

# VEGF mitigates bisphosphonate-induced apoptosis and differentiation inhibition of MC3T3-E1 cells

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**Abstract.** The present study aimed to investigate whether VEGF was involved in bisphosphonate (BP)-induced apoptosis and differentiation of osteoblasts. Murine MC3T3-E1 osteoblasts were stimulated with zoledronic acid (ZA) for 7 days. VEGF mRNA and protein expression levels were determined via reverse transcription-quantitative PCR and western blot analysis, respectively. Cell viability was evaluated using Cell Counting Kit-8 assay. In addition, the cell apoptotic rate and the expression levels of apoptosis-related proteins were measured using a TUNEL staining kit and western blot analysis, respectively. To evaluate mineralization, cells were stained with alizarin red, while the secretion levels of alkaline phosphatase (ALP) were measured using the corresponding assay kit. Finally, the expression levels of differentiation-related proteins and proteins of the Nod-like receptor family pyrin domain-containing 3 (NLRP3)/caspase 1/gasdermin D (GSDMD) pyroptosis pathway were measured by western blot analysis. VEGF expression level was notably decreased in ZA-stimulated MC3T3-E1 cells. However, the viability of these cells was enhanced following VEGF addition. Furthermore, VEGF attenuated apoptosis, promoted mineralization and increased ALP activity in ZA-stimulated

MC3T3-E1 cells. The ZA-mediated decrease in the protein expression of the osteogenic genes osteopontin, osteocalcin and runt-related transcription factor 2 was restored after MC3T3-E1 cell treatment with 10 ng/ml VEGF. The present study demonstrated that VEGF could attenuate BP-induced apoptosis and differentiation of MC3T3 cells by regulating the NLRP3/caspase 1/GSDMD pathway.

## Introduction

Bisphosphonates (BPs) are a series of recently developed drugs targeting musculoskeletal diseases and disorders of calcium metabolism. BPs act via binding with hydroxy-apatite in the bones to attenuate the activity of osteoclasts, thereby inhibiting bone resorption (1). Therefore, BPs have been used to treat osteoporosis, scleromalacia, metastatic bone cancer-induced hypercalcemia and itai-itai disease (2). Osteonecrosis of the jaw (ONJ) is a rare condition that occurs in patients with cancer treated with multimodality therapies, including intravenous or oral administration of BPs (3). The incidence of drug-induced ONJ is the highest among patients treated with third-generation BPs, such as zoledronic acid (ZA) (4,5).

VEGF regulates the proliferation and differentiation of osteoblasts, as well as attenuating blood vessel growth and bone regeneration by blocking the VEGF receptors VEGFR1 and VEGFR2 (6). Previous studies have demonstrated that exogenous VEGF could improve cell mineralization and osteogenesis *in vivo* in different bone defect animal models (7-9). Additionally, a recent study revealed a positive association between the occurrence of ONJ and treatment with bone resorption and VEGFR tyrosine kinase inhibitors (10). Based on the aforementioned findings, the present study hypothesized that VEGF could be involved in BP-induced ONJ. Therefore, the present study aimed to investigate whether VEGF could affect the apoptosis and differentiation of ZA-stimulated osteoblasts. The present results may provide new insights into the development of novel approaches for the treatment or prevention of BP-induced ONJ.

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## Materials and methods

**Cell culture and treatment.** The murine osteoblast cell line MC3T3-E1 was obtained from the American Type Culture Collection. The cells were grown in  $\alpha$ -minimal essential medium ( $\alpha$ -MEM) supplemented with ribonucleosides, deoxyribonucleosides, 2 mM L-glutamine, 1 mM sodium pyruvate (Gibco; Thermo Fisher Scientific, Inc.) and 10% FBS (Thermo Fisher Scientific, Inc.), without the addition of ascorbic acid. MC3T3-E1 cells were stimulated with different doses (0.1, 1 or 5  $\mu$ M) of ZA (Selleck Chemicals) which was dissolved in sterile distilled water to evaluate cell apoptosis and differentiation. Murine VEGF (R&D Systems, Inc.) at a concentration of 10 ng/ml in sterile PBS was selected to treat ZA-stimulated MC3T3-E1 cells, as previously described (11).

**Reverse transcription-quantitative PCR (RT-qPCR).** Total RNA was extracted from MC3T3-E1 cells using TRIzol<sup>®</sup> reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. RNA purity was quantified by determining the optical density (OD)<sub>260</sub>/OD<sub>280</sub> ratio using a spectrophotometer (Mettler-Toledo GmbH). RNA quality was estimated by agarose electrophoresis. Subsequently, RNA was reverse transcribed into complementary DNA (cDNA) with RevertAid First Strand cDNA Synthesis kit according to the manufacturer's instructions (Thermo Fisher Scientific, Inc.) a LightCycler<sup>®</sup> 480 system (Roche Diagnostics). The amplification of the target cDNAs were performed using the BeyoFast<sup>™</sup> SYBR Green One-Step RT-qPCR kit (Beyotime Institute of Biotechnology). The thermocycling conditions were as follows: Initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation for 15 sec at 95°C and annealing for 30 sec at 60°C. The primers were as follows: VEGF forward, 5'-CTGCCGTCC GATTGAGACC-3', reverse: 5'-CCCCTCCTTGATCCA CTGTC-3'; GAPDH, forward: 5'-AGGTCGGTGTGAACG GATTG-3', reverse: 5'-GGGGTCGTTGATGGCAACA-3'. Lastly, the relative expression levels of the target genes were measured using the  $2^{-\Delta\Delta C_q}$  method (12).

**Western blot analysis.** Proteins were extracted from MC3T3-E1 cells with RIPA lysis buffer (Absin Biotechnology Co., Ltd.) and the protein concentration was measured using a BCA kit (Beyotime Institute of Biotechnology). The protein samples were then boiled at 100°C for 3-5 min to achieve complete protein denaturation, followed by separation by 12% SDS-PAGE (30  $\mu$ g/lane; Beijing Solarbio Science & Technology Co., Ltd.). Subsequently, the proteins were transferred onto a PVDF membrane (Corning, Inc.). Following blocking with 5% bovine serum albumin at room temperature (Absin Biotechnology Co., Ltd.) for 2 h, the membranes were incubated with primary antibodies [Bcl-2, cat. no. 3498, 1:1,000; Bax, cat. no. 2772, 1:1,000; runt-related transcription factor 2 (RUNX2), cat. no. 12556, 1:1,000; NLRP3, cat. no. 15101, 1:1,000; caspase 1, cat. no. 83383, 1:1,000; GSDMD, cat. no. 39754, 1:1,000; GAPDH, cat. no. 5174, 1:1,000, Cell Signaling Technology, Inc.; VEGF, cat. no. ab214424, 1:1,000; osteocalcin, cat. no. ab133612, 1:1,000; osteopontin, ab283656, 1:1,000; all Abcam] diluted in TBS-Tween-20 (0.1% Tween-20, TBST) on a shaking bed at 4°C overnight. Membranes were washed

with TBST, then incubated with the corresponding horseradish peroxidase-conjugated secondary antibody (Anti-rabbit IgG; cat. no. 7074, 1:10,000, Cell Signaling Technology, Inc.) at room temperature for 2 h. Finally, the protein bands were visualized using an ECL kit (Beyotime Institute of Biotechnology). GAPDH served as the loading control.

**Cell Counting Kit-8 (CCK-8) assay.** The viability of ZA-treated MC3T3-E1 cells overexpressing VEGF or not was determined using a CCK-8 assay (Beyotime Institute of Biotechnology). Briefly, a 100- $\mu$ l cell suspension was added into a 96-well plate at a density of  $2 \times 10^3$  cells/well. Following treatment, 10  $\mu$ l of CCK-8 solution were added to each well and cells were incubated for 1 h at 37°C. Cell viability was assessed by measuring the OD value in each well at a wavelength of 450 nm using a spectrophotometer (Mettler-Toledo GmbH).

**TUNEL staining.** Adherent MC3T3-E1 cells were fixed with 4% paraformaldehyde at room temperature for 30 min (Shanghai Aladdin Bio-Chem Technology Co., Ltd.), and were first incubated with 0.3% Triton X-100 (Thermo Fisher Scientific, Inc.) at room temperature for 5 min and then with 0.3% H<sub>2</sub>O<sub>2</sub> (Merck KGaA) in PBS (Biofount; Beijing Biote Pharmaceutical Co., Ltd.) for an additional 20 min at room temperature. Prior to each step, cells were washed thrice with Hanks' balanced salt solution (HBSS; Sigma-Aldrich; Merck KGaA). Apoptotic cells were stained using TUNEL Apoptosis Assay kit by according to the manufacturer's guidance (Beyotime Institute of Biotechnology). The nuclei were stained using DAPI (0.1  $\mu$ g/ml) at room temperature for 5 min in the dark. The cells were observed under a fluorescence microscope (magnification, x200) and six random fields were chosen after Antifade Mounting Medium (Beyotime Institute of Biotechnology) was used to block sections.

**Alizarin Red S (ARS) staining.** The mineralization of MC3T3-E1 cells was evaluated using the ARS Staining kit for Osteogenesis (Sigma-Aldrich; Merck KGaA). Briefly, cells were seeded into a 24-well plate at a density of 250 cells/well and grown in complete culture medium for 3 days until 95% confluency. Subsequently, cell mineralization was induced following supplementation with osteogenic  $\alpha$ -MEM with 10% FBS, 20 mM  $\beta$ -glycerophosphate and 100 mg/ml ascorbic acid, 7.5 mM glycerol phosphate and 50 mg/ml ascorbic acid. After fixation at room temperature in 95% ethanol for 10 min and washing with sterilized water, ARS was utilized to stain cells at room temperature for 30 min. Finally, prior to an inverted light microscopy observation (Olympus), stained cells were washed with distilled water.

**Alkaline phosphatase (ALP) activity assay.** ALP activity was measured using Mouse ALP ELISA kit (cat. no. 69-50082, MSK Biotechnology Co., Ltd.) to evaluate the osteogenic ability of MC3T3-E1 cells. Briefly, 5  $\mu$ l cells ( $2.5 \times 10^5$  cells/well) were lysed using Cell Lysis Buffer (cat. no. P0013J, Beyotime Institute of Biotechnology) and added into a 96-well plate and mixed with the solutions prepared according to the manufacturer's instructions. Following incubation for 10 min at 37°C, the absorbance in each well was measured at a wavelength of 405 nm.

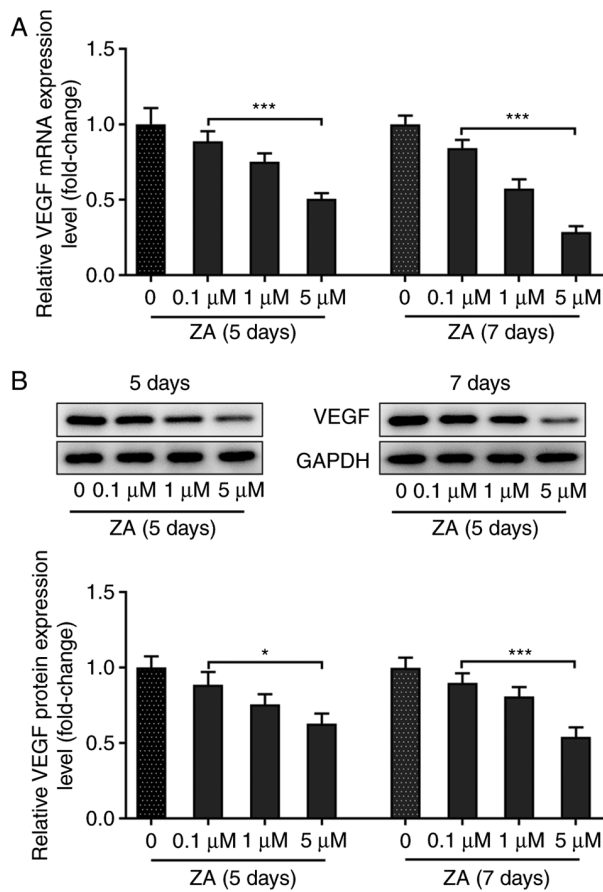


Figure 1. VEGF (A) mRNA and (B) protein expression after MC3T3-E1 cells were stimulated with ZA in different doses for 5 and 7 days, detected by reverse transcription-quantitative PCR and western blot assay. \* $P<0.05$ , \*\*\* $P<0.001$ . ZA, zoledronic acid.

**Statistical analysis.** The differences among different groups were compared by one-way ANOVA followed by Tukey's post hoc test. All data were analyzed and plotted with GraphPad Prism 6 (GraphPad Software, Inc.).  $P<0.05$  was considered to indicate a statistically significant difference. All data are expressed as the mean  $\pm$  SD. All experiments were performed in triplicate.

## Results

**MC3T3-E1 cell stimulation with ZA downregulates VEGF.** MC3T3-E1 cells were stimulated with ZA for 5 and 7 days, and the mRNA and protein expression levels of VEGF were assessed by RT-qPCR and western blot analysis, respectively. The results indicated that the expression level of VEGF in MC3T3-E1 cells was decreased following cell treatment with ZA in a dose-dependent manner at both the mRNA and protein levels (Fig. 1A and B). The expression of VEGF was not notably changed in MC3T3-E1 cells stimulated with ZA for 7 days compared with those stimulated for 5 days (Fig. 1A and B). Collectively, these results demonstrated that treatment of MC3T3-E1 cells with ZA reduced the expression of VEGF in a dose-dependent manner.

**VEGF improves the viability of ZA-stimulated MC3T3-E1 cells.** To reveal whether VEGF could affect cell viability,

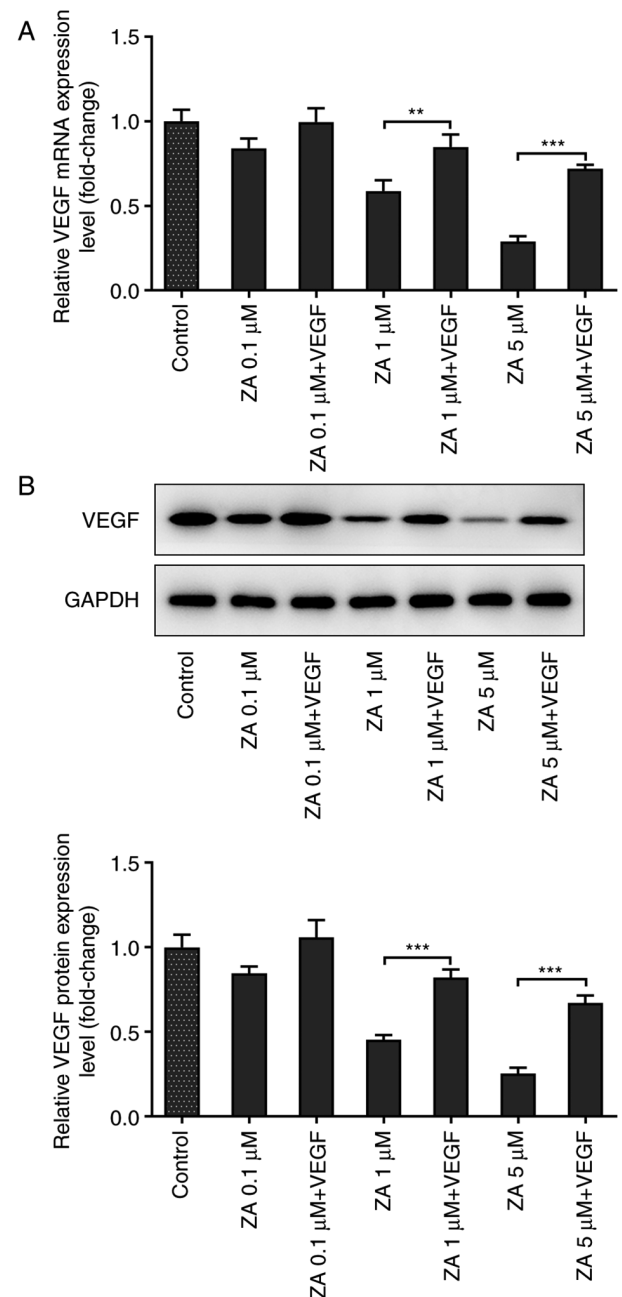


Figure 2. VEGF (A) mRNA and (B) protein expression before and after ZA-stimulated MC3T3-E1 cells were treated with exogenous mouse VEGF (10 ng/ml), detected by reverse transcription-quantitative PCR and western blot assay. \*\* $P<0.01$ , \*\*\* $P<0.001$ . ZA, zoledronic acid.

ZA-stimulated MC3T3 cells were treated with murine VEGF at a concentration of 10 ng/ml. RT-qPCR and western blot analysis confirmed that VEGF levels were increased in cells treated with exogenous VEGF (Fig. 2A and B). As presented in Fig. 3A, the OD values obtained by CCK-8 assays were increased in ZA-stimulated MC3T3 cells following VEGF addition. The present finding indicated that VEGF addition could enhance the viability of ZA-stimulated MC3T3-E1 cells.

**VEGF addition attenuates the apoptosis of ZA-stimulated MC3T3-E1 cells.** The effect of VEGF on cell apoptosis was evaluated via TUNEL staining. The results indicated that cell treatment with exogenous VEGF increased the number

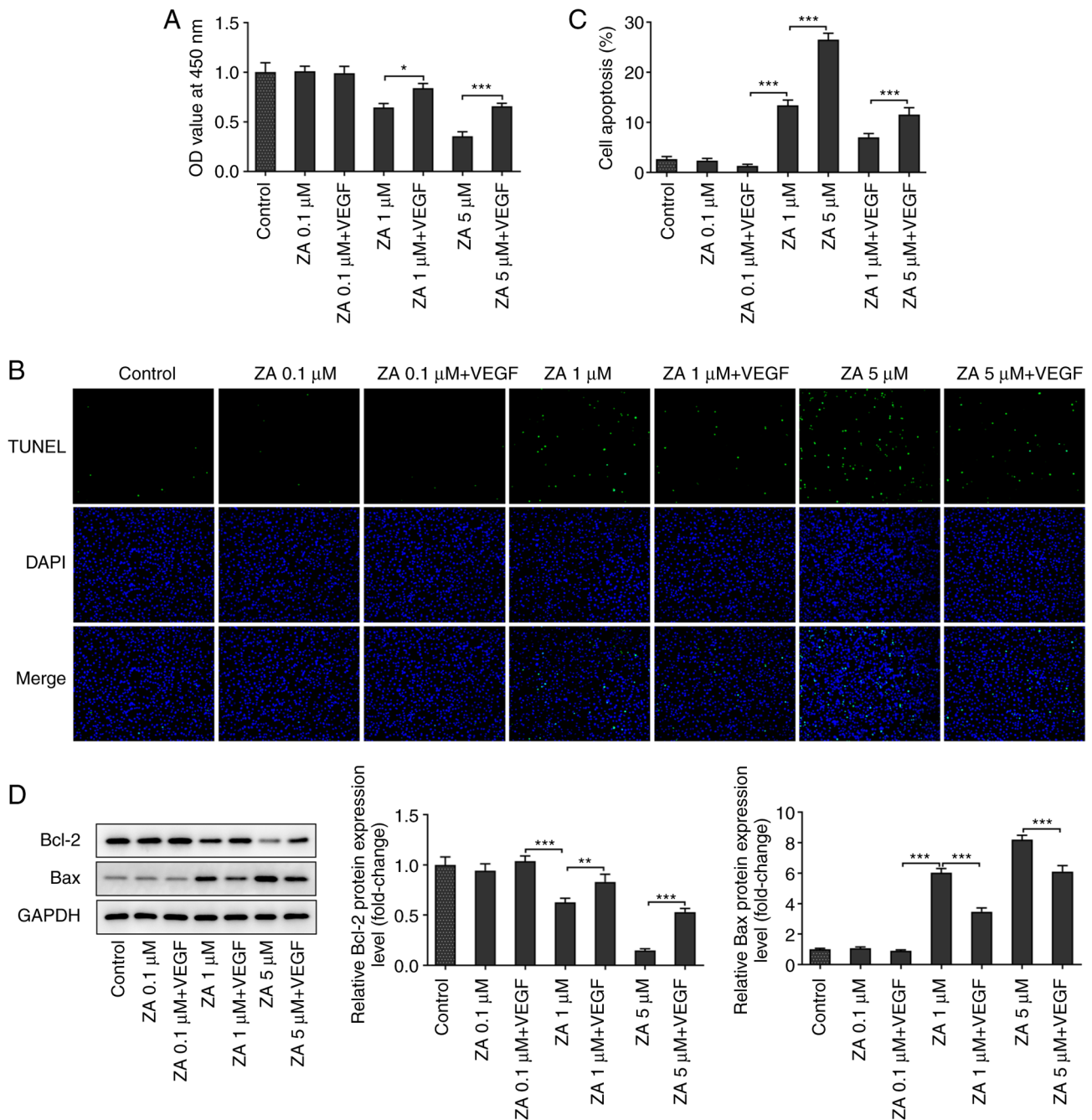


Figure 3. (A) Viability of ZA-stimulated MC3T3-E1 cells in the absence or presence of VEGF treatment, detected by Cell Counting Kit-8. (B) Apoptosis of ZA-stimulated MC3T3-E1 cells in the absence or presence of VEGF, detected by (C) TUNEL staining. (D) Expression levels of anti-apoptotic Bcl2 and pro-apoptotic Bax in ZA-stimulated MC3T3-E1 cells in the absence or presence of VEGF, detected using western blot assay. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . ZA, zoledronic acid. OD, optical density.

of viable ZA-stimulated MC3T3-E1 cells (Fig. 3B and C). In addition, western blot analysis was carried out to determine the expression levels of the apoptosis-related proteins Bcl2 and Bax. The analysis revealed that the expression levels of the anti-apoptotic protein Bcl2 were increased, while those of the pro-apoptotic protein Bax were decreased in ZA-stimulated MC3T3-E1 cells after VEGF addition (Fig. 3D). Taken together, the present results suggested that VEGF attenuated the apoptosis of ZA-stimulated MC3T3-E1 cells.

*VEGF addition promotes the differentiation of ZA-stimulated MC3T3-E1 cells.* The mineralization of ZA-stimulated

MC3T3-E1 cells was evaluated by ARS staining. As presented in Fig. 4A, red staining was more prominent in cells treated with exogenous VEGF compared with cells in the control group. ALP activity was also determined to further evaluate the mineralization of ZA-stimulated MC3T3-E1 cells. The results demonstrated that ALP activity was attenuated in a dose-dependent manner following cell treatment with ZA. This effect was restored after treatment with exogenous VEGF (Fig. 4B). Additionally, the expression levels of differentiation-related proteins were detected by western blot analysis and the results indicated that osteopontin, osteocalcin and RUNX2 were downregulated in ZA-stimulated MC3T3-E1

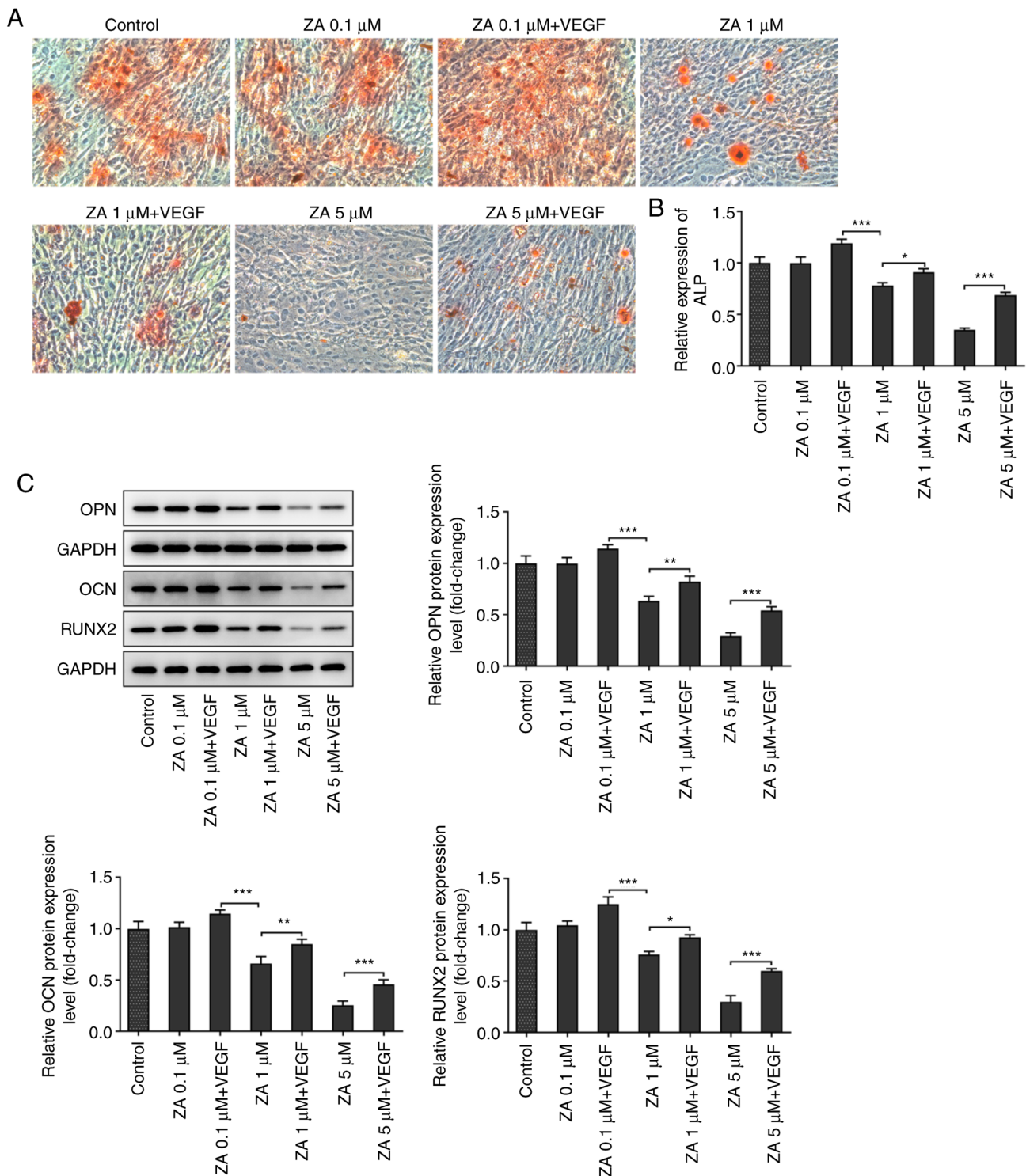


Figure 4. (A) Mineralization of ZA-stimulated MC3T3-E1 cells in the absence or presence of VEGF, detected by Alizarin red S staining. (B) ALP activity in ZA-stimulated MC3T3-E1 cells in the absence or presence of VEGF, detected by ALP assay kit. (C) Expression levels of differentiation-related proteins OPN, OCN and RUNX2 in ZA-stimulated MC3T3-E1 cells in the absence or presence of VEGF, detected by western blot assay, then quantified and analyzed. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . ZA, zoledronic acid; ALP, alkaline phosphatase; OPN, osteopontin; OCN, osteocalcin; RUNX2, runt-related transcription factor 2.

cells, and were then upregulated following VEGF addition (Fig. 4C and D). The aforementioned results suggested that VEGF addition promoted the differentiation of ZA-stimulated MC3T3-E1 cells.

*VEGF addition inhibits the expression of pyroptosis-related proteins in ZA-stimulated MC3T3-E1 cells.* The expression

levels of the pyroptosis-related proteins involved in the Nod-like receptor family pyrin domain-containing 3 (NLRP3)/caspase 1/gasdermin D (GSDMD) signaling pathway were detected in ZA-stimulated MC3T3-E1 cells to further investigate the effect of VEGF on BP-induced ONJ. As presented in Fig. 5, the expression of NLRP3 and caspase 1 was notably upregulated in ZA (1  $\mu$ M)-stimulated MC3T3-E1 cells when compared with

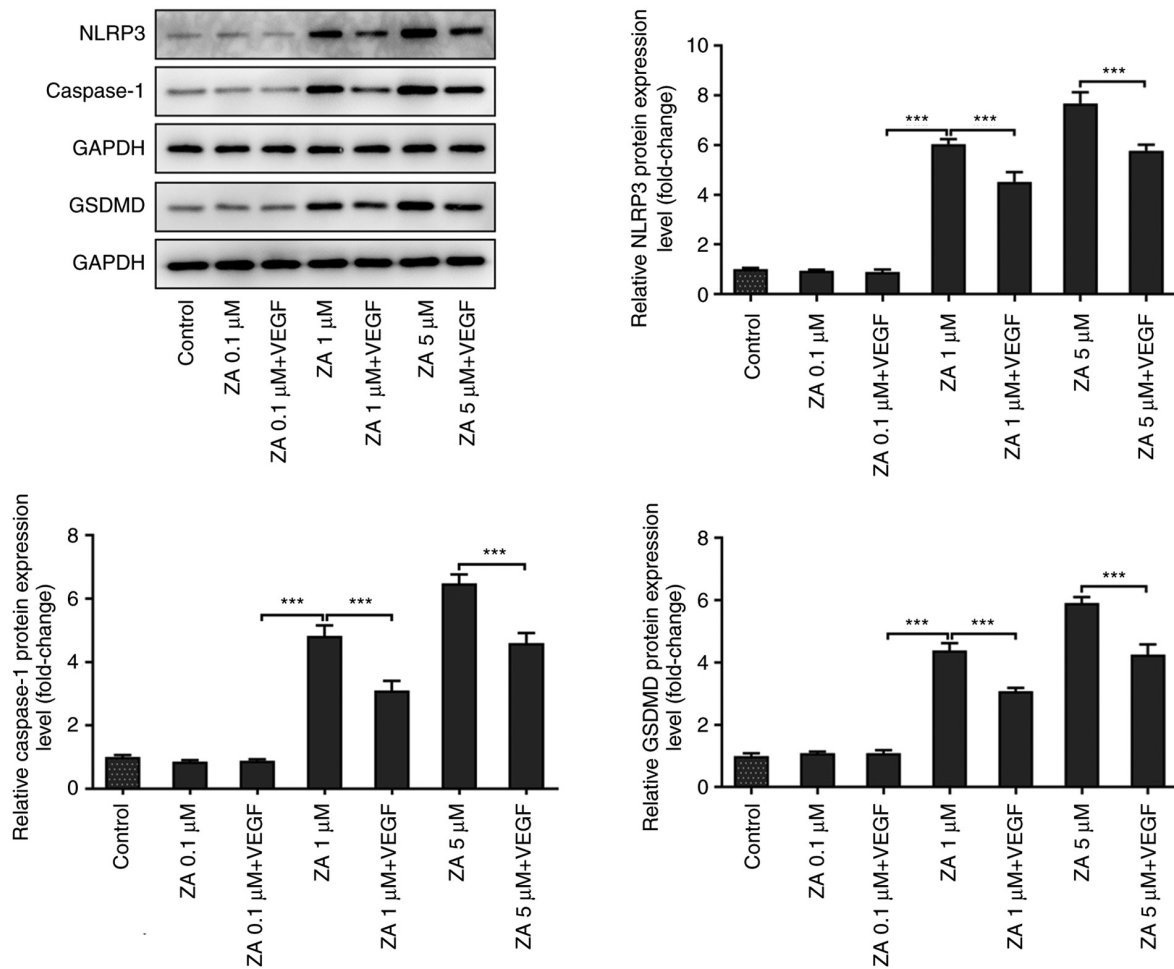


Figure 5. Expression levels of NLRP3, caspase-1 and GSDMD in ZA-stimulated MC3T3-E1 cells in the absence or presence of VEGF, detected by western blot assay. \*\*\* $P < 0.001$ . ZA, zoledronic acid; NLRP3, Nod-like receptor family pyrin domain-containing 3; GSDMD, gasdermin D.

Control group, and was restored after treatment with exogenous VEGF. However, the changes in the expression levels of GSDMD were not significantly different between the VEGF-treated and untreated ZA-stimulated MC3T3-E1 cells. Overall, the present findings suggested that VEGF addition could exert an inhibitory effect on the NLRP3/caspase 1/GSDMD signaling pathway in ZA-stimulated MC3T3-E1 cells.

## Discussion

BPs, including alendronate, risedronate, ZA and ibandronate, are a type of drug commonly used to treat patients with osteoporosis, since they can improve mineral density and reduce the risk of bone fractures (13). However, patients undergoing treatment with BPs may often develop ONJ, accompanied by jaw pain, tooth loss, oral infection or even osteomyelitis (14,15). Osteoblasts and osteoclasts interact with each other to renew bone tissues in order for the sequestrum to be resorbed and regenerated. It has been suggested that BP-induced ONJ may be associated with the accumulation of BP on the bone surface, thus attenuating the bone resorption ability via inhibiting osteoclast viability. Therefore, the reduced absorption capacity of the bones could be maintained for a long time after the withdrawal of BP therapy (16-18). It has been previously reported that *in vivo* treatment of osteocytes with ZA reduced

their number and function (19,20). Another study demonstrated that osteoblast treatment with 1-100  $\mu$ M of BP exerted a suppressive effect on their proliferation, differentiation and mineralization (21). Consistent with the aforementioned studies, the present study demonstrated that stimulation of MC3T3-E1 cells with  $\geq 1$   $\mu$ M ZA significantly inhibited cell viability and differentiation, and promoted MC3T3-E1 cell apoptosis.

VEGF serves a key role in regulating blood vessel growth and angiogenesis, but is also involved in osteogenesis (22). A previous study indicated that patients with BP-induced ONJ exhibited reduced VEGF serum levels. Therefore, VEGF levels are currently used as a biomarker for BP-induced ONJ (23). In the present study, the expression of VEGF was gradually decreased in MC3T3-E1 cells after treatment with increasing concentrations of ZA. It has been also reported that VEGF could regulate osteoblast survival (24). For example, a study on osteoarthritis revealed that the expression of VEGF was downregulated by ACY-1215, a histone deacetylase 6 inhibitor, to promote the apoptosis of osteoblasts (25). In the current study, VEGF revitalized ZA-stimulated MC3T3-E1 cells, as evidenced by the reduced apoptotic cell percentage and expression of the pro-apoptotic protein Bax, and the increased expression of the anti-apoptotic protein Bcl2. Another study revealed that, during the bone healing process, VEGF could

improve osteoblast differentiation and facilitate bone formation (26). The same osteogenic effects of VEGF were observed in the mineralization and differentiation of adipose-derived stem cells (27). The results of the present study indicated that ZA-stimulation impeded the mineralization of MC3T3-E1 cells and reduced ALP activity. However, VEGF treatment partially reversed the aforementioned effects. Furthermore, the reduced expression levels of differentiation-related proteins in ZA-stimulated MC3T3-E1 cells were also restored following cell treatment with exogenous VEGF. These findings indicated that VEGF could alleviate ZA-induced apoptosis and abrogate the inhibitory effect of ZA on the differentiation of murine osteoblasts.

To further examine the effects of VEGF, the present study also investigated whether VEGF could affect the NLRP3/caspase 1/GSDMD signaling pathway. Emerging evidence has reported that NLRP3 and its downstream target, caspase 1, are closely associated with disc degeneration (28). In addition, another study demonstrated that BPs could activate NLRP3/caspase 1 signaling to induce osteoporosis in diabetic mice (28,29). Likewise, the results of the present study indicated that the expression of both NLRP3 and caspase 1 was upregulated in ZA-stimulated MC3T3-E1 cells, which was suppressed following VEGF treatment. Furthermore, it has also been reported that increased pyroptosis in the alveolar bone was associated with reduced osteoblast differentiation capacity (30). However, the lack of evidence to support the role of ZA in NLRP3-mediated pyroptosis by VEGF requires more extensive studies in osteoblasts, and whether pyroptosis is involved in the occurrence of ONJ remains unknown. VEGF exerted a mitigatory effect on hepatocyte pyroptosis, thus protecting the liver from renal allograft ischemia-reperfusion injury (31); however, in the current study, ZA stimulation or VEGF treatment had no significant effects on the expression of the pyroptosis-related protein GSDMD in MC3T3-E1 cells.

Taken together, the present results suggested that VEGF addition could alleviate cell apoptosis, suppress the activation of the NLRP3/caspase 1 axis and reverse ZA-mediated inhibition of MC3T3-E1 cell differentiation. These findings could provide novel insights into the potential role of VEGF as a therapeutic target in the treatment of BP-induced ONJ.

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## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

YD, HJL, XHD, ZLG, YYX and YL contributed to the conception and design of the work and the acquisition, analysis and interpretation of data. YD, HJL and YL drafted the manuscript and revised it critically for important intellectual content. All authors read and approved the final version of the manuscript. YD, HJL and YL confirm the authenticity of all the raw data.

## Ethics approval and consent to participate

Not applicable.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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