

Original Article

Osteoblastic NF- κ B pathway is involved in 1α , $25(\text{OH})_2\text{D}_3$ -induced osteoclast-like cells formation *in vitro*

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Received February 10, 2015; Accepted April 12, 2015; Epub May 1, 2015; Published May 15, 2015

Abstract: 1α , 25-dihydroxyvitamin D_3 (1α , $25(\text{OH})_2\text{D}_3$) acts on the osteoblasts to enhance the expressions of receptor activator of nuclear factor κB ligand (RANKL) and macrophage-colony stimulating factor (M-CSF) and induce the formation of osteoclasts. However, the mechanism in osteoblasts by which 1α , $25(\text{OH})_2\text{D}_3$ promotes osteoclastogenesis has not yet been completely understood. This study aimed to select the first generation of murine osteoblasts to explore the underlying mechanism of 1α , $25(\text{OH})_2\text{D}_3$ -induced osteoclastic formation from bone marrow mononuclear cells (BMMNCs). We discovered the activation of osteoblastic NF- κB pathway under 10^{-8} mol/L 1α , $25(\text{OH})_2\text{D}_3$ treatment, as evidenced by the transfer of NF- κB p65 from cytoplasm to nuclei. Then, the NF- κB p65-siRNA was designed, constructed, and transfected into osteoblastic cells. Immunofluorescence assay confirmed the successfully silenced NF- κB p65 gene in osteoblasts. In the co-culture system of osteoblasts and BMMNCs with 1α , $25(\text{OH})_2\text{D}_3$ added, the multinucleated osteoclast-like cells containing 2-3 nuclei were observed in BMMNCs co-cultured with non-transfection osteoblasts, conversely, silencing osteoblastic NF- κB p65 resulted in failed differentiation of BMMNCs along with substantial vacuolar degeneration in cytoplasm. In addition, the expressions of RANKL and M-CSF were notably decreased in NF- κB p65-silenced osteoblasts. Taken together, our data indicated that osteoblastic NF- κB pathway was involved in 1α , $25(\text{OH})_2\text{D}_3$ -induced osteoclast-like cells formation from BMMNCs through regulating the expression of RANKL and M-CSF. Therefore, our findings further identified the mechanism of 1α , $25(\text{OH})_2\text{D}_3$ -induced osteoclastogenesis on the basis of prior studies.

Keywords: 1α , 25-dihydroxyvitamin D_3 , osteoclast-like cells, NF- κB pathway, receptor activator of nuclear factor κB ligand, macrophage-colony stimulating factor

Introduction

Osteoporosis, an age-related metabolic bone disease, begins as early as 30 years old with millions of patients worldwide [1]. Osteoporosis is also a multifactorial disease characterized by the low bone mass density (BMD), the deterioration of microarchitectural in bone tissue, and the increased risk of fractures [2, 3]. The maintenance of BMD and microarchitectural is closely associated with the dynamic process of bone remodeling mediated by osteoblasts and osteoclasts whereby osteoclasts resorb worn bone and osteoblasts synthesize new bone. Imbalance of bone resorption and formation would cause osteoporosis [4-6]. Hence, better understanding the interaction mechanism

between osteoclasts and osteoblasts is crucial for developing novel therapeutic methods and delaying the worsening of osteoporosis.

1α , 25-dihydroxyvitamin D_3 (1α , $25(\text{OH})_2\text{D}_3$) is the hormonally active form of vitamin D_3 produced in the proximal kidney tubule responsible for regulating calcium and phosphate homeostasis and cell differentiation [7-9]. Previous studies have found that 1α , $25(\text{OH})_2\text{D}_3$ could induce osteoblastic differentiation through traditional steroid hormone receptor-mediated gene transcription mechanisms or rapid membrane-associated signaling pathways [10-12]. Besides, both Fuller K et al. and Takahashi N et al. reported that osteoclast-like multinucleated cells were formed from bone marrow cells in

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response to $1\alpha, 25(\text{OH})_2\text{D}_3$ in a co-culture system [13, 14]. Recent evidences point to the fact that $1\alpha, 25(\text{OH})_2\text{D}_3$ upregulates the expressions of receptor activator of nuclear factor κ B ligand (RANKL) and macrophage-colony stimulating factor (M-CSF) in osteoblasts to interact with bone marrow cells and subsequently induces osteoclastogenesis [15, 16], ultimately leads to the release of ^{45}Ca from the pre-labeled bone and the stimulation of osteoclastic bone resorption [17]. However, the internal event of osteoblasts in the process of $1\alpha, 25(\text{OH})_2\text{D}_3$ -induced osteoclastic differentiation has not been fully understood.

In the present study, we detected the activated NF- κ B pathway upon $1\alpha, 25(\text{OH})_2\text{D}_3$ -treated osteoblasts, then osteoblastic NF- κ B p65 gene was silenced by siRNA interference, and the co-culture system of osteoblasts and bone marrow mononuclear cells (BMMNCs) containing $1\alpha, 25(\text{OH})_2\text{D}_3$ was established through a Transwell chamber to analyze the effect of osteoblastic NF- κ B pathway on osteoclast-like cells formation, immunofluorescence assay was further employed to examine the expression of RANKL and M-CSF after NF- κ B p65 silenced. We found that osteoblastic NF- κ B pathway regulated RANKL and M-CSF expression to induce osteoclast-like cells formation under $1\alpha, 25(\text{OH})_2\text{D}_3$ treatment.

Materials and methods

Osteoblast/BMMNC isolation and culture

The BALB/c mice were purchased from Laboratory Animal Center of China Medical University, Shenyang, China. All animal experiments were carried out in accordance with the Institutional Animal Care and Use Committee Guidelines of China Medical University.

For osteoblastic cells, calvaria were removed from neonatal mice within 1-day-old, and the loose connective tissue in it was shaved completely. Then the calvaria were digested by 0.25% trypsin for 30 min and 0.1% collagenase II for 90 min respectively at 37°C . After being centrifuged, cells were resuspended and grown in Roswell Park Memorial Institute-1640 (RPMI-1640, Gibco, Grand Island, NY, USA) supplemented with 15% fetal bovine serum (FBS, HyClone, Logan, UT, USA) and streptomycin/penicillin (100 U/mL) at 37°C in a 5% CO_2 incubator. The suspension was decanted and

replaced with fresh medium after 24 h, then every 2 to 3 days. When reached 90% confluences, cells were digested for subsequent experiments. To detect the differentiation of osteoblasts, each generation of osteoblasts from primary to the fifth was collected. The first generation of osteoblasts was collected for other experiments.

BMMNCs were isolated according to previously published methods with some modification [18]. Briefly, the 4-week old BALB/c mice were euthanized and bone marrow cells from each femur and tibia were flushed with RPMI-1640 medium containing 15% FBS and seeded in a plate. After being settled for 30 min in a 37°C , 5% CO_2 incubator, the non-adherent cells were harvested as BMMNCs.

Detection of osteoblastic differentiation

Osteoblastic differentiation was assessed by a modified Gomori-Takamatsu method. In brief, the differentiated and non-differentiated osteoblasts were separated depending on differential adhesion speeds and inoculated onto coverslips in 6-well plates respectively. After being cultured overnight, osteoblasts on each coverslip were fixed in 3.7% formaldehyde for 30 min and washed with phosphate buffer salines (PBS, pH 7.4) for three times, followed by incubated with alkaline phosphatase (ALP) solution (5 ml 3% β -glycerol sodium phosphate, 5 ml 2% pentobarbital sodium, 10 ml 2% CaCl_2 , 1 ml 2% MgSO_4 , and 10 ml ddH_2O , pH 9.4) at 37°C for 4 h and 2% cobalt nitrate for 5 min. Then the cells were treated with 1% ammonium sulfide for 5 min, and stained with Giemsa solution for 30 min. The coverslips were mounted inversely onto slides with neutral gum and observed under a fluorescence microscope.

Drug treatment

$1\alpha, 25(\text{OH})_2\text{D}_3$ (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in RPMI-1640 medium. Osteoblasts were planted in 6-well plates. When reached to 50% confluences, cells were exposed to 10^{-8} mol/L $1\alpha, 25(\text{OH})_2\text{D}_3$ for 6 h, 12 h, 24 h, 36 h, and 48 h respectively, and untreated cells were served as control. For induction of osteoclast-like cells formation, osteoblasts and BMMNCs were co-cultured with 10^{-8} mol/L $1\alpha, 25(\text{OH})_2\text{D}_3$ for 6 h before NF- κ B p65 interference. 10^{-8} mol/L $1\alpha,$

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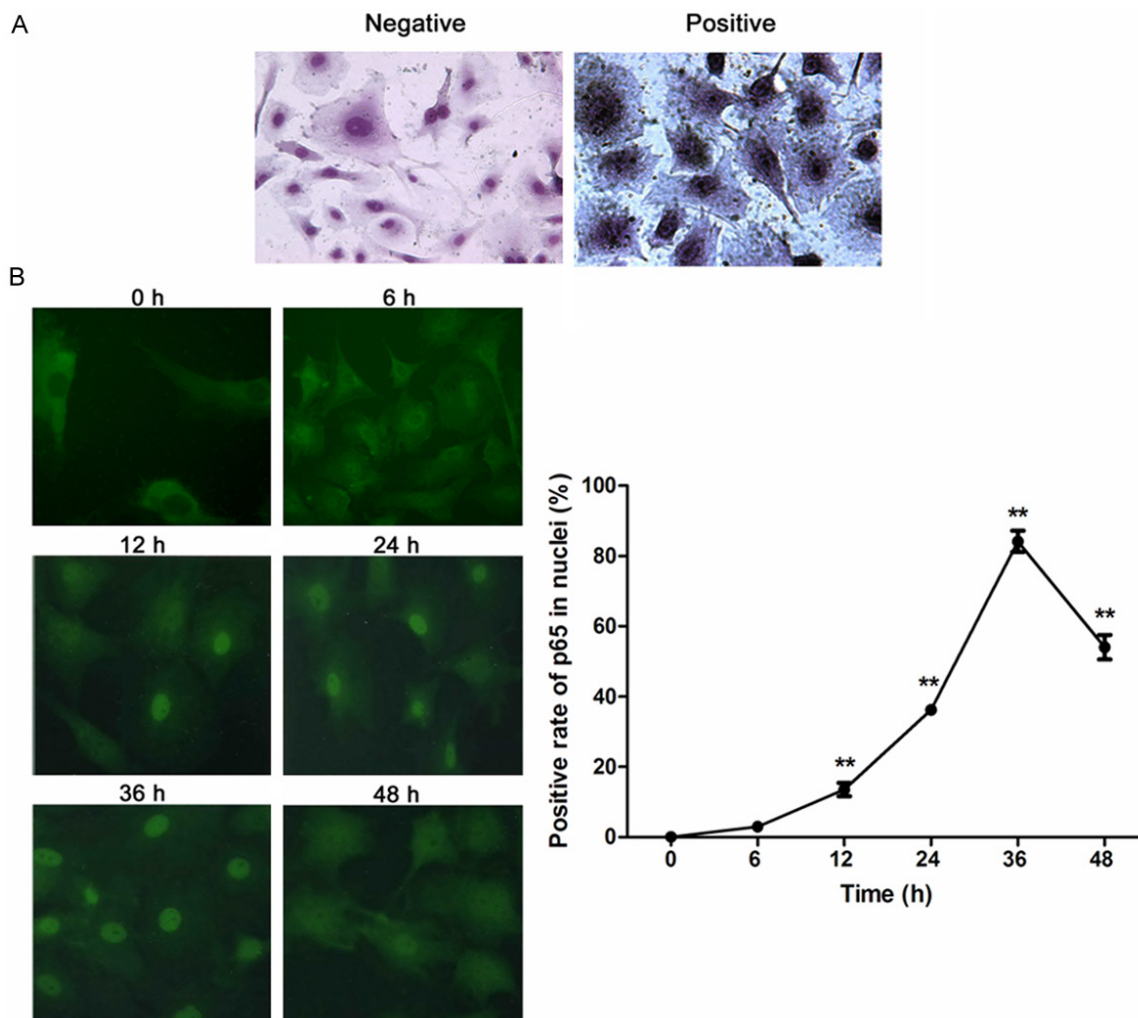


Figure 1. $1\alpha, 25(\text{OH})_2\text{D}_3$ induces the activation of NF- κ B pathway in osteoblasts. A. Identification of differentiated and non-differentiated osteoblasts using Gomori-Takamatsu method. Representative examples of images are shown. The cytoplasm of non-differentiated osteoblasts appeared to be uniform purple (negative, left). Substantial gray or black particles existed in cytoplasm of differentiated osteoblasts (positive, right). Magnification 400 \times for all panels. B. Immunofluorescence assay was performed to investigate the distribution of osteoblastic NF- κ B p65. Osteoblastic cells were seeded in 6-well plates and exposed to 10^{-8} mol/L $1\alpha, 25(\text{OH})_2\text{D}_3$ for 6 h, 12 h, 24 h, 36 h and 48 h respectively. The distribution of NF- κ B p65 stained with FITC-labeled goat anti-rabbit IgG (green) was observed at 400 \times magnification. Cells with nuclei-transferred NF- κ B p65 were calculated in five representative random microscopic fields. Experiments were done in triplicates for statistical significance, and the results are expressed as mean \pm SD. ** $P < 0.01$ vs control (0 h).

$25(\text{OH})_2\text{D}_3$ was added into the medium after transfection for further experiments.

NF- κ B p65-siRNA interference

siRNA for NF- κ B p65 were designed on siRNA Target Finder software (Ambion, Austin, Texas, USA). The scrambled sequences as negative control were obtained through the program of GenScript Inc. (New Jersey, USA). Sequences were as follows: NF- κ B p65-siRNA: 5'-GCUAU-AACUCGCCUGGUGAAU-3' (Sense); 5'-UCACCAG-

GCGAGUUAUAGCUU-3' (Antisense); control siRNA: 5'-GAUUACG-AUAGCGGUUCCUU-3' (Sense); 5'-CGAACGCG-CUAUCGUAAUCUU-3' (Antisense). NF- κ B p65-siRNA and control siRNA were constructed with a Silencer siRNA Construction Kit (Ambion) according to the manufacturer's directions. Osteoblastic cells were seeded and maintained in 6-well plates at a density of 5×10^5 per well until reached to 50% confluences. Then the NF- κ B p65-siRNA and control siRNA were transfected into osteoblasts respectively using the siPORT Lipid Kit

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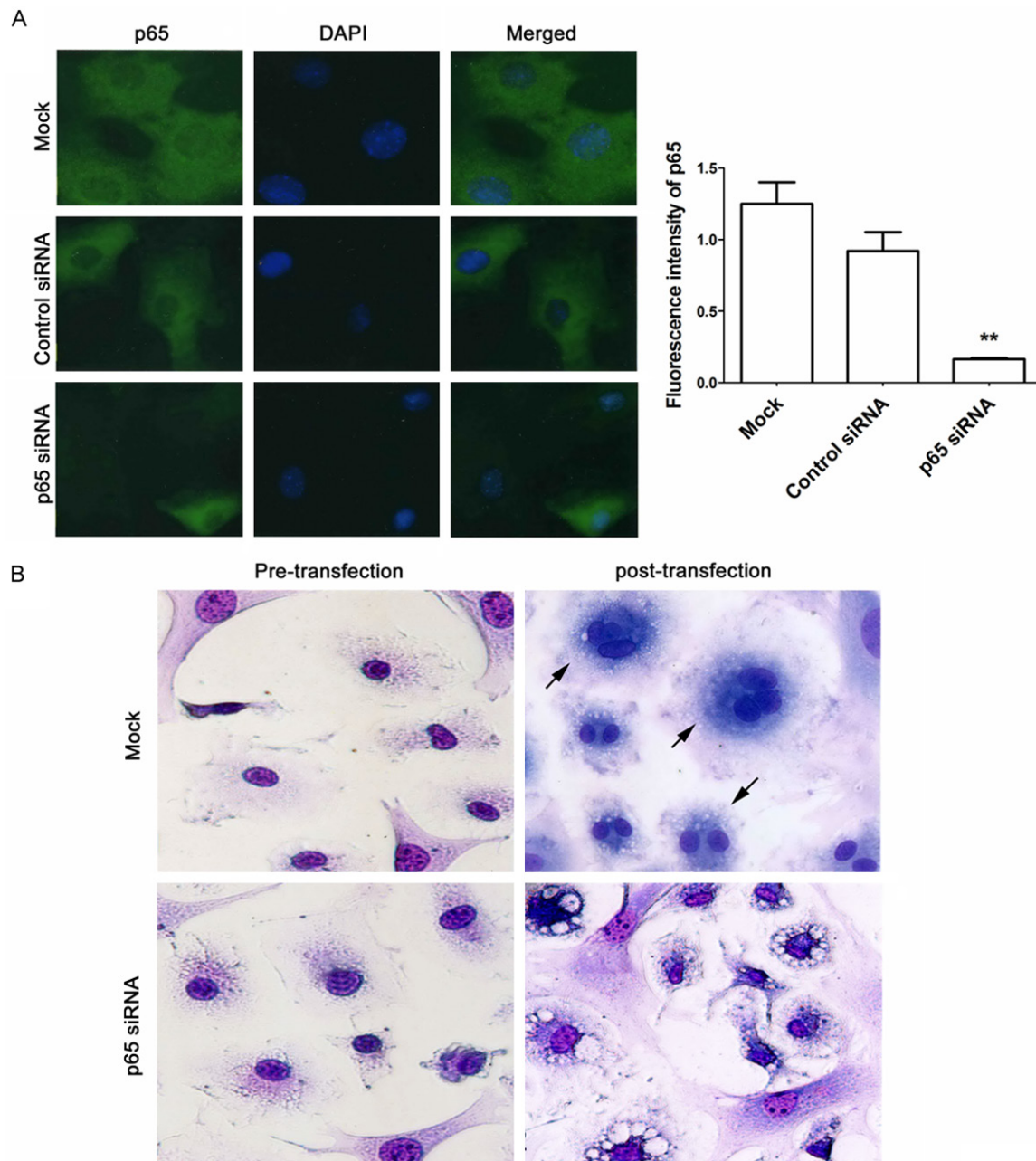


Figure 2. $1\alpha, 25(\text{OH})_2\text{D}_3$ is involved in osteoclast-like cell formation through osteoblastic NF- κ B pathway. A. Detection on the expression of osteoblastic NF- κ B p65 by immunofluorescence staining under a laser scanning confocal microscope (600 \times). Osteoblastic NF- κ B p65 was visualized with FITC-labeled goat anti-rabbit IgG as green, and cells nuclei were stained with DAPI as blue (left). Fluorescence optical density results are shown on the right. Data are given as mean \pm SD. ** $P < 0.01$ vs control siRNA. B. Osteoblastic cells with or without p65 siRNA transfection were separated from BMMNCs (lower chambers) using Transwell inserts in 6-well plates and co-cultured with 10^{-8} mol/L $1\alpha, 25(\text{OH})_2\text{D}_3$ for 4 days, then the BMMNCs were stained with Giemsa staining solution. The arrows indicate multinucleated osteoclast-like cells. Magnification, 400 \times . The above two experiments were repeated three times, and the representative results are shown.

(Ambion) strictly according to the manufacturer's instructions, and cells without siRNA transfection were used as Mock. Cells were harvested at 2 days after transfection for NF- κ B p65

expressed detection, and at 4 days with $1\alpha, 25(\text{OH})_2\text{D}_3$ incubation after transfection for osteoclast-like cells formation and RANKL/M-CSF expression assay.

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The formation of osteoclast-like cells from BMMNC

Osteoblasts and BMMNCs were co-cultured with $1\alpha, 25(\text{OH})_2\text{D}_3$ for internal time at densities of 1×10^5 and 1×10^7 respectively, and separated based on differential adhesion speeds. The osteoclast-like cells formation assay was carried out in a transwell system (0.4 μm pore size, Corning Costar Corp., Cambridge, MA, USA) as described previously [19, 20]. Briefly, the isolated BMMNCs were planted on coverslips in the lower chambers of a 6-well Transwell plate. The isolated osteoblasts were seeded in Transwell inserts and performed NF- κ B p65-siRNA interference. Thereafter, osteoblasts were added to the upper chamber of the Transwell plate and the co-cultures were continued for additional 4 days in the presence of $1\alpha, 25(\text{OH})_2\text{D}_3$. The removed coverslips were fixed with 3.7% formaldehyde for 30 min, stained with Giemsa for 30 min, and mounted with neutral gum. The formation of osteoclast-like cells was observed under an inverted microscope.

Immunofluorescence staining

Osteoblastic cells were grown on coverslips with appropriate treatment and fixed in 3.7% formaldehyde for 15 min. After washed with PBS for three times, cells were blocked with 10% bovine serum albumin (BSA) at room temperature for 1 h. Thereafter, cells were incubated with primary antibodies against NF- κ B p65, RANKL and M-CSF (all 1:100 diluted, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight and subsequently incubated with FITC-labeled goat anti-rabbit IgG (H+L) secondary antibody (Santa Cruz) at a dilution of 1:200 for 1 h at room temperature. Unbound antibodies in each step were removed by washing with PBS. After staining with 4', 6-diamidino-2-phenylindole (DAPI) and a final rinse with PBS, the coverslips were mounted inversely onto slides with 95% glycerol and observed under a laser scanning confocal microscope and photographed. Fluorescence optical density values were analyzed by Image-Pro Plus (IPP) software.

Statistical analysis

Statistical analysis was performed with GraphPad Prism 5.0 software. Immunofluorescence staining results are presented as

mean \pm standard deviation (SD), and the differences between two groups were analyzed with unpaired Student's *t* test. $P < 0.05$ was considered to be statistically significant.

Results

$1\alpha, 25(\text{OH})_2\text{D}_3$ induces the activation of NF- κ B pathway in osteoblasts

To explore the effect of $1\alpha, 25(\text{OH})_2\text{D}_3$ on osteoblastic NF- κ B pathway, We conducted Gomori-Takamatsu method to detect osteoblastic differentiation and immunofluorescence staining to observe the alterations of NF- κ B p65 in $1\alpha, 25(\text{OH})_2\text{D}_3$ -treated osteoblasts. We discovered negatively stained primary osteoblasts, slightly stained second generation of osteoblasts, and dispersed stained fifth generation of mature osteoblasts (**Figure 1A**). Hence, we employed the first generation of osteoblasts for subsequent experiments. Further immunofluorescence study revealed that the population of osteoblasts with nuclear-transferred NF- κ B p65 was increased significantly after $1\alpha, 25(\text{OH})_2\text{D}_3$ treatment for 12 h to 48 h compared with 0 h control, and positive cell counts elevated to the peak at the point of 36 h treatment (**Figure 1B**, $P < 0.01$). The above results demonstrated that $1\alpha, 25(\text{OH})_2\text{D}_3$ could activate osteoblastic NF- κ B pathway.

$1\alpha, 25(\text{OH})_2\text{D}_3$ is involved in osteoclast-like cells formation through osteoblastic NF- κ B pathway

To evaluate whether the $1\alpha, 25(\text{OH})_2\text{D}_3$ -activated osteoblastic NF- κ B pathway affects the formation of osteoclast-like cells, we first silenced NF- κ B p65 gene by siRNA interference, as shown in **Figure 2A**, NF- κ B p65 expression levels in p65-silenced osteoblasts was decreased significantly by 5.57-fold than those in control siRNA cells ($P < 0.01$), confirming successfully silenced NF- κ B p65 in osteoblasts. We then investigated the formation of osteoclast-like cells through a co-culture system of osteoblasts and BMMNCs. The results showed that BMMNCs were differentiated into osteoclast-like cells with 2-3 nuclei in Mock under $1\alpha, 25(\text{OH})_2\text{D}_3$ treatment. However, after silencing osteoblastic NF- κ B p65, BMMNCs existed substantial vacuolar degeneration in cytoplasm and failed to form osteoclast-like cells under the same condition (**Figure 2B**), which lasted for one week until died, suggesting that $1\alpha,$

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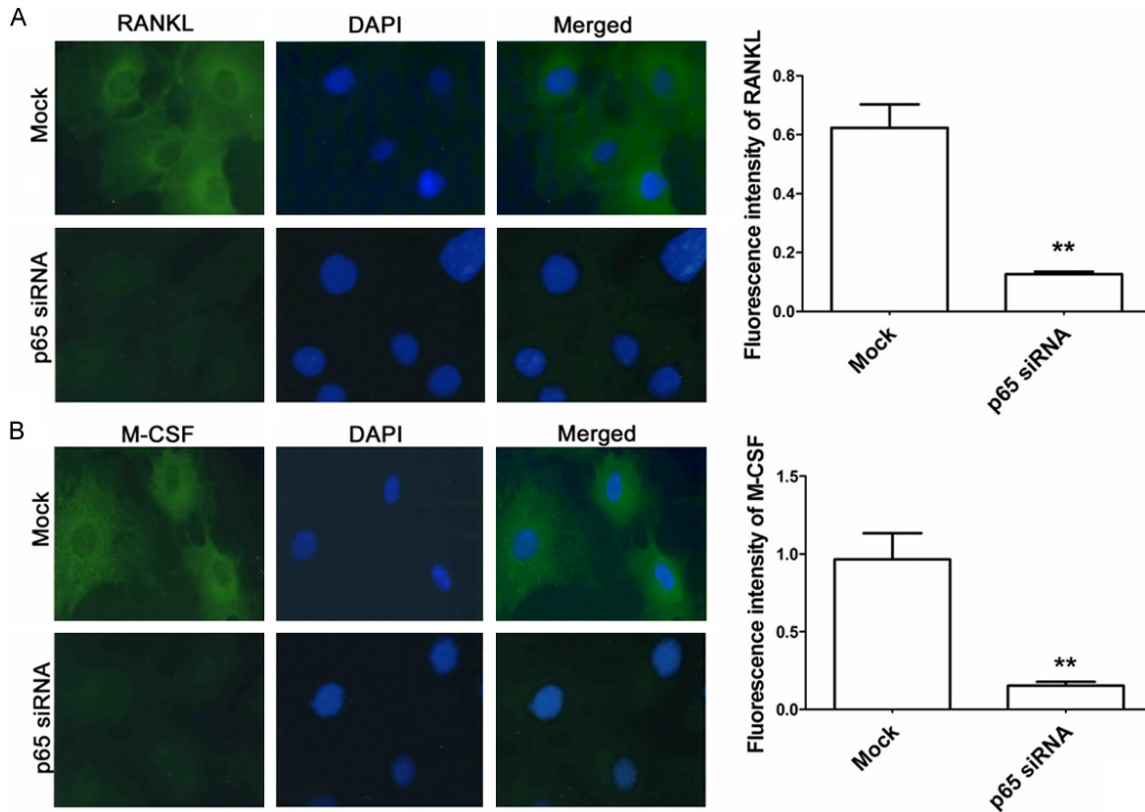


Figure 3. NF- κ B pathway regulates the expression of RANKL and M-CSF in osteoblasts. Representative photomicrographs of osteoblasts with or without p65 siRNA interference stained by antibody to RANKL or M-CSF. Nuclei labeled with DAPI were observed to be blue. The green fluorescence in cytoplasm was target proteins. Magnification 600 \times , ** $P < 0.01$ vs Mock. A. RANKL expression at 4 days after transfection and incubation with 10^{-8} mol/L $1\alpha, 25(\text{OH})_2\text{D}_3$ followed with respective fluorescence intensity. B. M-CSF expression at 4 days after transfection and incubation with 10^{-8} mol/L $1\alpha, 25(\text{OH})_2\text{D}_3$ followed with respective fluorescence intensity. The above results are expressed as mean \pm SD, and the error bars represent the SD of three independent experiments.

$25(\text{OH})_2\text{D}_3$ was involved in osteoclast-like cells formation through osteoblastic NF- κ B pathway.

NF- κ B pathway regulates the expression of RANKL and M-CSF in osteoblasts

Immunofluorescence assay was performed to examine the expressions of RANKL and M-CSF after osteoblastic NF- κ B p65 silenced under $1\alpha, 25(\text{OH})_2\text{D}_3$ treatment. Fluorescence optical density analysis showed that the expression levels of RANKL and M-CSF in p65-silenced osteoblasts were 4.90-fold and 6.31-fold lower than those in Mock respectively (**Figure 3**, $P < 0.01$), indicating that NF- κ B pathway could regulate osteoblastic RANKL and M-CSF expression.

Discussion

$1\alpha, 25(\text{OH})_2\text{D}_3$ stimulates osteoclasts formation from osteoclast progenitors by upregulat-

ing the osteoblastic RANKL and M-CSF expression. However the detailed molecular mechanism in osteoblasts remains unclear. Here, we detected the $1\alpha, 25(\text{OH})_2\text{D}_3$ -mediated activation of NF- κ B pathway in osteoblasts. Silencing osteoblastic NF- κ B p65 caused failure of osteoclast-like cells formation from $1\alpha, 25(\text{OH})_2\text{D}_3$ -treated BMMNCs, which may because of the inhibition of RANKL and M-CSF expression regulated by NF- κ B pathway.

Inactivated NF- κ B is a heterodimer of p50 and p65 subunits and binds to I κ B in the cytoplasm. Signal-dependent phosphorylation of I κ B leads to the transportation of p50/p65 heterodimer to nucleus to regulate various gene expressions [21]. The present study observed the transferred NF- κ B p65 from cytoplasm to nuclei during $1\alpha, 25(\text{OH})_2\text{D}_3$ -treated osteoblasts at the concentration of 10^{-8} mol/L, which was demonstrated as the most effectively dose to stimulate osteoclasts formation [22]. Our data

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proved that $1\alpha, 25(\text{OH})_2\text{D}_3$ could activate osteoblastic NF- κ B pathway. Yet Doroudi et al. reported that $1\alpha, 25(\text{OH})_2\text{D}_3$ could activate PKC α to stimulate osteoblastic differentiation by NF- κ B pathway and platelet secretion pathway [10]. Hence, further experiments were required to address the effect of osteoblastic NF- κ B pathway on osteoclastic formation.

It is a well-established fact cytokines stimulate osteoclastic formation through the microenvironment between osteoblasts and osteoclast progenitors. Yasuda et al., Yang et al., and Okada et al. all successfully developed osteoclast-like cells in different $1\alpha, 25(\text{OH})_2\text{D}_3$ -treated co-culture systems [23-25]. In our experiments, we developed a mouse Transwell co-culture system of osteoblasts and BMMNCs to investigate the role of osteoblastic NF- κ B pathway in $1\alpha, 25(\text{OH})_2\text{D}_3$ -induced osteoclastogenesis *in vitro*. We found that the formation of osteoclast-like cells was inhibited significantly on $1\alpha, 25(\text{OH})_2\text{D}_3$ treatment after NF- κ B p65 silenced, indicating that osteoblastic NF- κ B pathway is involved in the process of $1\alpha, 25(\text{OH})_2\text{D}_3$ -induced osteoclastogenesis.

RANKL, a membrane-anchored cytokine belongs to the tumor necrosis factor (TNF) family, is mainly distributed in osteoblasts/osteocytes of bone marrow [26]. RANKL could bind to receptor activator of nuclear factor κ B (RANK) for the induction of osteoclastogenesis and the activation of mature osteoclasts [16, 27]. Previous studies have found that the expression of RANKL was elevated in $1\alpha, 25(\text{OH})_2\text{D}_3$ -treated osteoblastic cells [9, 16, 18, 28]. Blocking RANKL expression with siRNAs resulted in inhibition of osteoclast differentiation and osteolysis [29-31]. In this study, we found that silenced NF- κ B p65 decreased the expression of RANKL in $1\alpha, 25(\text{OH})_2\text{D}_3$ -treated osteoblasts, suggesting that NF- κ B pathway could regulate RANKL expression. M-CSF is another important molecular capable of activating c-Fms to promote multinucleated osteoclastic formation and osteoclastic absorption [32, 33]. Loss M-CSF decreases the number of osteoclast progenitors and arrests the formation of osteoclasts in the presence of $1\alpha, 25(\text{OH})_2\text{D}_3$ [15]. Our experiment confirmed that M-CSF expression was sharply decline after silencing NF- κ B p65 in $1\alpha, 25(\text{OH})_2\text{D}_3$ -treated osteoblasts. Overall, our results reflected that osteoblastic NF- κ B pathway may participate in $1\alpha,$

$25(\text{OH})_2\text{D}_3$ -mediated osteoclastic formation through regulating RANKL and M-CSF expression.

In summary, our present study revealed that osteoblastic NF- κ B pathway was involved in $1\alpha, 25(\text{OH})_2\text{D}_3$ -induced osteoclast-like cells formation from BMMNCs by regulating the expression of RANKL and M-CSF. Our data further explained the mechanism of $1\alpha, 25(\text{OH})_2\text{D}_3$ -mediated osteoclastogenesis in osteoblasts and BMMNCs co-culture system.

Acknowledgements

Study was supported by a grant from the National Natural Science Foundation of China (No. 81201419).

Disclosure of conflict of interest

None.

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