Vitamin K2 stimulates MC3T3-E1 osteoblast differentiation and mineralization through autophagy induction

WEIWEI LI[1], SHAOKUN ZHANG[1], JIE LIU[2], YONGYI LIU[1] and QINGWEI LIANG[3]

[1]Department of Orthopedics, The First Hospital of China Medical University, Shenyang, Liaoning 110000;
[2]Science Experiment Center of China Medical University, Shenyang, Liaoning 110122;
[3]Department of Sports Medicine, The First Hospital of China Medical University, Shenyang, Liaoning 110000, P.R. China

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Abstract. Vitamin K2 likely exerts its protective effects during osteoporosis by promoting osteoblast differentiation and mineralization. However, the precise mechanism remains to be fully elucidated. Autophagy maintains cell homeostasis by breaking down and eliminating damaged proteins and organelles. Increasing evidence in recent years has implicated autophagy in the development of osteoporosis. The aim of the present study was to verify whether vitamin K2 (VK2) can induce autophagy during the differentiation and mineralization of osteoblasts. In the present study, MC3T3-E1 osteoblasts were treated with various doses of VK2 (10^{-8}-10^{-3} M) for 1-5 days. The results revealed no cytotoxicity at concentrations below 10^{-5} M, but cell viability was reduced in a dose-dependent manner at concentrations above 10^{-5} M. Furthermore, MC3T3-E1 osteoblasts were seeded in 6-well plates in complete medium supplemented with dexamethasone, β-glycerophosphate and vitamin C (VC) for osteogenic differentiation. MC3T3-E1 osteoblasts treated with different concentrations (10^{-5}, 10^{-6} and 10^{-7} M) of VK2 for 24 h on days 1, 3, 5 and 7 of the differentiation protocol. It was confirmed that VK2 promoted osteoblast differentiation and mineralization by using alkaline phosphatase (ALP) and alizarin red staining. Using western blotting, immunofluorescence, monodansylcadaverine staining and reverse transcription-quantitative polymerase chain reaction, it was observed that VK2 induced autophagy in osteoblasts. The results revealed that VK2 (1 μM) significantly increased ALP activity and the conversion of microtubule associated protein 1 light chain 3-α (LC3) II to LC3I in MC3T3-E1 osteoblasts ($P<0.05$) at every time point. The number of fluorescent bodies and the intensity increased with VK2, and decreased following treatment with 3-MA+VK2. There was an increase in the mRNA expression levels of ALP, osteocalcin (OCN) and Runt-related transcription factor 2 in VK2-treated cells ($P<0.01$). The present study further confirmed the association between autophagy and osteoblast differentiation and mineralization through treatment with an autophagy inhibitor [3-methyladenine (3-MA)]. Osteoblasts treated with 3-MA exhibited significant inhibition of ALP activity and osteogenic differentiation (both $P<0.05$). In addition, ALP activity and osteogenesis in the VK2+3-MA group was lower compared with VK2-treated cells ($P<0.05$ for both). The present study confirmed that VK2 stimulated autophagy in MC3T3 cells to promote differentiation and mineralization, which may be a potential therapeutic target for osteoporosis.

Correspondence to: Professor Qingwei Liang, Department of Sports Medicine, The First Hospital of China Medical University, 155 North Nanjing Street, Shenyang, Liaoning 110000, P.R. China
E-mail: cmulqw@163.com

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Introduction

Osteoporotic fractures are increasingly gaining attention due to their high incidence rates and economic burden (1). Osteoporosis is a pathological condition characterized by a decrease in bone mineral density or bone mass, which can reduce bone strength and lead to fractures. Clinical osteoporosis is classified into two types: Type I, or postmenopausal osteoporosis; and type II, i.e., osteoporosis in the elderly (1,2). Bone homeostasis depends on the synergistic activities of osteoclasts and osteoblasts, and any imbalance between bone formation and resorption may lead to a number of diseases, including osteoporosis, Paget's bone disease and osteodystrophy (3). Apart from decreased bone mass, osteoporosis is associated with a slower rate of bone differentiation following an increase in the number of adipocytes and osteoclasts, decreased osteoblasts and enhanced bone resorption (4,5). In clinical terms, the prevention of osteoporosis is particularly significant and includes resistance exercises to increase bone density, adequate calcium in the diet, and calcium supplement medications (6). In recent years, vitamin K2 (VK2) has attracted attention as an auxiliary drug for preventing osteoporosis (7).

VK2 is a fat-soluble vitamin that is structurally similar to other members of the same family, including phylloquinone, menaquinones and menadione (7). It is a critical factor in the blood clotting process (8-10), and epidemiological studies have demonstrated that a lack of VK2 may lead to osteoporosis and osteoarthritis in older individuals (10,11). In addition, VK2

has been clinically utilized to prevent osteoporosis, and likely exerts its protective effects by promoting osteoblast differentiation and mineralization (12-14). However, the precise mechanism remains to be fully elucidated (7).

Increasing evidence in recent years has implicated autophagy in the development of osteoporosis. Autophagy is an essential process that maintains homeostasis in eukaryotic cells by breaking down and eliminating damaged proteins and organelles (15,16). When a cell is subjected to external stress, it may initiate autophagy to adjust to the associated stimulus by degrading cytoplasmic substances to provide energy for cell survival (17,18). Autophagy has a critical role in physiological conditions, and is associated with certain diseases, including cancer, diabetes and leukemia. A lack of autophagy in osteoblasts can decrease mineralizing capacity, and induce an imbalance in the population of osteoblasts and osteoclasts, resulting in a low bone mass phenotype (19). The aim of the present study was to verify whether VK2 is able to promote the differentiation and mineralization of osteoblasts by inducing autophagy.

Materials and methods

Chemical reagents. α-Modification minimum essential medium (α-MEM) was purchased from HyClone (GE Healthcare Life Sciences, Logan, UT, USA). Fetal bovine serum (FBS) was acquired from Clark Bioscience (Claymont, DE, USA). The MTT Cell Proliferation and Cytotoxicity Assay kit was purchased from Nanjing Keygen Biotech Co., Ltd. (Nanjing, China). The Alkaline Phosphatase (ALP) Assay kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). VK2, vitamin C (VC), and dexamethasone (Dex) were acquired from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). β-Glycerophosphate and alizarin red were purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). The autophagy inhibitor 3-methyladenine (3-MA) and agonist rapamycin were purchased from Selleck Chemicals (Houston, TX, USA). Rabbit anti- microtubule associated protein 1 light chain 3-α (LC3)b antibody was purchased from Abcam (Cambridge, UK; cat. no. ab192890). Rabbit anti-β-actin antibody and goat anti-rabbit IgG antibody were purchased from BIOSS (Beijing, China; cat. nos. bs-0061R and bs-0295G, respectively). Anti-beclin 1 antibody was purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA; cat. no. 3738S) and Cy3-conjugated Affinipure Goat Anti-Rabbit IgG (H+L) was obtained from Wuhan Sanying Biotechnology (Wuhan, China; cat. no. SA00009-2).

Cell culture and differentiation. MC3T3-E1 is a preosteoblast cell line. It may be used to study differential and mineral induction in an experimental system that mimics a bone-like extracellular matrix (ECM) environment. The appearance of the ECM is crucial to the bone formation process. The mouse cranial osteoblast (MC3T3-E1 Subclone 14; American Type Culture Collection CRL-2594) cell line was obtained from Cell Bank of the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). A subclone with high differentiation potential was derived from the parental cell line for studying critical events during osteoblast differentiation and mineralization. The cells were seeded in 15 cm^2 flasks in α-MEM containing 1% antibiotics and 10% FBS (complete medium), and the medium was changed every 2 days. Cells were passaged when they reached 90% confluence. For osteogenic differentiation, the osteoblasts were first seeded in 6-well plates at a density of $1x10^4$ cells/well in complete medium and cultured for 3 days until the cells reached 70% confluence. To initiate the differentiation, 3 ml complete medium supplemented with Dex (10^{-7} M), β-glycerophosphate (10 mM) and VC (50 μg/ml) was added to each well, and the differentiation medium was replaced every 2 days.

Cell viability assay. The cell viability assay was performed to analyze the possible cytotoxic effects of different concentrations of VK2 (10^{-8}-10^{-3} M) on MC3T3-E1 cells. The MC3T3-E1 osteoblasts were seeded into 96-well plates. When 70% confluence was achieved, cells were treated with VK2 at different concentrations (10^{-8}-10^{-3} M) for different durations (1-5 days). At least six replicated wells per sample were prepared. The cell viability was measured at 48 h using the MTT Cell Proliferation and Cytotoxicity Assay kit, according to the manufacturer's protocol.

Quantitative assay of ALP activity. MC3T3-E1 osteoblasts were plated in 6-well plates at a density of $2.5x10^5$ cells/well and treated with different concentrations (10^{-5}, 10^{-6} and 10^{-7} M) of the drugs (3MA, VK2 and 3MA+VK2; untreated controls were also included) for 24 h during the differentiation protocol, and harvested after 1, 3, 5 and 7 days. Total protein was extracted from the cells using radioimmunoprecipitation assay (RIPA) buffer [300 mM NaCl, 50 mM Tri-HCL (pH 7.6), 0.5% Triton X-100, 2 mM phenylmethylsulfonyl fluoride] and quantified using the bicinchoninic acid (BCA) assay. ALP activity was measured using a fluorescence detection kit per the manufacturer's protocol. The standard curve was plotted using p-nitrophenol, and the ALP activity of each sample was normalized to the total protein concentration.

Mineralization analysis. MC3T3-E1 osteoblasts were treated with the different drugs as described above, during the 7-day differentiation. On the 7th day, the supernatant was discarded, and the cells were washed twice with PBS and fixed with 4% paraformaldehyde at room temperature (20˚C) for 5 min. Calcium nodes were stained with 0.1% alizarin red at 37˚C for 1 h. For quantitative analysis, cells were destained with ethylpyridinium chloride for 30 min at room temperature and transferred to a 96-well plate to measure the absorbance at 550 nm using a microplate reader.

Western blotting. MC3T3-E1 osteoblasts were seeded into 100 mm dishes and cultured in complete α-MEM to 70% confluency, following which the cells were treated with the different drugs as described above. VK2 was used at the optimal concentration of 10^{-6} M (1 μM). Total proteins were extracted following lysis of the cells in RIPA buffer at 4˚C for 1 h. The lysates were centrifuged at 20,000 x g for 30 min, and the protein concentration was determined using a BCA protein quantification kit. Equal amounts of protein (30 μg) were loaded into each well of a 7.5-15% SDS-PAGE gel and

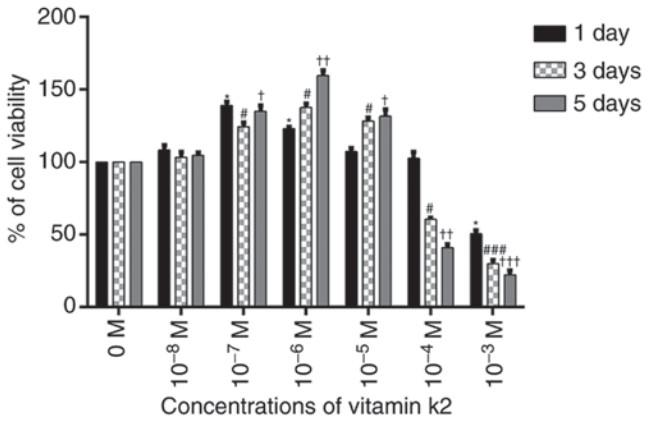

Figure 1. Cytotoxic effect of VK2 on MC3T3-E1 cells. Different concentrations of VK2 (10^{-8}-10^{-3} M) were used to stimulate the MC3T3-E1 cells for 1-5 days. Concentrations of VK2 between 10^{-7}-10^{-5} M did not cause cytotoxicity. However, concentrations above 10^{-5} M significantly reduced cell viability in a dose-dependent manner. Compared with the control, the concentration of 10^{-8} M had no significant effect on the cell. The experimental data are expressed as the mean ± standard deviation. Significance analysis of the experimental data for each group was performed using one-way analysis of variance and Tukey's multiple comparisons test. *P<0.05 vs. control group at 1 day; #P<0.05, ###P<0.001 vs. control group at 3 days; †P<0.05, ††P<0.01, †††P<0.001 vs. control group at 5 days. VK2, vitamin K2.

separated by electrophoresis. The separated protein bands were transferred to a polypropylene fluoride membrane and, following incubation overnight at 4˚C with primary antibody against LC3b (1:2,000), beclin 1 (1:1,000) and β-actin (1:2,000), respectively. Next, the membrane was incubated with goat anti-rabbit secondary antibody (1:5,000) for 2 h at room temperature. The specific bands were visualized using an enhanced chemiluminescence detection system (MF-ChemiBIS 3.2, DNR Bio-Imaging Systems, Ltd., Neve Yamin, Israel) and imaged with an Alpha Imager HP (ProteinSimple, San Jose, CA, USA). The band density was quantified using the ImageJ image processing program (National Institutes of Health, Bethesda, MD, USA. software version 1.5b).

Immunofluorescence assay. MC3T3-E1 osteoblasts were seeded in 24-well plates and upon reaching 50% confluency, were treated with 3-MA, VK2 and VK2+3MA for 1 h. The cells were washed twice with PBS, fixed with 4% paraformaldehyde at room temperature for 15 min, and permeabilized with 0.5% Triton X-100 for 2 min at room temperature. After blocking for 2 h with 5% bovine serum albumin (cat. no. ST023-50 g, Beyotime Institute of Biotechnology, Shanghai, China) at room temperature, the cells were incubated overnight with anti-LC3b antibody (1:200) at 4˚C. After 1 h incubation with Cy3-conjugated secondary antibody (1:3,000), the cells were counterstained with DAPI for 10 min at room temperature. The stained cells were imaged using confocal fluorescence microscopy (magnification, x200, DS-U3 Nikon Eclipse CI; Nikon Corporation, Tokyo, Japan).

Monodansylcadaverine (MDC) staining. The cells were seeded in 24-well plates at a density of $4.0x10^4$ cells/well, and VK2 (1 μM) and the autophagy inhibitor 3-MA (0.5 mM) were added. The supernatant was removed and after washing the cells once, they were stained with MDC (a fluorescent marker of autophagic vacuoles) for 45 min at 37˚C. The stained cells were imaged using an Olympus fluorescence microscope (magnification, x200, IX71; Olympus Corporation, Tokyo, Japan).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). MC3T3-E1 osteoblasts were seeded at a density of $5x10^4$ cells/well in a 6-well plate for 3 days until the cells reached 70% confluence. The cells were treated with the different drugs as described, and total RNA was extracted using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocol. A total of ~1 μg of RNA was used in the reverse transcription reaction using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. For gene-specific primed cDNA synthesis, the reaction was conducted for 60 min at 42˚C. Then, for random hexamer primed synthesis, incubate for 5 min at 25˚C. RT was terminated by heating at 70˚C for 5 min. Real-time PCR was performed using the SYBR Green PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) on the ABI 7500 Fast Real-Time PCR System (Applied Biosystems 7500 System Sequence Detection System, software version 2.6.2; Thermo Fisher Scientific, Inc.). The PCR conditions were as follows: Initial denaturation at 95˚C for 15 min, followed by 40 cycles of denaturation at 94˚C for 20 sec, annealing at 60˚C for 30 sec and extension at 72˚C for 60 sec. The primer sequences were as follows: Runt related transcription factor 2 (Runx2) forward, 5'-CCCTGAACT CTGCACCAAGT-3'; Runx2 reverse, 5'-TGGAGTGGA TGGATGGGGAT-3'; osteocalcin (OCN) forward, 5'-AGC AGCTTGGCCCAGACCTA-3'; OCN reverse, 5'-TAGCGC CGGAGTCTGTTCACTAC-3'; β-actin forward, 5'-TTC GTTGCCGGTCCACACCC-3'; and β-actin reverse, 5'-GCT TTGCACATGCCGGAGCC-3'.

Statistical analysis. All experiments were repeated at least three times and the results are expressed as the mean ± standard deviation. Statistical analysis was performed using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). Different groups were compared by one-way analysis of variance followed by Tukey's multiple comparisons test. P<0.05 was considered to indicate a statistically significant difference.

Results

VK2 is not cytotoxic to MC3T3-E1 cells at concentrations below 10^{-5} M. An MTT assay was used to analyze the possible cytotoxic effects of different concentrations of VK2 (10^{-8}-10^{-3} M) on MC3T3-E1 cells. The cells were stimulated for different durations (1-5 days). No cytotoxicity was observed at concentrations below 10^{-5} M, but cell viability was reduced in a dose-dependent manner at concentrations above 10^{-5} M. Furthermore, compared with the control group, 10^{-8} M VK2 did not have a significant effect on the induction of differentiation and mineralization. Therefore, all subsequent experiments were performed with VK2 at concentrations between 10^{-7} and 10^{-5} M (Fig. 1).

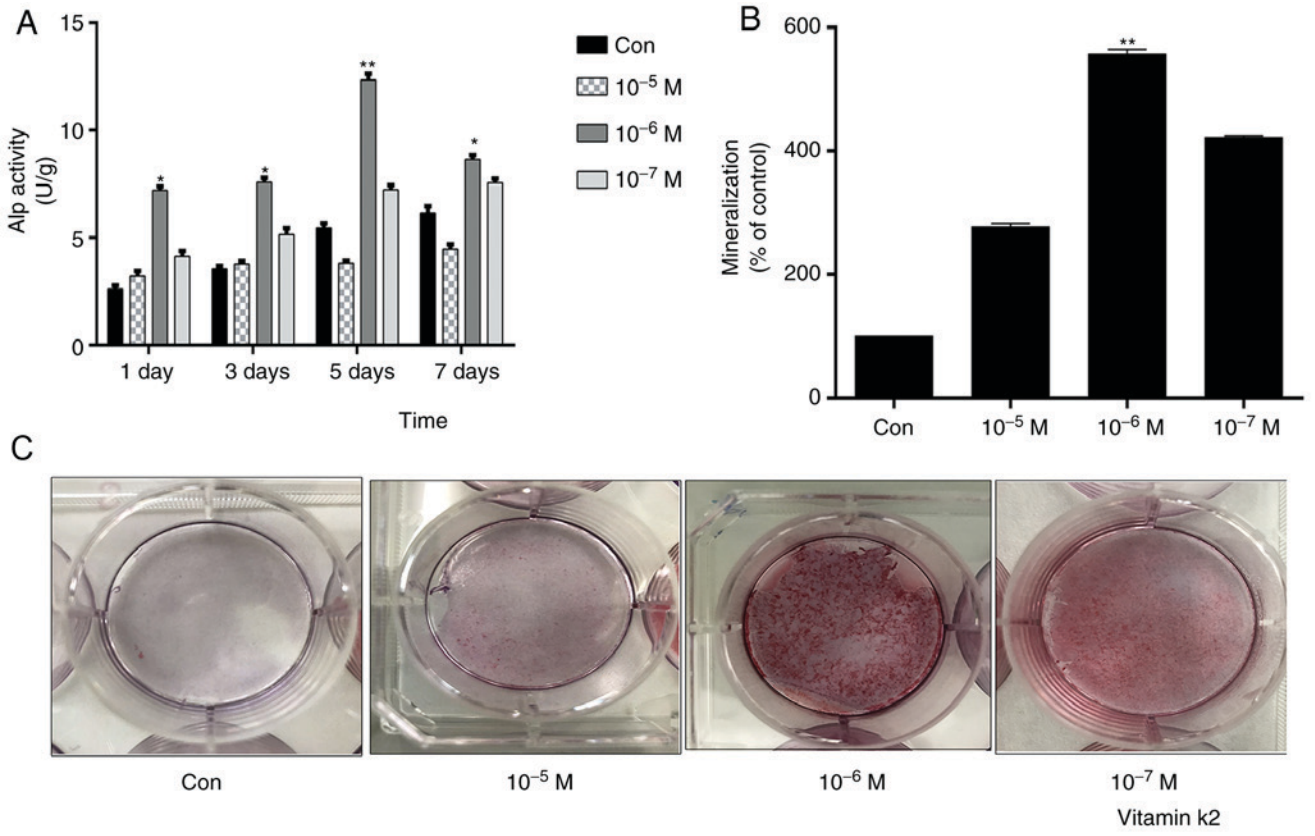

Figure 2. VK2 enhances MC3T3-E1 osteoblast differentiation and mineralization. (A) ALP activity was significantly increased in MC3T3-E1 osteoblasts treated with 1 μM VK2 compared with the untreated control, and this was not observed with the other concentrations. ALP activity was even more significantly enhanced in the VK2-treated cells on day 5 compared with the untreated control. (B) Osteoblasts treated with 1 μM VK2 had a significantly higher number of alizarin red-stained mineralized nodules compared with untreated control cells. (C) Representative images of alizarin red staining. The experimental data are expressed using the mean ± standard deviation. Significance analysis of the experimental data for each group was performed using one-way analysis of variance and Tukey's multiple comparisons test. *P<0.05, **P<0.01 vs. respective control group. VK2, vitamin K2; ALP, alkaline phosphatase; Con, control.

VK2 enhances ALP activity and promotes MC3T3-E1 osteoblast differentiation and mineralization. To determine the optimal concentration of VK2 that promotes MC3T3-E1 osteoblast differentiation and mineralization, an ALP assay and alizarin red staining were performed, respectively, following treatment of the cells with 10^{-7}-10^{-5} M VK2 for 1-7 days. Treatment with 1 μM VK2 significantly increased ALP activity, while other concentrations were not notably effective. In addition, maximum ALP activity with 1 μM VK2 was observed on the 5th day (Fig. 2A). Bone formation is accompanied by osteogenic differentiation and mineralization, i.e., calcium deposition. MC3T3-E1 osteoblasts treated with VK2 (1 μM) for 7 days exhibited significant calcium deposition, as detected by alizarin red staining (Fig. 2B and C).

VK2 stimulates autophagy in MC3T3-E1 osteoblasts. To analyze the effects of VK2 on autophagy in MC3T3-E1 osteoblasts, the cells were treated with VK2 (1 μM) for 0.5-1.5 h (each day for 7 days), various autophagy markers, including LC3 and Beclin-1, were detected on days 1, 3, 5 and 7 of differentiation. The ratio of LC3II/LC3I is frequently analyzed to determine the extent of autophagy. Treatment with VK2 for 0.5-1.5 h steadily increased LC3I conversion rates in the osteoblasts on days 1, 3, 5 and 7 (Fig. 3A). In addition, VK2 treatment for 1 h significantly increased the conversion of LC3I at 1, 3, and 5 days (Fig. 3B-D). Furthermore, the LC3II/LC3I ratio and Beclin-1 levels were higher on day 5, corresponding to the strongest ALP activity (Fig. 3G). Autophagy was also evaluated using immunofluorescence and MDC staining assays. The number of fluorescent bodies and the intensity increased with VK2 and decreased following treatment with 3-MA+VK2. MDC is a marker for autolysosomes; the results demonstrated that the VK2 group had stronger fluorescence, and the fluorescence intensity of the 3-MA+VK2 group was markedly reduced (Fig. 3H). The inhibitory effect of 3-MA on VK2-induced autophagy was confirmed with the significantly decreased LC3II/LC3I ratio in cells treated with 3-MA, and significantly higher ratio in cells treated with rapamycin. Notably, the conversion rate of LC3II/LC3I in the VK2+3-MA group was lower compared with the VK2 group (Fig. 4A). These results demonstrated that VK2 stimulated autophagy in MC3T3-E1 osteoblasts.

3-MA inhibits the osteogenic differentiation and mineralization induced by VK2. To confirm the role of autophagy in VK2-induced osteogenic differentiation and mineralization, the ALP assay and alizarin red staining were performed on cells treated additionally with 3-MA. The 3-MA treated osteoblasts (3-MA+VK2 group) exhibited significantly reduced ALP activity compared with the VK2 group (Fig. 4B). In addition, 3-MA slightly inhibited the mRNA expression of osteogenic differentiation markers compared with the control

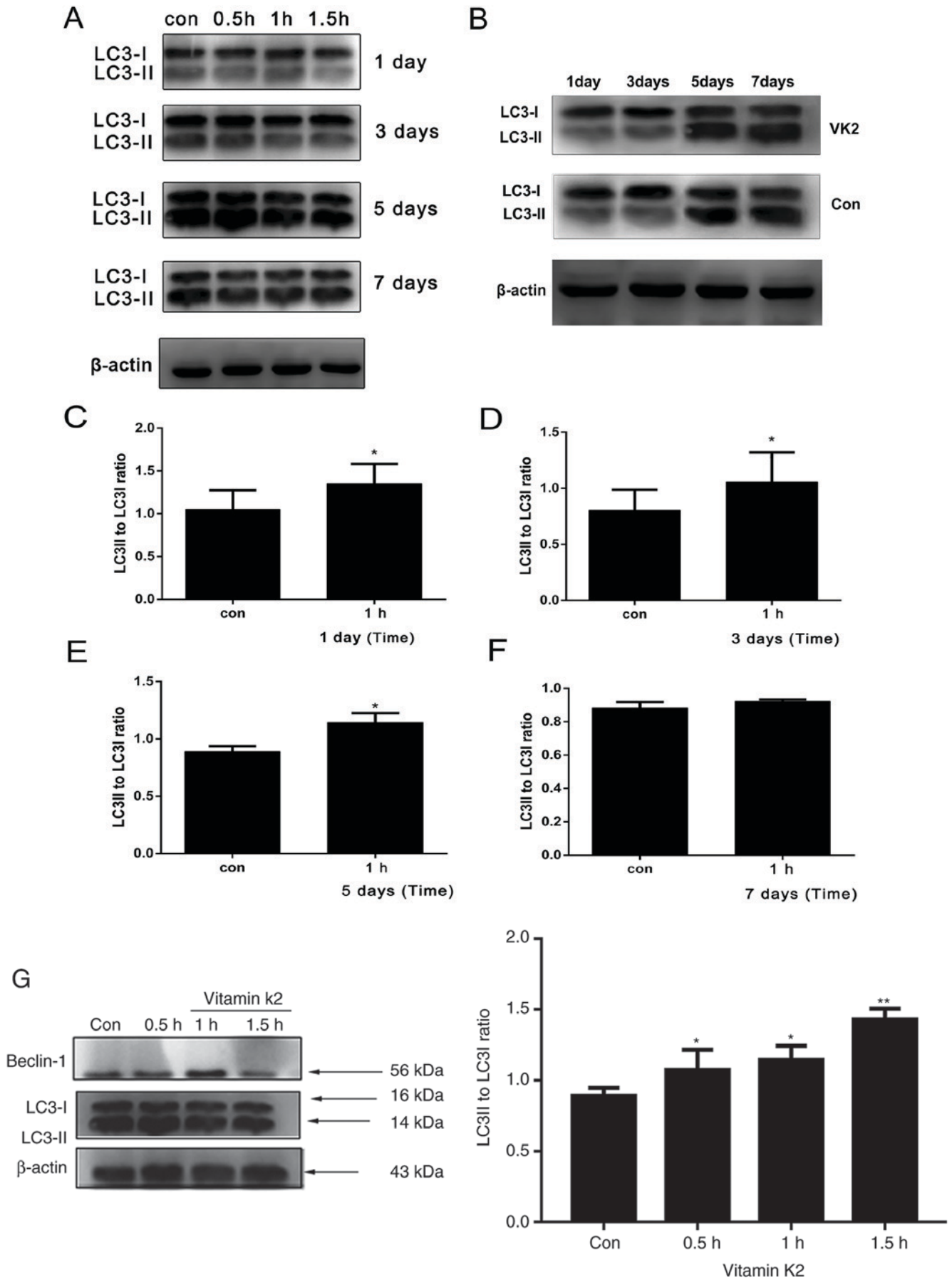

Figure 3. VK2 stimulates autophagy in MC3T3-E1 osteoblasts. The degree of autophagy was evaluated on the basis of LC3I to LC3II conversion (LC3II/LC3I). (A) Treatment with 1 μM VK2 for 0.5-1.5 h steadily increased LC3I conversion rates in MC3T3-E1 osteoblasts over (B) 1, 3, 5 and 7 days. Compared with the untreated control cells, those treated with VK2 for 1 h exhibited a significantly higher degree of autophagy at (C) 1, (D) 3 and (E) 5 days, although not at (F) 7 days. (G) Higher LC3II/LC3I conversion rates were observed on day 5 after 0.5, 1 and 1.5 h of treatment compared with the untreated controls.

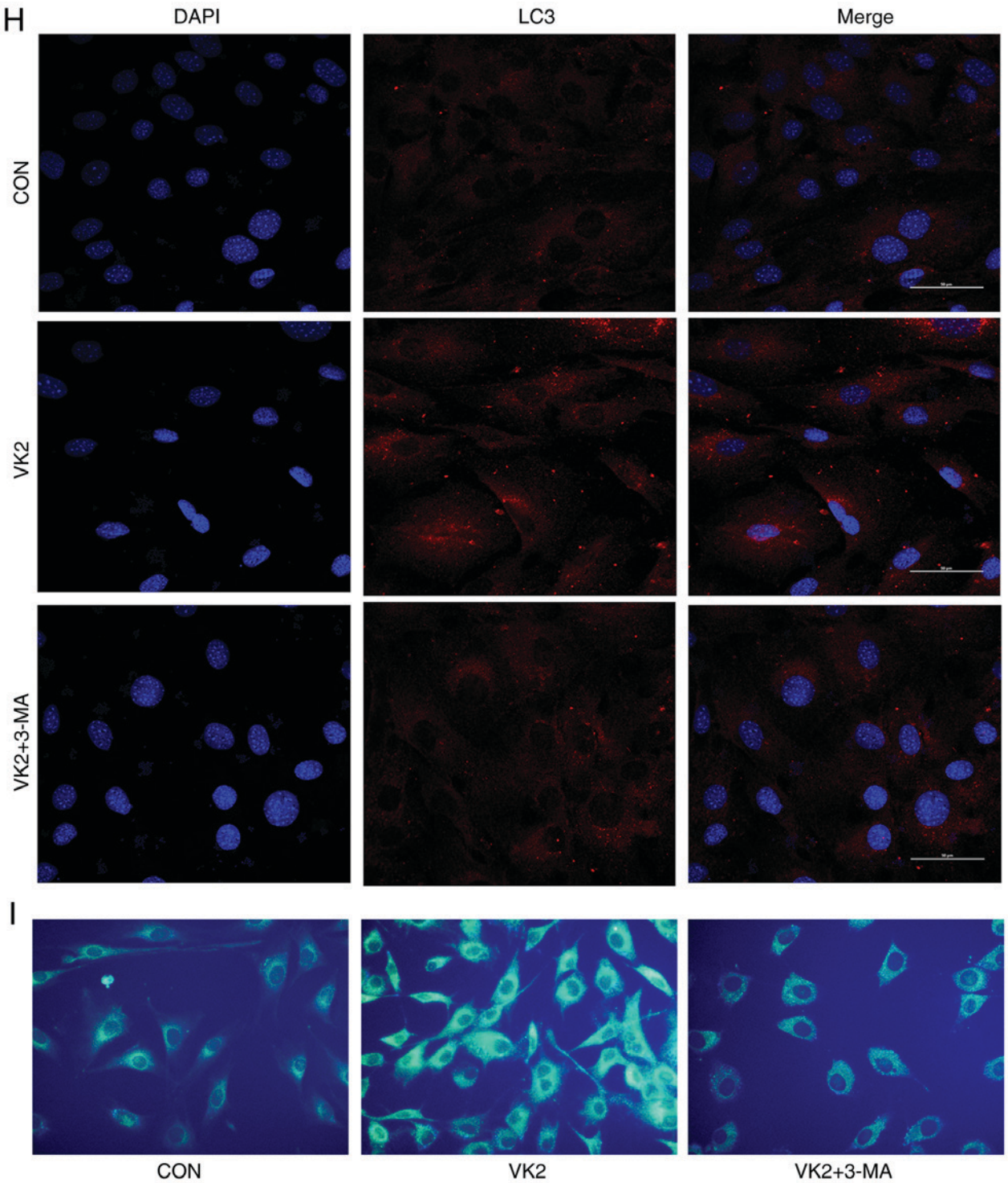

Figure 3. Continued. (H) To further verify the effect of vitamin K2 on autophagy in MC3T3-E1 osteoblasts, cells were stained in situ with fluorescence labeled anti-LC3b. The number of fluorescent bodies and the fluorescence intensity increased with VK2 and decreased following treatment with 3-MA+VK2 compared with the untreated controls. (I) MDC is a marker for autolysosomes. MDC staining was used to confirm the abundance of autophagic vacuoles in VK2-treated cells. In the control group, weak and diffuse MDC staining was observed throughout the cytoplasm, and very little punctate staining. In VK2-treated cells, the MDC staining was visibly enhanced, and its distribution pattern was altered from diffuse to punctate accumulation. The punctate staining in the 3-MA+VK2 treatment group was markedly reduced. Scale bars, 50 μm. The experimental data are expressed as the mean ± standard deviation. Significance analysis of the experimental data for each group was performed using one-way analysis of variance and Tukey's multiple comparisons test. *P<0.05, **P<0.01 vs. respective control group. VK2, vitamin K2; ALP, alkaline phosphatase; 3-MA, 3-methyladenine; LC3, microtubule associated protein 1 light chain 3-α; Con, control; MDC, monodansylcadaverine.

(Fig. 4C). 3-MA inhibited the mineralization induced by VK2 (Fig. 4D). These results demonstrated that autophagy induced by VK2 stimulation is involved in osteogenic differentiation and mineralization.

Discussion

MC3T3-E1 osteoblasts were used to study the mechanism of VK2-mediated amelioration of osteoporosis

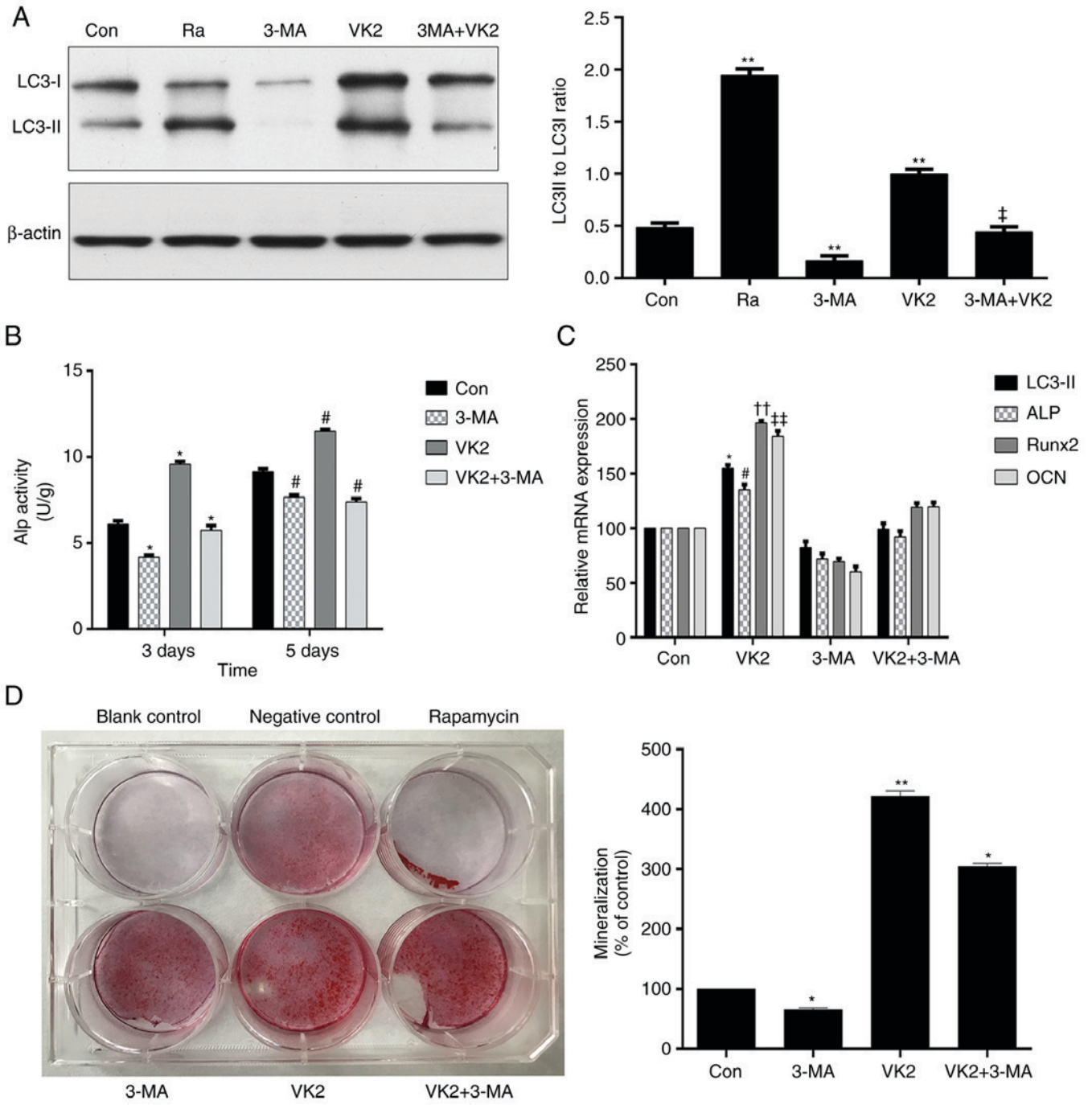

Figure 4. Association between autophagy and differentiation in MC3T3-E1 osteoblasts. (A) To further verify the inhibitory effect of 3-MA on autophagy mediated by VK2, the western blotting results demonstrated that the conversion rate of LC3II/LC3I in cells treated with the autophagy inhibitor 3-MA was significantly decreased, while that of rapamycin was significantly higher. The conversion rate of LC3II/LC3I in VK2+3-MA group was lower compared with the VK2 group. **P<0.01 vs. control group and ‡P<0.05 vs. VK2 group. (B) Compared with the untreated control cells, the 3-MA treated osteoblasts exhibited a significant inhibition of ALP activity, and the ALP activity of the VK2+3-MA treated cells was significantly lower compared with the VK2-treated cells. *P<0.05 vs. control group at 3 days; $^{\#}$P<0.05 vs. control group at 5 days. (C) The mRNA expression levels of LC3II, ALP, OCN and Runx2 in VK2-treated cells increased. *P<0.05 vs. LC3II control; $^{\#}$P<0.05 vs. ALP control; ††P<0.01 vs. Runx2 control; ‡‡P<0.01 vs. OCN control. (D) In addition, alizarin red staining indicated that 3-MA markedly inhibited the osteogenic differentiation induced by VK2. The experimental data are expressed as the mean ± standard deviation. Significance analysis of the experimental data for each group was performed using one-way analysis of variance and Tukey's multiple comparisons test. *P<0.05, **P<0.01 vs. control group. VK2, vitamin K2; 3-MA, 3-methyladenine; Ra, rapamycin; Con, control; ALP, alkaline phosphatase; Runx2, runt related transcription factor 2; OCN, osteocalcin; LC3, microtubule associated protein 1 light chain 3-α.

symptoms, as this cell line is routinely used to study the osteogenic characteristics of bone differentiation and mineralization *in vitro*. Biomarkers including ALP, RUNX2 and OCN are upregulated in the bone matrix during osteogenic differentiation and mineralization, and are thus used to assess the these processes in *in vitro* models (20,21). Osteoblasts were stimulated with different concentrations of VK2, and osteogenic differentiation and mineralization were evaluated by ALP activity and alizarin red staining, respectively; the concentration of 1 μM induced the most

significant effect on osteoblast differentiation. The present results are consistent with previous studies that have correlated VK2 with increased osteoblast differentiation and mineralization (22-24). However, the specific mechanism of VK2 in promoting bone metabolism has not yet been elucidated. It was demonstrated that VK2 induced autophagy during osteoblast differentiation and mineralization using the markers LC3 and beclin 1. Studies have increasingly demonstrated a role for autophagy in the occurrence and development of osteoporosis. Studies on human genome-wide association data report that autophagy-related genes are associated with osteoporosis (15,25). In addition, the degree of autophagy decreases along with the expression of LC3-II, beclin 1 and unc-51 like autophagy activating kinase 1, while apoptosis and p62 expression increases during osteoporosis (16,26,27). Decreased autophagy likely promotes osteoporosis by increasing oxidative stress (28,29). Furthermore, certain studies have demonstrated that autophagy is associated with the differentiation of osteoblasts. It has been demonstrated that autophagy-associated proteins are involved in osteoblast differentiation and bone formation, and other studies have reported that autophagy affects osteoblast mineralization and bone cell network structure (30,31). Finally, mouse knockout models of autophagy genes illustrate a significant decrease in trabecular bone volume, trabecular number and trabecular thickness (30,32). The present study also confirmed that VK2 may stimulate autophagy in MC3T3 cells to promote differentiation and mineralization.

The use of MC3T3 cells is the primary limitation of the present study; these cells are appropriate for the purpose of this research and numerous studies have used MC3T3-E1 to assess differentiation and mineralization (33-36). However, there are limitations associated with performing experiments with a single cell line. The present results may be confirmed by using an additional cell line, such as mesenchymal stem cells, and additional factors may be used to evaluate autophagy in the future, including other types of autophagy inhibitors to exclude off-target effects, and *in vivo* experiments. Finally, the specific pathways associated with autophagy were completely explored. Therefore, more in-depth mechanistic studies are required.

In conclusion, the present findings indicated that VK2 promotes autophagy during the differentiation and mineralization of osteoblasts, which may be a potential therapeutic target for osteoporosis.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

WL and QL conceived and designed the study. WL, SZ and YL performed the experiments. WL and JL analyzed the data. WL was a major contributor in writing the manuscript. All authors read and approved the final manuscript.

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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