Protective effect of VK_2 on glucocorticoid-treated MC3T3-E1 cells

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Abstract. Glucocorticoids (GCs) contribute to the increased incidence of secondary osteoporosis and osteonecrosis, and medications for the prevention and treatment of these complications have been investigated for many years. Vitamin K_2 (VK_2) has been proven to promote bone formation both *in vitro* and *in vivo*. In this study, we examined the effects of VK_2 on dexamethasone (DEX)-treated MC3T3-E1 osteoblastic cells. We observed that VK_2 promoted the proliferation and enhanced the survival of dexamethasone-treated MC3T3-E1 cells. In addition, VK_2 upregulated the expression levels of osteogenic marker proteins, such as Runt-related transcription factor 2 (Runx2), alkaline phosphatase (ALP) and osteocalcin, which were significantly inhibited by dexamethasone. On the whole, our findings indicate that VK_2 has the potential to antagonize the effects of GCs on MC3T3-E1 cells, and may thus prove to be a promising agent for the prevention and treatment of GC-induced osteoporosis and osteonecrosis.

Introduction

Glucocorticoids (GCs) have been extensively used in the treatment of a variety of diseases, due to their potent anti-inflammatory effects. However, the long-term and excessive use of GCs is one of the most common causes of atraumatic osteonecrosis of the femoral head and likely increases the incidence of secondary osteoporosis (1). These complications are partially attributed to modifications in the bioactivity of bone marrow-derived stem cells, osteoblasts/osteocytes and osteoclasts (2-4). Previous studies have indicated that GCs antagonize Runt-related transcription factor 2 (Runx2) during the osteoblast differentiation of mesenchymal cells and inhibit the osteogenesis of bone marrow-derived stem cells (3,5).

GCs have also been reported to directly suppress the osteogenic differentiation of osteoblasts (6), induce osteoblast and osteocyte apoptosis, and decrease the number of bone-forming cells (7-9). Another study indicated that GC-induced bone resorption is caused by a direct effect of the GCs on extending the lifespan of osteoclasts (10).

Vitamin K (VK), whose active form has been demonstrated to be a coenzyme for γ-carboxylase, plays an important role in bone metabolism (11,12). There are two types of VK in nature, VK_1 (phylloquinone) and VK_2 (menatetrenone). VK_1 is a single compound and is primarily found in plants, while VK_2 is a series of vitamers with multiple isoprene units at the 3-position of the naphthoquinone and is named according to the number of these prenyl units (13,14). Studies have indicated that VK_2 has a more pronounced osteoprotective effect than VK_1 (15,16). In addition to the γ-carboxylation of osteocalcin (OCN), VK_2 has been proven to promote osteoblast proliferation (13) and the osteoblast-to-osteocyte transition *in vitro* (15,17-19), including OCN accumulation in the extracellular matrix, the upregulation of Runx2 and alkaline phosphatase (ALP), and the transcription of osteogenic genes. Additional studies also revealed the osteoprotective effects of VK_2 *in vivo*. Akiyama *et al* and Iwamoto *et al* observed that VK_2 prevented bone loss in rats with ovariectomy or sciatic neurectomies (20,21); bone healing was also promoted in the osteotomy model in the study by Iwamoto *et al* (22). Based on these findings, VK_2 has been used in the treatment of osteoporosis in Asian countries for a number of years (23,24).

Several studies have reported the protective effects of VK_2 on prednisolone-treated rats (22,25,26); however, few studies have reported similar findings *in vitro*. Thus, the purpose of this study was to examine the effects of VK_2 on GC-treated osteoblasts.

Materials and methods

Chemicals. The cell culture medium, Dulbecco's modified Eagle's medium (DMEM; low glucose, 1 g/l), was obtained from HyClone, Logan, UT, USA. Fetal bovine serum (FBS) and the penicillin-streptomycin solution (10,000 U/ml penicillin; 10 mg/ml streptomycin) were purchased from Gibco Laboratories (Grand Island, NY, USA). VK_2, L-ascorbic acid and β-glycerophosphate disodium salt hydrate were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dexamethasone (DEX) was obtained from Sigma and was

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used at a concentration of 1 μM in all the experiments in this study. VK$_2$ was dissolved in anhydrous ethanol and all other chemicals were dissolved in PBS.

Cell culture. Mouse osteoblastic MC3T3-E1 cells (GNM15) were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (10 μg/ml). Osteogenic differentiation was induced in DMEM supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (10 μg/ml), L-ascorbic acid (50 μg/ml) and β-glycerophosphate disodium salt hydrate (10 mM). All cells were cultured at 37°C in a humidified atmosphere containing 5% CO$_2$.

Cell proliferation assay. The MC3T3-E1 cells (5,000/well) were plated in 96-well plates and incubated overnight. Ten microliters of Cell Counting Kit-8 (CCK-8) solution were then added to 100 μl of culture medium and the wells were incubated for an additional 2 h. The absorbance values at 450 nm measured using a microplate reader (Bio-Rad, Hercules, CA, USA) were recorded as the initial values (0 day), and the cells were then treated with both DEX and various concentrations of VK$_2$ (10^{-5}, 10^{-6} and 10^{-7} M), with medium changes every 2 days. CCK-8 detection was performed again at appropriate time points (48, 96 and 144 h) following incubation with the different chemicals, and the absorbance values were recorded and analyzed.

Cell apoptosis and viability assay. The Annexin V-FITC cell apoptosis detection kit (Beyotime Biotechnology, Shanghai, China) was used to detect cell apoptosis. The MC3T3-E1 cells were incubated with or without DEX and various concentrations of VK$_2$ (10^{-5}, 10^{-6} and 10^{-7} M) for 6 days, collected, resuspended in 200 μl of Annexin V-FITC and 10 μl of propidium iodide, and incubated for 20 min at room temperature. Subsequently, flow cytometry was used to evaluate the number of apoptotic cells. The early apoptotic cells are labeled green and the dead and late apoptotic cells are labeled red, while the live cells are not stained.

Trypan blue staining was performed to evaluate cell viability. The MC3T3-E1 cells were treated with both DEX and various concentrations of VK$_2$ (10^{-5}, 10^{-6} and 10^{-7} M) in FBS-free medium for 6 days and then collected. Ten microliters of trypan blue (Invitrogen, Carlsbad, CA, USA) were mixed with 10 μl of the cell suspension, and 10 μl of the mixture were then added to the cell counting plate. The cell death rates were automatically calculated with a cell counter (Invitrogen). A ReadyProbes® Cell Viability Imaging kit (Life Technologies, Gaithersburg, MD, USA) was also used to detect cell viability at 6 days after the MC3T3-E1 cells were incubated under the different conditions. The blue dye stained all living cells, and the green dye stained the dead cells.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The cells were cultured in osteogenic differentiation medium and treated with DEX or DEX with various concentrations of VK$_2$ (10^{-5}, 10^{-6}, and 10^{-7} M). Total RNA was extracted using TRIzol reagent (Invitrogen) at 1, 3 and 7 days following treatment, and the RNA was then reverse transcribed into cDNA using the EasyScript one-step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech Co., Ltd.,

Beijing, China), according to the manufacturer's instructions. RT-qPCR for ALP, OCN and Runx2 was performed using the TransStart Tip Green qPCR SuperMix (TransGen Biotech Co., Ltd.) with ABI Prism 7900 (Invitrogen). The reaction conditions were 1 cycle of 95°C for 30 sec and 40 cycles of 95°C for 5 sec and 60°C for 30 sec. Subsequently, a 65-95°C solubility curve was constructed. The relative amount of each mRNA was normalized to the β-actin mRNA. The primer sequences of each cDNA are presented in Table I.

Determination of ALP activity and staining. To assay the ALP activity in the cells subjected to the different treatments, the total protein was harvested at 1, 3 and 7 days after the different treatments, as described above. ALP activity was evaluated using the ALP assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), according to the manufacturer's instructions. The values were measured at 520 nm and normalized to the protein concentration determined using the BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA). In addition, ALP staining was performed 7 days following incubation with the conditioning medium using the BCIP/NBT ALP Color Development kit (Beyotime Biotechnology), according to the manufacturer's instructions.

Alizarin Red staining. Following incubation with DEX or DEX plus various concentrations of VK$_2$ (10^{-5}, 10^{-6}, and 10^{-7} M), the cell cultures were rinsed 3 times with PBS, fixed with 4% paraformaldehyde for 30 min, and then stained with Alizarin Red (Beyotime Biotechnology) for a further 30 min. The cultures were then evaluated under a light microscope (CKX31; Olympus, Tokyo, Japan).

Immunofluorescence staining for Runx2 and OCN. Following 7 days of incubation with the different conditioning media, the MC3T3-E1 cells were fixed with 4% paraformaldehyde for 20 min, treated with 0.1% Triton X-100 for 15 min, and blocked with 10% FBS for 30 min at 37°C. The cells were then incubated with a rabbit anti-Runx2 monoclonal antibody (1:1,000 dilution; #12556; Cell Signaling Technology, Danvers, MA, USA) or an anti-OCN antibody (1:200 dilution; AB10911; Millipore, Billerica, MA, USA), followed by an anti-rabbit Alexa Fluor™ 488 secondary antibody (1:500 dilution; A32731; Invitrogen) for 1 h at 37°C. Finally, the MC3T3-E1 cells were stained with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen) for a further 30 sec, rinsed with PBS and then examined under a fluorescence microscope (Leica DM IL LED; Leica, Wetzlar, Germany).

Western blot analysis. To examine the effects of DEX or DEX and VK$_2$ on the differentiation of the MC3T3-E1 cells, total protein was harvested from the cells cultured in the osteogenic medium described above for 1, 3 and 7 days. The protein concentrations were measured using the BCA protein assay kit (Thermo Fisher Scientific). The protein samples were then separated on a 10% SDS-PAGE gel and transferred onto a PVDF membrane. The membrane was blocked with 5% BSA and incubated with the primary antibodies overnight at 4°C, followed by incubation with a horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h. After rinsing 3 times with PBST, the membrane was scanned in an Odyssey scanner (Li-COR

Table I. Sequences of primers used for RT-qPCR.

Gene	Forward primer	Reverse Primer
Runx2	TGGCCGGGAATGATGAGAAC	TGAAACTCTTGCCTCGTCCG
ALP	CACTCTGTCCCGTTGGTGTC	TTGACGTTCCGATCCTGCAC
OCN	TCTGACAAAGCCTTCATGTCCA	AGCCCTCTGCAGGTCATAGA
β-actin	GTCGAGTCGCGTCCACC	GTCATCCATGGCGAACTGGT

Runx2, Runt-related transcription factor 2; ALP, alkaline phosphatase; OCN, osteocalcin.
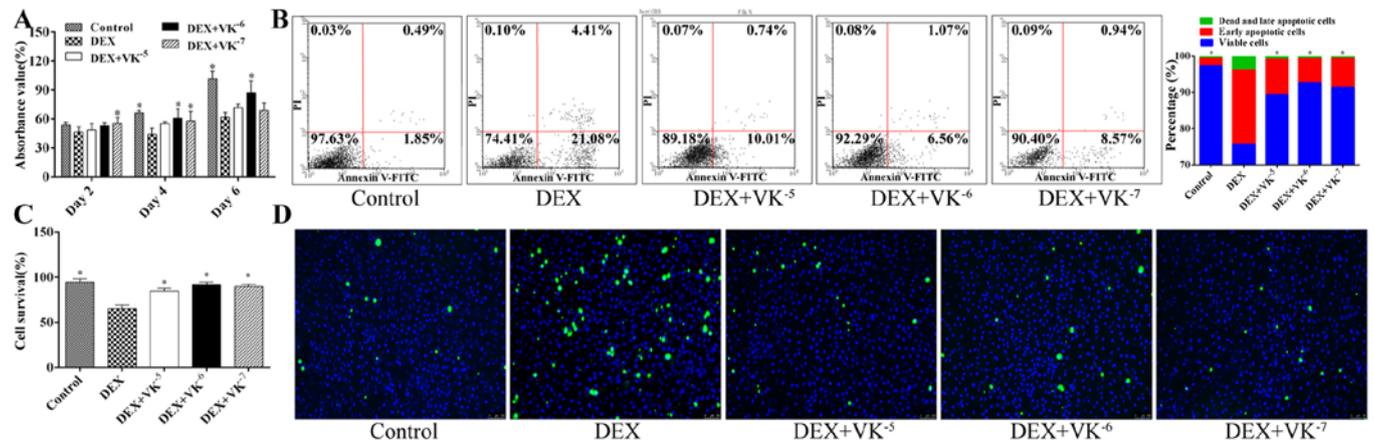

Figure 1. Effects of dexamethasone (DEX) and DEX plus vitamin K$_2$ (VK$_2$) on the proliferation, apoptosis and survival of MC3T3-E1 cells. (A) MC3T3-E1 cell proliferation was detected using Cell Counting Kit-8 (CCK-8) assays. The results revealed that DEX inhibited cell proliferation, particularly at day 6, while VK$_2$ promoted cell proliferation. (B) Detection of MC3T3-E1 cell apoptosis; the values in the right bottom quadrant indicate the apoptotic cells. (C and D) Trypan blue staining and live/dead cell staining showed that significantly fewer MC3T3-E1 cells in the DEX group survived and more cells in the DEX + VK group survived (*P<0.05, significant difference vs. the DEX group).

Biosciences, Lincoln, NE, USA). The antibodies used for the western blot analysis were as follows: monoclonal rabbit anti-rat GAPDH antibody (1:1,000 dilution; #2118), monoclonal rabbit anti-rat Runx2 antibody (1:1,000 dilution; #12556), and an HRP-conjugated rat anti-rabbit antibody (1:2,000 dilution; #7074) (all from Cell Signaling Technology, Danvers, MA, USA). The bands were quantified using Quantity One software and normalized to GAPDH.

Enzyme-linked immunosorbent assay (ELISA) for OCN in the media. Following incubation in the conditioning medium for 1, 3 and 7 days, the MC3T3-E1 cells were incubated with regular medium for a further 24 h. The media were then harvested and the concentrations of OCN in the media were detected using an ELISA kit (Mlbio, Shanghai, China); the values were normalized to the total protein concentration, which was determined using a BCA kit (Invitrogen).

Statistical analysis. SPSS 20.0 software (Microsoft, SPSS, Inc., Chicago, IL, USA) was used to analyze the values in each group. All the experiments in this study were performed in triplicate and the data are expressed as the means and standard deviation (SD). A statistical comparison of the data between the groups was performed using one-way analysis of variance (ANOVA) with a Student-Newman-Keuls (SNK) post hoc test. A P-value <0.05 was considered to indicate a statistically significant difference.

Results

VK$_2$ promotes MC3T3-E1 cell proliferation and enhances MC3T3-E1 cell survival in the DEX-treated cultures. A CCK-8 assay was performed at 48, 96 and 144 h following treatment with DEX alone or with DEX and VK$_2$. The results revealed that MC3T3-E1 cell proliferation was significantly suppressed at 96 and 144 h by DEX, although no significant change was observed at 48 h. However, the addition of VK$_2$ promoted cell proliferation at these 3 time points, particularly following treatment with 10^{-6} and 10^{-7} M VK$_2$. We did not observe a dose-dependent effect of VK$_2$ (Fig. 1A).

The results of cell apoptosis assay indicated that VK$_2$ inhibited apoptosis and enhanced the survival of the DEX-treated cells, which was also demonstrated by trypan blue staining. In this experiment, only 65.3% of the MC3T3-E1 cells in the DEX group survived after being treated with DEX in FBS-free medium for 6 days, while significantly more cells survived in the other groups. Cell viability imaging also yielded similar results (Fig. 1B-D).

VK$_2$ improves the osteogenic differentiation potential of DEX-treated MC3T3-E1 cells. To verify whether VK$_2$ enhances the osteogenic differentiation potential of DEX-treated MC3T3-E1 cells, we also detected the mRNA expression levels of both early and mature osteogenic markers in the MC3T3-E1 cells. The results revealed that, following incubation, the
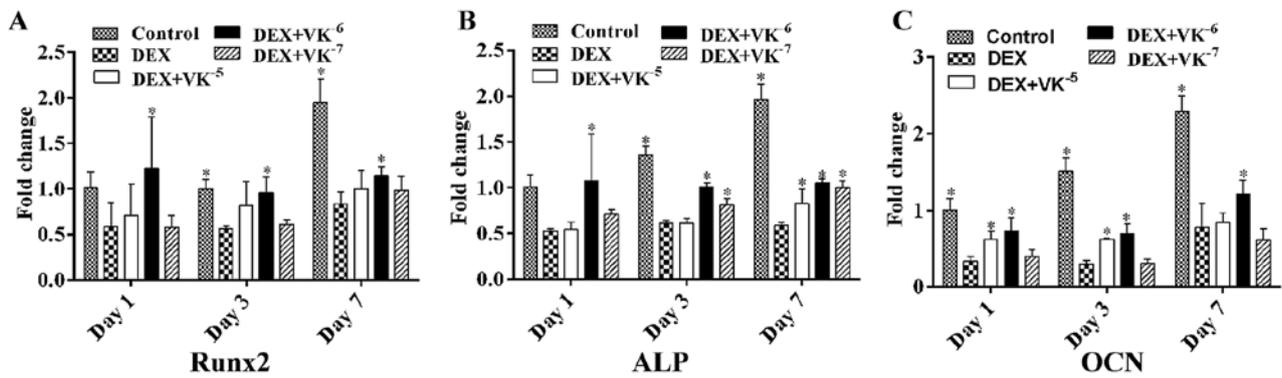
Figure 2. Expression of osteogenesis-related mRNAs in the MC3T3-E1 cells. The mRNA levels of (A) Runx2, (B) ALP and (C) OCN were downregulated by dexamethasone (DEX) and upregulated by vitamin K_2 (VK_2), particularly 10^{-6} M VK_2 (*P<0.05, significant difference vs. the DEX group).

mRNA levels of Runx2, ALP and OCN were downregulated by DEX and upregulated by VK_2, particularly following treatment with 10^{-6} M VK_2 (Fig. 2).

VK_2 upregulates Runx2 expression in DEX-treated MC3T3-E1 cells. We performed immunofluorescence staining and western blot analysis to detect the expression of Runx2, an early osteogenic marker. We observed that the Runx2 level in the DEX-treated MC3T3 cells was significantly decreased, while the Runx2 protein levels were significantly upregulated in the presence of VK_2, particularly 10^{-6} M VK_2 (Fig. 3).

VK_2 promotes ALP expression in the DEX-treated MC3T3-E1 cells. Following 7 days of incubation with DEX or DEX and various concentrations of VK_2, we performed ALP staining to detect osteogenesis in the MC3T3-E1 cells. As shown in Fig. 4, treatment with 10^{-5} M DEX markedly decreased the number of ALP-positive cells, while VK_2 antagonized this effect, showing more bluish violet-coloured cells. ALP activity was also detected following 1, 3 and 7 days of incubation. The results revealed that ALP activity increased over time. The MC3T3-E1 cells in the DEX group displayed less ALP activity than those of the control group, particularly on days 3 and 7, while the MC3T3-E1 cells in the VK_2 groups exhibited an increased ALP expression, particularly the cells treated with 10^{-6} M VK_2 on day 7 (Fig. 4).

VK_2 promotes OCN expression in the DEX-treated MC3T3-E1 cells. OCN is a mature stage osteogenic marker; therefore, the OCN levels in the media of the MC3T3-E1 cells treated with DEX and VK_2 were detected on days 1, 3 and 7. The results revealed that the OCN levels in the media increased over time. The DEX-treated MC3T3-E1 cells secreted evidently less OCN than the controls at all time points, while the MC3T3-E1 cells treated with DEX and VK_2 had more OCN in the media, particularly the cells treated with 10^{-6} M VK_2 (Fig. 5). We also detected OCN expression in the MC3T3-E1 cells using immunofluorescence staining, and observed less OCN expression in the DEX-treated MC3T3-E1 cells and increased OCN expression in the VK_2 groups. In addition, Alizarin Red staining revealed a similar antagonism of VK_2 toward the DEX-treated MC3T3-E1 cells (Fig. 5).

Discussion

GC-induced osteoporosis greatly increases the risk of fracture, and pharmacological therapy is recommended as soon as possible and has been studied for many years (27). VK_2 has been reported to be a promising therapy for GC-induced osteoporosis (28). Studies have indicated that VK_2 inhibits bone loss and increases bone formation in GC-treated rats (25,29). Clinical studies have also reported increased serum levels of carboxylated OCN and lumbar bone mass volume in GC-treated patients (26,30). This study further confirmed the protective effects of VK_2 against GC-induced damage in osteoblasts *in vitro*.

Several studies have indicated that GCs inhibit osteoblast proliferation *in vivo* (31,32), and VK_2 has also been reported to promote osteoblast proliferation (13). In this study, we further confirmed the proliferative-promoting effect of VK_2 on GC-treated MC3T3-E1 cells. This effect may be related to growth arrest-specific gene 6 (Gas6), which is a VK-dependent protein that is involved in cell proliferation by activating Axl (33,34). GC-induced osteoblast apoptosis has been clearly demonstrated in both *in vivo* and clinical studies (7,35). In this study, we observed significant apoptosis of the DEX-treated MC3T3-E1 cells and an anti-apoptotic effect of VK_2. Studies have demonstrated that VK_2 has some biological effects as a co-factor, including anti-apoptotic effects in erythroid progenitors (36), maintaining endothelial cell survival (37) and protecting neurons from methylmercury-induced cell death (38). The anti-apoptotic effects of VK_2 are believed to be related to its role as an electron carrier in the regulation of mitochondrial function (39).

The critical role of Runx2 in osteoblasts has been well described by previous studies (40,41). Runx2 is a master transcription factor in osteoblast differentiation and bone formation, and strongly impacts the expression of osteoblast marker genes and related proteins, such as ALP. The study by Koromila *et al* demonstrated that GC markedly antagonized Runx2 and Runx2-mediated ALP activity in mesenchymal cells (3). In this study, we also observed that Runx2 was downregulated by GCs in osteoblastic cells, similar to a previous study by Kim *et al* (6). Furthermore, we observed a higher expression of Runx2 in the MC3T3-E1 cells treated
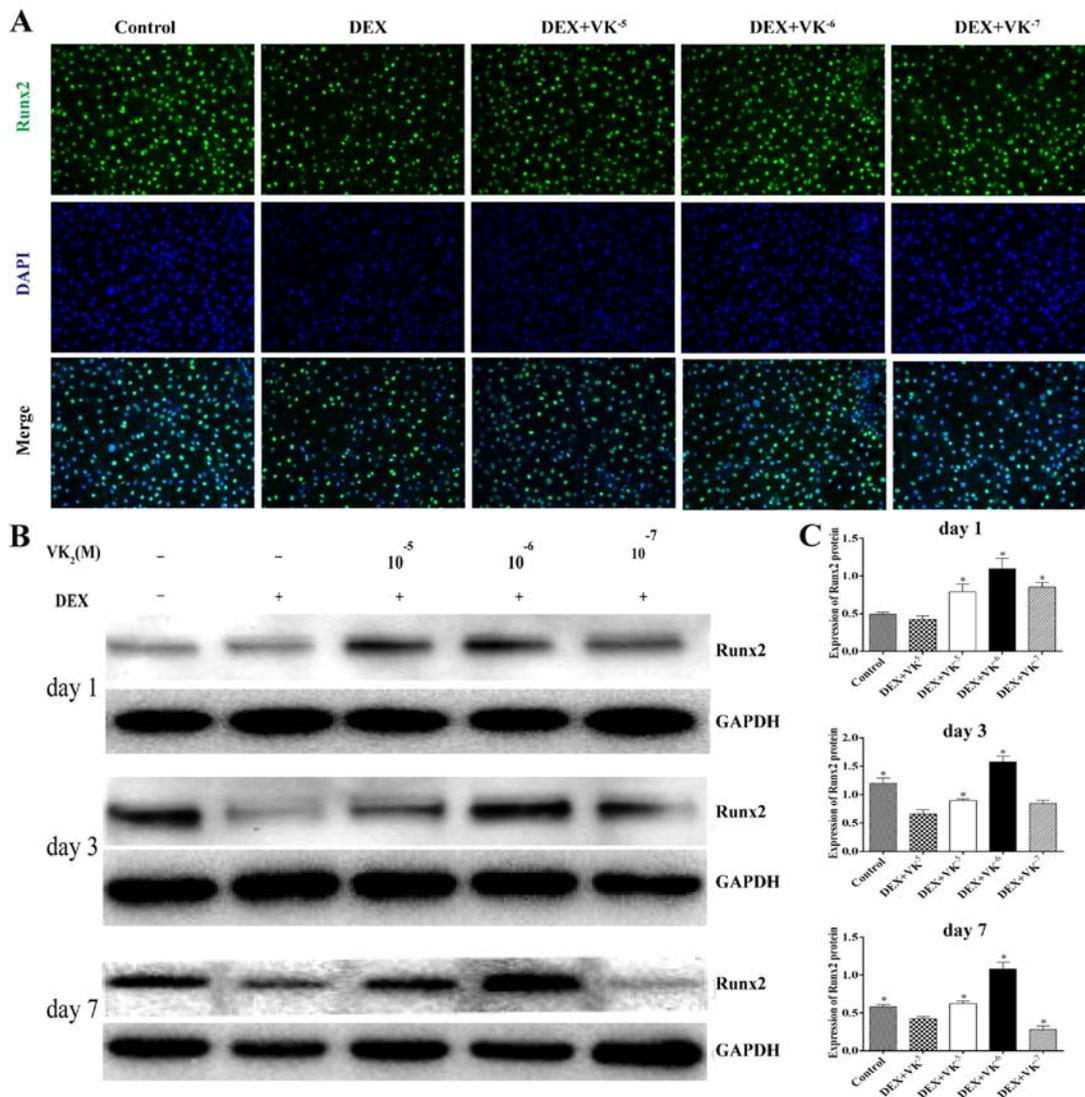
Figure 3. Runx2 protein expression in the MC3T3-E1 cells. (A) Immunofluorescence staining of the MC3T3-E1 cells after 7 days of incubation with dexamethasone (DEX) or DEX and various concentrations of vitamin K_2 (VK$_2$). Little green staining was observed in the DEX group. By contrast, more green staining was observed in the DEX + VK groups, particularly the DEX + VK^{-6} group. (B and C) Western blot analysis results of the Runx2 protein. More protein expression was observed in the DEX + VK groups, particularly the group treated with 10^{-6} M VK$_2$ (*P<0.05, significant difference vs. the DEX group).
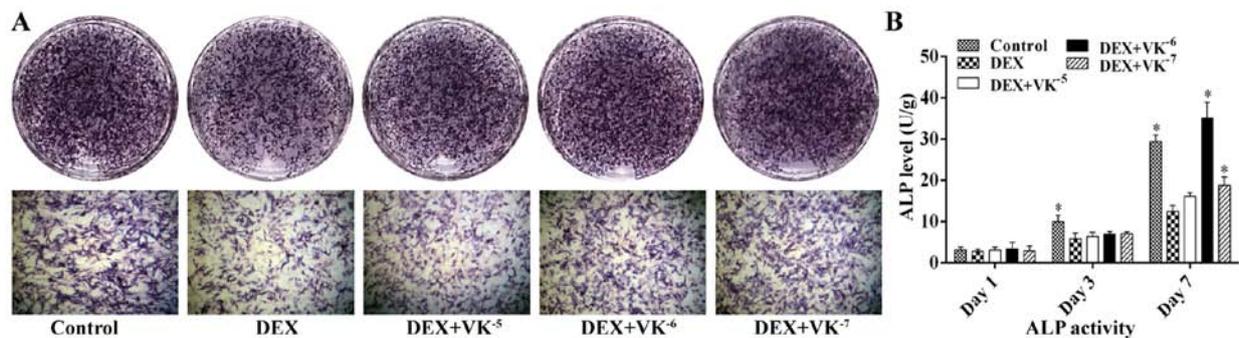

Figure 4. Quantitative and qualitative analysis of the expression of the alkaline phosphatase (ALP) protein. (A) ALP staining of MC3T3-E1 cells treated with dexamethasone (DEX) or DEX combined with vitamin K_2 (VK$_2$). Fewer bluish violet cells were observed in the DEX group than the control group, and more were observed in the DEX + VK groups. (B) ALP activity in the MC3T3-E1 cells. The MC3T3-E1 cells in the DEX plus 10^{-6} M VK$_2$ group exhibited obviously upregulated ALP activity (*P<0.05, significant difference vs. the DEX group).

with both GCs and VK$_2$, which indicated that VK$_2$ promoted the osteoblast differentiation of GC-treated osteoblastic cells partly by upregulating Runx2.

OCN is a non-collagenous, VK-dependent protein that is secreted in the late stage of osteoblast differentiation. One role of VK$_2$ in bone metabolism is to act as a coenzyme for the
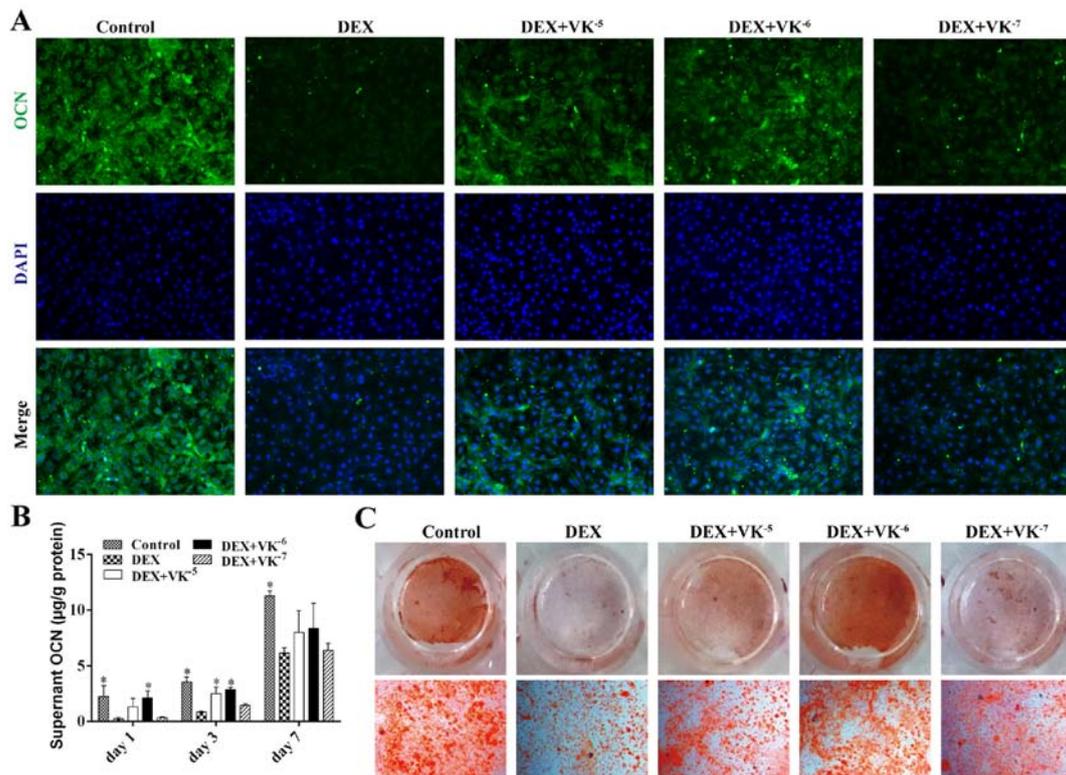
Figure 5. Protein expression of osteocalcin (OCN) and Alizarin Red staining. (A) Immunofluorescence staining of the MC3T3-E1 cells after a 7-day incubation with dexamethasone (DEX) or DEX and various concentrations of vitamin K$_2$ (VK$_2$). (B) Levels of OCN secretion from the MC3T3-E1 cells. VK$_2$ markedly promoted increased OCN secretion by the MC3T3-E1 cells treated with 10^{-5} M DEX. (C) Alizarin Red staining of the MC3T3-E1 cells that were induced for 7 days. Increased numbers of calcified nodules were observed in the DEX + VK groups than in the DEX group, although they were still fewer than the control group (*P<0.05, significant difference vs. the DEX group).

γ-carboxylation of OCN, which combines with hydroxyapatite to ultimately promote bone mineralization (42). With the exception of its role as a regulator of bone mineralization, OCN has also been reported to regulate bone remodeling by modulating osteoblast and osteoclast activity (43). Runx2 has been recognized as a key regulator of OCN transcription, and both Runx2 and OCN transcription are inhibited by GCs (3,43). Studies have also shown that VK$_2$ enhances OCN accumulation in human osteoblasts (43,19). In this study, we observed an evident downregulation in the proteins levels of Runx2 and OCN in the DEX-treated MC3T3-E1 cells and further confirmed the stimulatory effect of VK$_2$ on osteoblast differentiation. Furthermore, we observed the upregulated expression of osteogenesis-related genes in the MC3T3-E1 cells treated with DEX and VK$_2$; this may be attributed to the transcriptional effect of VK$_2$ as VK$_2$ can activate steroid and xenobiotic receptor (SXR) and regulate the transcription of extracellular matrix-related genes and collagen accumulation in osteoblastic cells (44-46).

Studies have demonstrated that treatment with 10^{-5} M VK$_2$ alone promotes mineralization and increases the Ca^{2+} concentrations more effectively than lower concentrations (17,18). However, in this study, we found that treatment with 10^{-6} M VK$_2$, which was comparable to the serum concentrations in patients treated with VK (47), exerted the most effective protection against DEX. Some other studies have observed similar results, where 10^{-6} M VK$_2$ had a stimulatory effect on colony formation and the proliferation of hematopoietic progenitors, producing fewer apoptotic cells than those treated with 10^{-5} M VK$_2$ (36). Koshihara and Hoshi (19) observed more OCN in the medium

of cells treated with 0.5 μM and 1.5 μM VK$_2$, and OCN release in the medium was significantly enhanced by 0.5 μM VK$_2$ in the presence of 1,25 (OH)$_2$-D3. Therefore, we speculate that 10^{-6} M VK$_2$ may be the most appropriate concentration to antagonize DEX *in vitro*.

In conclusion, in this study, we demonstrated that VK$_2$ promoted osteoblast proliferation and osteogenic differentiation, inhibited cellular apoptosis and enhanced cellular survival, supporting the view that VK$_2$ is a promising option for the prevention and treatment of GC-induced osteoporosis and osteonecrosis.

Acknowledgements

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