and western blot analysis were performed to examine the mRNA and protein expressions of ERK and ERK1/2 phosphorylation at day 14 post induction. The data in each group were normalized to that in the agomiR-126 + empty group. (C,D) qPCR and western blot analysis were performed to examine the mRNA and protein expressions of Bcl-2 at day 14 post induction. The data in each group were normalized to that in the agomiR-126 + empty group. (E) qPCR was employed to examine the expressions of proinflammatory cytokines (IL-1β, IL-6, and TNF-α) at day 14 post induction. The data in each group were normalized to that in the agomiR-126 + empty group. (F) Flow cytometry was performed to detect the percentage of Annexin V-positive and PI-negative cells in each group. The percentage of early apoptotic cells is displayed in the right panel. (G) Caspase cleavage was examined by western blot analysis. $n = 4$. $^{*}P < 0.05$, $^{**}P < 0.01$ vs agomiR-NC + empty groups.

ALP activity assays and AR staining, compared with those in the pCDNA3-empty vector control-transfected cells (Figure 7G,H). Overall, these findings suggest that ERK1/2 and Bcl-2 are associated with miR-126-mediated repression of OD in hBMMSCs.

Discussion

Increasing incidences of bone disorders and ageing populations have indicated the emerging demands of more efficient treatments. MicroRNAs have been documented to impact OD through various regulatory mechanisms, including chromatin modification and association with transcription factors and competing endogenous RNAs [25]. To illustrate the molecular mechanisms mediating the OD of BMMSCs, in the present study we explored the role of miR-126 in the OD of hBMMSCs by assessing whether miR-126 mediates OD by regulating ERK1/2 and Bcl-2. *In vitro* OD cell models were successfully established with hBMMSCs and the subsequent results showed that upregulated miR-126 inhibited the OD-induced development of hBMMSCs, as evidenced by the reduced expressions of OD-associated genes and ALP activity, as well as impaired matrix mineralization. Moreover, miR-126 upregulation induced

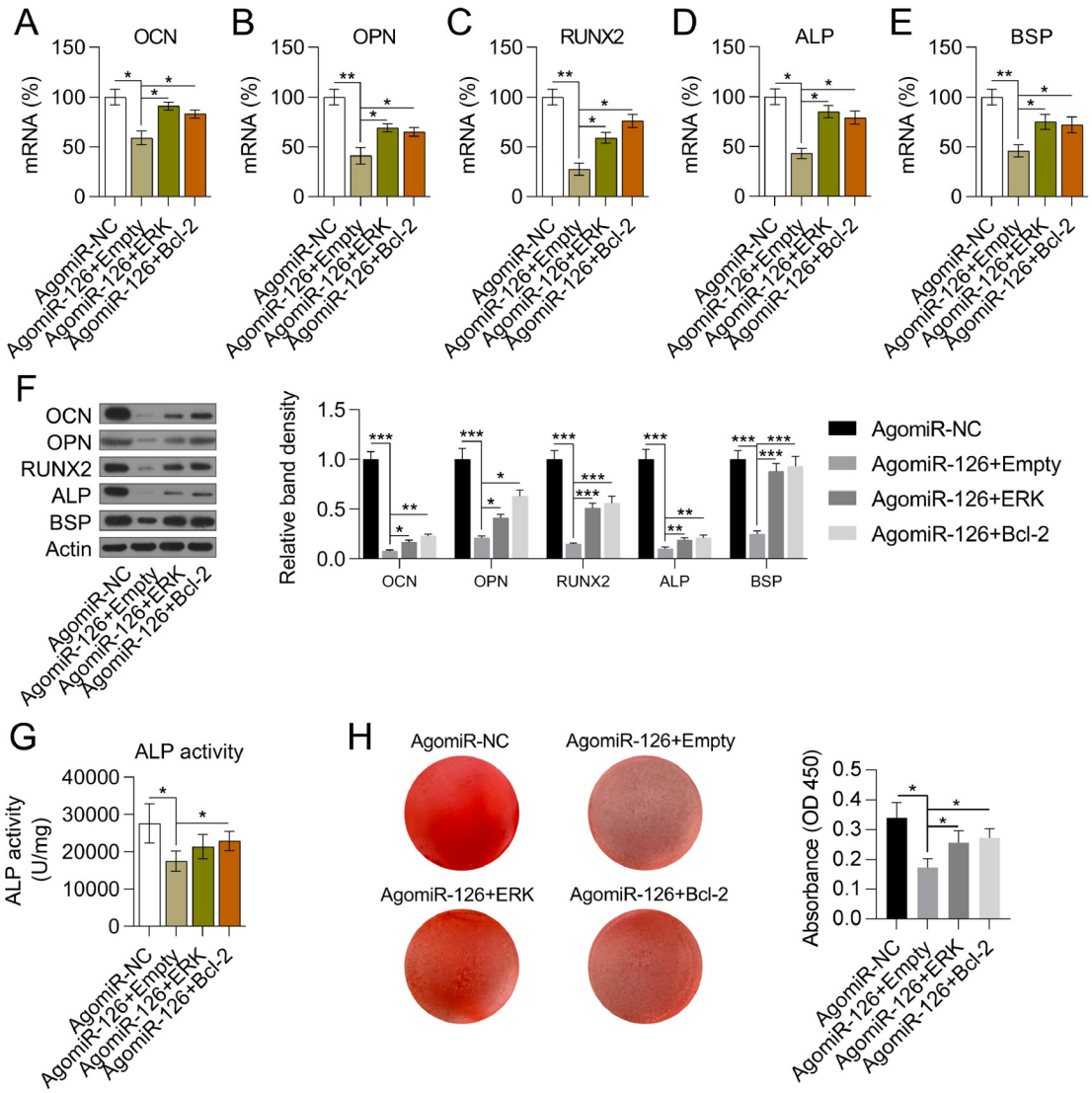

Figure 7. Role of Bcl-2 expression and ERK1/2 phosphorylation in miR-126-mitigated OD of hBMMSCs hBMMSCs were treated with 100 μM ascorbic acid, 2 mM β-glycerophosphate, and 10 nM dexamethasone for the induction of osteogenic differentiation. At day 3 post induction, the cells were transfected with agomiR-126/NC and pCDNA3-ERK1/2 or pCDNA3-Bcl-2 for 36 h. (A-E) qPCR was performed to examine the mRNA expressions of OCN, OPN, RUNX2, ALP, and BSP at day 14 post induction. The data in each group were normalized to that in the agomiR-126 + empty group. (F) Western blot analysis was performed to detect the protein levels of OCN, OPN, RUNX2, ALP, and BSP at day 14 post induction. (G) ALP activity was determined as day 14 during OD. (H) Representative AR staining images showing extracellular matrix mineralization at day 14 post induction. $n = 4$. $^{*}P < 0.05$, $^{**}P < 0.01$.

inflammation and apoptosis of hBMMSCs by suppressing Bcl-2 expression and ERK1/2 phosphorylation, thereby inhibiting the differentiation of hBMMSCs. Thus, increased apoptosis and inflammation may attenuate the viability of hBMMSCs, resulting in impaired extracellular matrix mineralization.

Mitogen-activated protein kinases (MAPKs) are a group of serine/threonine kinases that play a critical role in signal transduction by regulating gene transcription in the nucleus in response to alterations in cells [26]. The role of ERK signaling pathways in cell proliferation and differentiation has been widely studied in various cell systems, such as osteoblasts [27]. Furthermore, rapid increases in the phosphorylation of ERK and c-Jun N-terminal kinase (JNK) have been reported in alendronate-exposed bone marrow stromal cells (BMSCs). In addition, PD98059 and SP600125 (MAPK-specific inhibitors) were reported to completely disrupt the regulation of alendronate-mediated cell differentiation of BMSCs. These findings suggest that both the ERK and JNK pathways are relevant to the alendronate-mediated regulation of osteogenic and adipogenic differentiation of BMSCs [18]. Furthermore, ERK1/2 has been documented to affect early osteoblast-specific gene expression, thereby promoting OD [28,29].

As a well-documented anti-apoptotic indicator [30], the influence of Bcl-2 on osteoblast proliferation was not examined in transgenic mice in a previous study [31], but osteoblast proliferation in Bcl-2 transgenic mice was promoted, whereas Bcl-2 introduction could not promote the proliferation of wild-type primary osteoblasts [17]. Furthermore, previous reports have suggested that Bcl-2 could repress cell proliferation by promoting G0 arrest and preventing the

G0 to S phase transition in fibroblasts and hematopoietic cells [32]. Moreover, the levels of p27 and p130 were elevated in Bcl-2-overexpressing cells during G0 arrest [33,34]. However, Bcl-2 overexpression promoted the proliferation of myocytes [35]. The ERK1/2-Bcl-2 signaling pathway regulates a group of essential cellular processes [36], and its activation has been reported to facilitate the survival and suppress the apoptosis of pancreatic tumor cells [37].

Our findings further corroborate the results of two similar studies [16,19] on the role of miR-126 in the OD of hBMMSCs and inflammation. Further deliberation on these findings revealed some similarities and differences between our study and these two studies. Kong *et al.* [19] demonstrated that miR-126 promotes proliferation, migration, invasion, and endothelial differentiation but inhibits the apoptosis and OD of BMMSCs. Yu *et al.* [16] showed that miR-126 protects chondrocytes from IL-1β-induced inflammation via upregulation of Bcl-2. First and most importantly, our findings on the effect of miR-126 on OD were similar to those of Kong *et al.* [19]. They showed that miR-126 upregulation resulted in reduced OD and endothelial differentiation, while our data showed that agomiR-126 transfection led to reduced OD. Moreover, both Kong *et al.* and we examined the expressions of OD-associated genes, including *ALP*, *OPN*, and *RUNX2*, to indicate the progression of OD. However, in our study, we additionally examined the expressions of OCN and BSP at both the mRNA and protein levels, in addition to ALP activity and matrix mineralization. The experimental evidence from our study suggests an inhibitory role of miR-126 on the OD of hBMMSCs. Second, Kong *et al.* [19] reported that miR-126 inhibition caused apoptosis, while our results demonstrated that miR-126 elevation induced apoptosis of hBMMSCs. Moreover, while they focused on the role of miR-126 in the migration and invasion of BMSCs, we aimed to investigate the role of miR-126 in the inflammation of hBMMSCs during OD. These major differences urged us to report our findings here. We believe that the inconsistency of miR-126 efficiency on differentiation and apoptosis may be attributed to the differences in the cell lines used. Kong *et al.* [19] purchased human BMSCs for their study, while we isolated and telomerase-immortalized hBMMSCs from human tissue. Similarly, our study demonstrated the involvement of the miR-126/Bcl-2 axis in hBMMSCs but not in chondrocytes, as reported by Yu *et al.* [16]. Notably, Yu *et al.* [16] also found that miR-126 inhibition enhanced cell viability and ameliorated inflammation and apoptosis, which also supports our present findings. We showed that upon upregulation or downregulation of miR-126 in hBMMSCs via transfection with agomiR or antagomiR, ALP activity and the expressions of OD-relevant genes were also reduced or promoted, respectively. Meanwhile, enhanced expression of inflammatory cytokines and induction of apoptosis were also detected in hBMMSCs with miR-126 upregulation. Likewise, Bcl-2 expression and ERK1/2 phosphorylation showed an inverse association with the cellular level of miR-126. However, we do not have data to explain why miR-126 overexpression can reduce ERK phosphorylation. Nevertheless, a previous study indicated that miR-126 was able to reduce ERK phosphorylation [13], which is consistent with our findings. Notably, ERK1/2 overexpression counteracted the effect of miR-126 upregulation on inflammation, whereas Bcl-2 restoration abrogated the influence of miR-126 on apoptosis, indicating that the role of miR-126 in OD probably involves ERK1/2 deactivation and Bcl-2 suppression. Thus, our study highlights a novel mechanism for the interaction of the miR-126-ERK1/2/Bcl-2 pathway during the OD of hBMMSCs.

In summary, our data demonstrated that upregulation of miR-126 could inhibit the OD of hBMMSCs by downregulating Bcl-2 expression and ERK1/2 phosphorylation. Further *in vivo* and clinical experiments are needed to demonstrate the role of the miR-126/ERK1/2/Bcl-2 axis in bone formation/regeneration, and the exact mechanisms regulating the interplay between miR-126 and other relevant signaling pathways remain to be explored.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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