

C-C chemokine receptor type 6 modulates the biological function of osteoblastogenesis by altering the expression levels of Osterix and OPG/RANKL

Lisha Li^{1,2,3,§}, Jing Zhou^{1,2,3,§}, Yingping Xu⁴, Zengshu Huang^{1,2,3}, Na Zhang^{1,2,3}, Xuemin Qiu^{1,2,3}, Ling Wang^{1,2,3,*}

¹Obstetrics and Gynecology Hospital of Fudan University, Shanghai, China;

²The Academy of Integrative Medicine of Fudan University, Shanghai, China;

³Shanghai Key Laboratory of Female Reproductive Endocrine-related Diseases, Shanghai, China;

⁴Reproductive Medicine Centre, Taizhou Hospital Affiliated to Wenzhou Medical University, Linhai, Zhejiang, China.

SUMMARY Circulating inflammatory factors affect osteoblast and osteoclast formation and activity in osteoporosis. Estrogen affects the migration of Th17 cells *via* the C-C chemokine receptor type 6 (CCR6) and C-C chemokine ligand 20 (CCL20) signaling pathways to modulate bone metabolism; however, it is unclear whether and how CCR6 modulates bone homeostasis. In the present study, CCR6 knockout (CCR6^{-/-}) mice were selected to investigate the effects of CCR6 in the regulation of homeostasis of osteoblasts and osteoclasts. Primary osteoblasts were isolated from the calvarium of newborn CCR6^{-/-} or wild-type mice, followed by osteoblastic differentiation culture *in vitro*. CCR6 deletion reduced osteoblast activity in terms of alkaline phosphatase (ALP) activity and inhibited osteoblast mineralization according to the results of Alizarin Red S staining, whereas it did not affect the proliferation of osteoblasts. CCR6 deletion inhibited *Osterix* mRNA expression in osteoblasts during the late stage of mineralization *in vitro*, while it did not affect mRNA expression levels of runt-related transcription factor 2 (*Runx2*) and *Collagen-1*. The ratio of osteoprotegerin (OPG) / receptor activator of nuclear factor κ-B ligand (RANKL) mRNA level in osteoblasts was decreased by CCR6 deficiency in the culture treated with 1,25(OH)2D3/PGE2, while there was no effect observed in the normal culture environment. The results provide novel insights, such as that CCR6 deletion suppresses osteoblast differentiation by downregulating the expression levels of the transcription factor *Osterix*, and indirectly promotes osteoclast production by increasing transcription of RANKL. This may be one of the mechanisms *via* which CCR6 deletion regulates bone metabolism.

Keywords osteoporosis, C-C chemokine receptor type 6, osteoprotegerin, receptor activator of nuclear factor κ-B ligand, osteoblast, osteoclast

1. Introduction

Bone metabolism balance is an important factor in maintaining the health of the body. Osteoporosis is a group of systemic skeletal diseases characterized by low bone mass, degeneration of the bone microstructure, increased bone fragility and fracture sensitivity (1,2). The main pathogenesis is the imbalance of bone remodeling. Bone remodeling mainly includes bone formation and bone resorption activities, both of which are initiated and modulated by a number of factors, including inflammation, hormone levels and mechanical stimulation (3,4). A decrease in estrogen level is the main cause of osteoporosis in postmenopausal women. Estrogen reduction affects the biological behavior of

osteoblasts, osteoclasts and T cells by altering the levels of cytokines, such as TNF-α, IL-1, IL-6 and IL-17, which affects bone metabolism (5-8).

During the early stages of collagen-induced arthritis (CIA), estrogen treatment can increase the number of Th17 cells in lymph nodes and decrease the number of Th17 cells in joints of CIA mice (9,10). Studies have demonstrated that C-C chemokine receptor type 6 (CCR6) serves an important role in the differentiation of B cells driven by antigens, and can regulate the migration of dendritic cells and T cells in inflammatory and immune responses (11-13). In addition, estrogen can increase the expression levels of CCR6 and CCL20, which play a role as the ligand of CCR6 in Th17 cells of the lymph nodes. Furthermore, the increase in CCR6

and C-C chemokine ligand 20 (CCL20) expression in the lymph nodes impels Th17 cells to stay in the lymph nodes and hinders the migration of Th17 cells to the joints, thus reducing the recruitment of neutrophils into joints, and alleviating arthritis and erosion providing potential treatment targets (14). In the physiological state, osteoclasts are involved in bone resorption and form local bone resorption lacunae. Additionally, osteoclasts release cytokines and chemotaxins, recruit osteoblasts to local bone resorption lacunae, and participate in new bone formation, thus maintaining the balance of bone metabolism (15-17). Previous studies have revealed that global loss of CCR6 in mice markedly decreases trabecular bone mass coincident with reduced osteoblast numbers. CCL20 and CCR6 are co-expressed in osteoblast progenitors and the levels increase during osteoblast differentiation, indicating the potential for CCL20/CCR6 signaling to influence osteoblasts *via* both autocrine and paracrine pathways. CCL20/CCR6 signaling may serve an important role in regulating bone mass accrual, potentially by modulating osteoblast maturation, survival and the recruitment of osteoblast-supporting cells (18). Further studies investigating the role of CCR6 in the pathogenesis of bone metabolism-related diseases could provide novel ideas and methods for the treatment of osteoporosis.

2. Materials and Methods

2.1. Mice

CCR6^{-/-} and wild-type (WT) mice (C57BL/6 mice), aged 10-12 weeks and weighing 20-30 g, were purchased from Jackson Laboratory. Mice were raised in carbonate plastic cages in the animal room (clean grade) of the Institute of Gynecology and Obstetrics of the Hospital Affiliated to Fudan University, Shanghai, China. The genotype of the *CCR6*^{-/-} mice was identified according to the standard protocol provided by Jackson Laboratory, and the identified primer sequences are listed in Table 1. The experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals published by the US National Institutes of Health and were approved by the Fudan ethics committee. Throughout the study period, the mice were housed in a temperature-controlled (23 ± 0.5°C) and humidity-controlled (43 ± 8%) environment with a 12-h light-dark cycle and *ad libitum* access to food and water.

2.2. Genomic DNA isolation and genotyping

Genotypes of *CCR6*^{-/-} mice were confirmed by PCR analyses of genomic DNA. Generally, tissue samples were collected and used for further PCR analyses with 2X GC rich PCR MasterMix (Tiangen Biotech Co., Ltd.). The PCR reaction was performed according to

Table 1. Summary of oligonucleotide primers for *CCR6*^{-/-} mice genotyping

Oligonucleotide	Sequence* (5'-3')
Common forward primer	AAAACCCAAGTGTGGTGGCATGAG
Wild-type reverse primer	CCCTAGAAGAGGTCAGAAACTTCAC
Mutant reverse primer	GGGTGGGATTAGATAAATGCCTGCTCT

*The oligonucleotide sequences for genotyping were obtained from the Jackson Laboratory website: <https://www.jax.org/search?q=+005793>

the protocol provided by Jackson Laboratory: 94°C for 3 min, followed by 10 cycles of 94°C for 30 sec, 65°C for 15 sec and 68°C for 10 sec, followed by another 28 cycles of 94°C for 15 sec, 60°C for 15 sec and 72°C for 10 sec, and finally an additional step of 72°C for 2 min prior to the end of the program.

2.3. Primary osteoblast isolation and induced differentiation culture

Osteoblasts were collected from the calvarium of newborn mice after 2 days as previously described (19). Skull bones were extracted and digested (five times; 10 min each time) in α -minimum essential medium (α -MEM) containing 0.1% collagenase and 0.2% dispase. The supernatant from the first 10-min digestion was discarded. Cells obtained from the remainder of the digestions were pooled, and 5×10^5 cells were seeded onto 10% FBS (Gibco) and phenol red-free α -MEM supplemented with 10 U/mL penicillin and 10 μ g/mL streptomycin in 6-well culture plates until they reached 80% confluence. The osteogenic differentiation medium consisted of 10% serum and phenol red-free α -MEM, 20 mM ascorbic acid, 1 M β -glycerophosphate disodium salt hydrate and 1 mM dexamethasone.

2.4. Cell culture

MC3T3-E1 cells were acquired commercially from American Type Culture Collection (ATCC, Rockville, MD, USA) and maintained in Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich, St. Louis, MO, USA). Cell growth medium (GM) was supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich) and 1% antibiotic (Gibco, Rockville, MD, USA) in a humidified atmosphere of 5% CO₂ at 37°C. After culturing MC3T3-E1 cells until sub-confluence, osteogenic differentiation was ensured by changing medium to osteogenic differentiation medium.

2.5. Cell transfection

The *CCR6*-specific small interfering RNA (siRNA) and negative control (si-NC) were purchased from

GenePharma (Shanghai, China). Cell transfection was conducted using Lipofectamine 2000 (Invitrogen, CA, USA). Cell transfection was performed independently at least three times.

2.6. Western blot assay

Cell protein samples were isolated, quantified and diluted in loading buffer to the same concentration. After denaturation, proteins were separated by electrophoresis on 10% sodium dodecyl sulphate (SDS) polyacrylamide gels, followed by transfer to Hybond membrane. Membranes were blocked in 5% skimmed milk at room temperature for 2 h and exposed to primary antibodies against Actin (ab8226, Abcam, USA), CCR6 (PA1-21615, Invitrogen, USA), runt-related transcription factor 2 (Runx2) (ab92336, Abcam, USA), Osterix (ab209484, Abcam, USA), Collagen-1 (ab270993, Abcam, USA) overnight at 4°C. Following rinsing three times in Tris-buffered saline (TBST), membranes were incubated with secondary antibodies for 2 h. Proteins in the membranes were visualized using a chemiluminescence (ECL) system (Amersham Biosciences, USA). Actin (ab197345, Abcam, USA) was used as an internal reference. The experiment was independently repeated 3 times.

2.7. Alkaline phosphatase (ALP) staining and quantitative detection of ALP activity

For ALP detection, 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitro blue tetrazolium (NBT) was the preferred staining substrate. After 7 days of osteogenic induction medium treatment in 24-well plates, cells were washed with 500 µL PBS and fixed with 4% paraformaldehyde for 20 min at room temperature, followed by three washes with 500 µL PBS. The fixed cells were incubated in BCIP/NBT buffer, which was prepared according to the kit's protocol (3 ml ALP staining buffer, 10 µL 300X BCIP buffer and 20 µL 150X NBT buffer) for > 30 min at room temperature avoiding light until ALP-positive differentiated osteoblasts appeared blue-violet. The reaction was stopped by adding excess deionized water. The results were visualized using an HP scanner and recorded. A total of 5×10^5 primary osteoblasts were seeded

into 6-well culture plates and cultured until they reached 90% confluence. The cells were digested with pancreatin and collected into a 1.5 mL Eppendorf tube. ALP activity in cell lysate was quantitated by using an Alkaline Phosphatase Assay kit (Beyotime Institute of Biotechnology) according to the manufacturer's protocol.

2.8. Alizarin Red S staining

The primary osteoblasts were isolated and the cell density was adjusted to 1×10^5 cells/mL. After 21 days of osteogenic induction medium treatment in 24-well plates, Alizarin Red S stain was added to each well and the plate was incubated in the dark for 10 min at room temperature. The staining buffer was removed carefully when mineralized osteoblasts appeared bright orange-red and undifferentiated cells were slightly red or colorless.

2.9. RNA isolation and reverse transcription-quantitative PCR (RT-qPCR)

For PCR analysis, total RNA was isolated using an RNA extraction kit (Axygen; Corning Inc.) according to the manufacturer's protocol. The concentration of total RNA was measured using a NanoDrop 2000c (Thermo Fisher Scientific, Inc.). RNA (1 µg) was reverse transcribed into cDNA using a reverse transcriptase kit (Promega Corporation). qPCR was performed using SYBR Premix Ex Taq (Takara Bio, Inc.). The cDNA levels were quantified using the housekeeping gene GAPDH. Gene expression was normalized to the level of housekeeping gene GAPDH and analyzed using the standard $2^{-\Delta\Delta CT}$ method. Primer sequences are listed in Table 2.

2.10. Cell viability assay

Primary osteoblasts were isolated according to the method "Primary osteoblast isolation and induced differentiation culture", and the cell density was adjusted to 2×10^5 cells/mL. Subsequently, 100 µL medium containing 2,000 cells was added to each well of a 96-well cell culture plate. After 24 h, when the cells had adhered completely, the medium was exchanged with medium containing 25 µg/mL vitamin C, 10 mM

Table 2. The primers used in the study

Gene	Forward Primer	Reverse Primer
<i>Osterix</i>	GCTCGTAGATTTCTATCCTC	CTTAGTGACTGCCTAACAGA
<i>Collagen-1</i>	TGACTGGAAGAGCGGAGAGTA	GACGGCTGAGTAGGGAACAC
<i>Runx2</i>	GACAGTCCCAACTTCCTGTG	GCGGAGTAGTTCATCATTC
<i>OPG</i>	CCTTGCCCTGACCACTCTTAT	CGCCCTTCCTCACACTCAC
<i>RANKL</i>	CAAGATGGCTTCTATTACCTGT	TTGATGCTGGTTTTAACGAC
<i>GAPDH</i>	GTTGTCTCCTGCGACTTCA	GGTGGTCCAGGGTTTCTTA

Runx2: runt-related transcription factor 2; *OPG*: osteoprotegerin; *RANKL*: nuclear factor-κ B ligand.

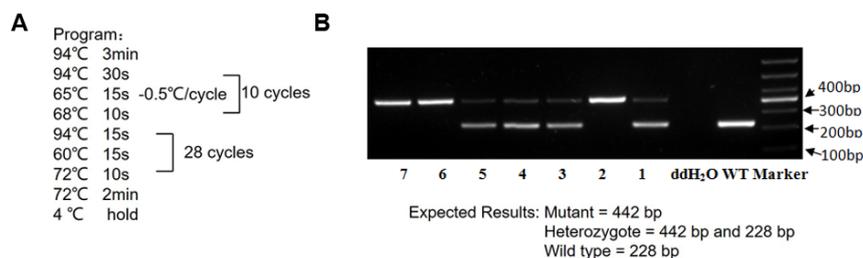


Figure 1. Gene identification of *CCR6*^{-/-} mice. (A) Master protocol details provided by Jackson Laboratory. **(B)** Gel image of PCR products and the expected results of mice. Mutant, 442 bp; heterozygote, 442 and 228 bp; wild-type, 228 bp. *CCR6*, *C-C chemokine receptor type 6*.

β -glycerol phosphate and 100 nM dexamethasone (differentiation medium). Each well contained 100 μ L medium, and six replicate wells were analyzed for each group. After 12, 24, 48 and 96 h, 10 μ L MTT solution (5 mg/mL; 0.5% MTT) was added to each well, followed by incubation in a cell incubator for 4 h. Subsequently, 100 μ L formazan solution was added to each well, followed by incubation in a cell culture box until the formazan was completely dissolved as observed under an ordinary optical microscope. The absorbance of each well was measured at a wavelength of 570 nm using an ELISA plate reader. The activity of OB after 12 h in WT mice was calculated and expressed as a percentage of that in the control group.

2.11. Treated primary osteoblasts with 1,25(OH)₂D₃/PGE₂ *in vitro*

The primary osteoblasts were isolated according to the method "Primary osteoblast isolation and induced differentiation culture" and the cell density was adjusted to 1×10^5 cells/mL. A 24-well plate was used to inoculate 500 μ L/well or a 6-well plate was used to inoculate 2 ml/well. The cells were cultured in an incubator at 37°C with 5% CO₂. After 48 h, the medium containing 1×10^{-8} M 1,25-dihydroxyvitamin D₃ and 1×10^{-6} M prostacyclin E₂ (co-culture medium) was used to replace the medium, which simulated a microenvironment of co-culture of osteoblasts and osteoclasts (20). Subsequently, medium containing 1,25(OH)₂D₃/PGE₂ was changed once every 48 h.

2.12. Statistical analysis

All data are presented as the mean \pm SEM. Differences were assessed by Student's *t*-test using SPSS software (IBM Corp.). All experiments were repeated more than three times. $p < 0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. Identification of *CCR6*^{-/-} mice

The transgene identification of *CCR6*^{-/-} mice was

confirmed according to the standard protocol provided by Jackson Laboratory (stock number, 005793; strain name, B6.129P2-Ccr6tm1Dgen/J). The master protocol details are presented in Figure 1A. The expected product size of mutant (*CCR6*^{-/-}) was 442 bp, the sizes of heterozygote (*CCR6*^{+/-}) were 228 and 442 bp, and the size of WT was 228 bp (Figure 1B).

3.2. Deletion of *CCR6* inhibits the differentiation of osteoblasts *in vitro*

The mRNA expression of *CCR6* in primary osteoblasts from *CCR6*^{-/-} mice was decreased obviously compared to WT controls (Figure 2A). The present study first examined the effect of *CCR6* deficiency on ALP activity of differentiated primary osteoblasts 7 days after osteogenic induction medium treatment. ALP staining revealed reduced osteoblastic differentiation in *CCR6*^{-/-} mice compared with WT controls (Figure 2B). Additionally, ALP activity in the cell lysate was markedly decreased in *CCR6*^{-/-} osteoblasts compared with WT controls (Figure 2C).

3.3. *CCR6* deficiency inhibits mineralization of differentiated primary osteoblasts while it has no effect on cell viability

Osteoblasts of WT and *CCR6*^{-/-} mice were treated with induction differentiation medium for 21 days, and the number of calcium nodules was compared using Alizarin Red staining. Calcium nodules appeared orange-red following Alizarin Red staining. The results of Alizarin Red staining demonstrated that the number of osteoblastic calcium nodules in *CCR6*^{-/-} osteoblasts cultured *in vitro* was lower than that in osteoblasts from WT mice (Figure 3A). Quantitative comparison of the number of calcium nodules per pore revealed that the number of osteoblastic calcium nodules cultured in *CCR6*^{-/-} osteoblasts was markedly lower than that in WT osteoblasts (Figure 3B). Therefore, the present study suggests that *CCR6* deletion may weaken osteoblast activity and inhibit osteoblast mineralization *in vitro*. Furthermore, the proliferation activity of primary osteoblasts from WT and *CCR6*^{-/-} mice was assessed. The results demonstrated that there was no significant

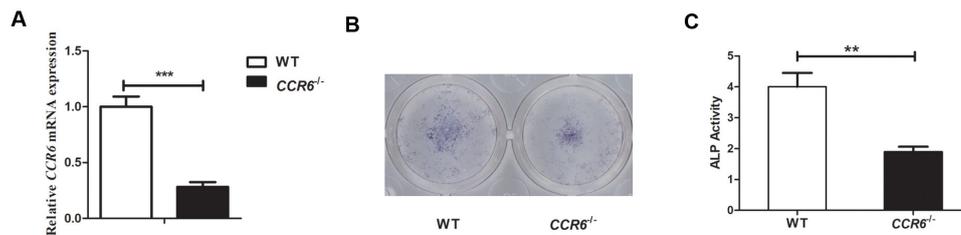


Figure 2. *CCR6* deficiency inhibits ALP activity in differentiated primary osteoblasts. (A) The mRNA expression of *CCR6* was detected in primary osteoblasts of WT and *CCR6*^{-/-} mice. (B) Primary osteoblasts from *CCR6*^{-/-} or WT mice were treated with osteogenic induction medium for 7 days, and ALP levels and activity were examined. ALP staining revealed reduced osteoblastic differentiation in *CCR6*^{-/-} mice compared with WT controls. (C) ALP activity was decreased in *CCR6*^{-/-} osteoblasts compared with WT controls. **p* < 0.05. All data are presented as the mean ± SEM and representative of at least three experiments.

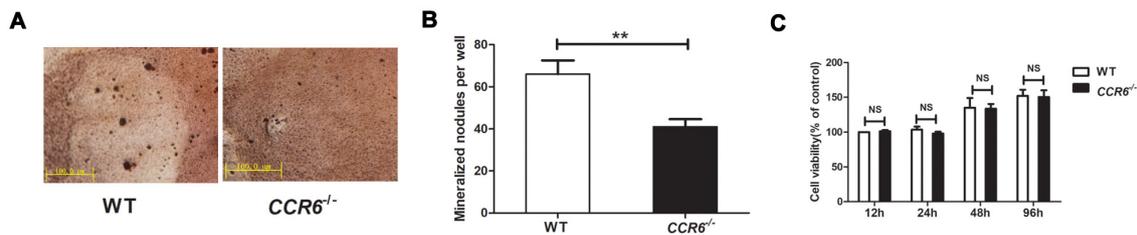


Figure 3. Mineralization of differentiated primary osteoblasts is inhibited in *CCR6*^{-/-} mice. Following Alizarin Red S staining of primary osteoblasts isolated from *CCR6*^{-/-} or WT mice, the number of calcium nodules was compared. (A) The numbers of osteoblastic calcium nodules in *CCR6*^{-/-} osteoblasts were markedly lower than those in osteoblasts from WT mice *in vitro*. (B) The number of mineralized nodules per well was calculated for *CCR6*^{-/-} and WT control osteoblasts. (C) *CCR6* deficiency did not affect the viability activity of primary osteoblasts. **p* < 0.05. All data are presented as the mean ± SEM and representative of at least three experiments.

difference in cell proliferation activity between WT and *CCR6*^{-/-} osteoblasts at 12, 24, 48 and 96 h (Figure 3C). This indicated that *CCR6* deletion had no effect on the proliferation of osteoblasts in mice.

3.4. *CCR6* deficiency decreases *Osterix* expression in differentiated primary osteoblasts

Runx2 and Osterix are important transcription factors in osteoblast growth and differentiation. Runx2 is expressed in the early stage of osteoblast differentiation, whereas Osterix is only expressed in the late stage of osteoblast differentiation. The primary osteoblasts of WT and *CCR6*^{-/-} mice were induced to differentiate and cultured *in vitro*. RT-qPCR was used to analyze the expression levels of *Runx2* and *Osterix* in the two groups. The results revealed that with the prolongation of cell culture time *in vitro* there was no significant difference in the mRNA expression levels of *Runx2* (Figure 4A). The mRNA expression levels of *Osterix* in *CCR6*^{-/-} osteoblasts were markedly lower than those in WT osteoblasts after 21 days of culture (Figure 4B). Additionally, the present study analyzed the mRNA expression levels of the functional factor *Collagen-1* during osteoblastic differentiation *in vitro*. There was no significant difference in the mRNA expression levels of collagen-1 between *CCR6*^{-/-} and WT osteoblasts (Figure 4C). We also transfected MC3T3-E1 cells with siRNA-*CCR6* to detect the expression of related proteins.

The MC3T3-E1 cells were induced to differentiate, after 21 days of differentiation culture, we transfected the MC3T3-E1 with siRNA-*CCR6* and si-NC, then detected the protein expression of Runx2, Osterix and Collagen-1 in cells. There was no significant difference in the expression levels of Runx2 and Collagen-1 between si-NC and si-*CCR6* groups, while the expression level of Osterix in si-*CCR6* group was markedly lower than those in NC group (Figure 4D).

3.5. Osteoprotegerin (*OPG*)/receptor activator of nuclear factor κ -B ligand (RANKL) levels decreased in *CCR6*^{-/-} osteoblasts treated with 1,25(OH)2D3/PGE2

The OPG/RANKL/receptor activator of nuclear factor κ B (RANK) system is an important signaling pathway in osteoclast differentiation. Osteoblasts can regulate osteoclastogenesis by expressing OPG and RANKL. The primary osteoblasts of WT and *CCR6*^{-/-} mice were divided into a common culture group and a treated group. The common culture group was cultured with common culture medium, whereas treated group was supplemented with 1,25(OH)2D3/PGE2 to common culture medium, which was used to simulate the co-culture environment. Total RNA was collected after 0, 1 and 2 days of culture, and the mRNA expression levels of OPG and RANKL in osteoblasts were analyzed by RT-qPCR. The results demonstrated that the mRNA expression levels of OPG in *CCR6*^{-/-} osteoblasts were

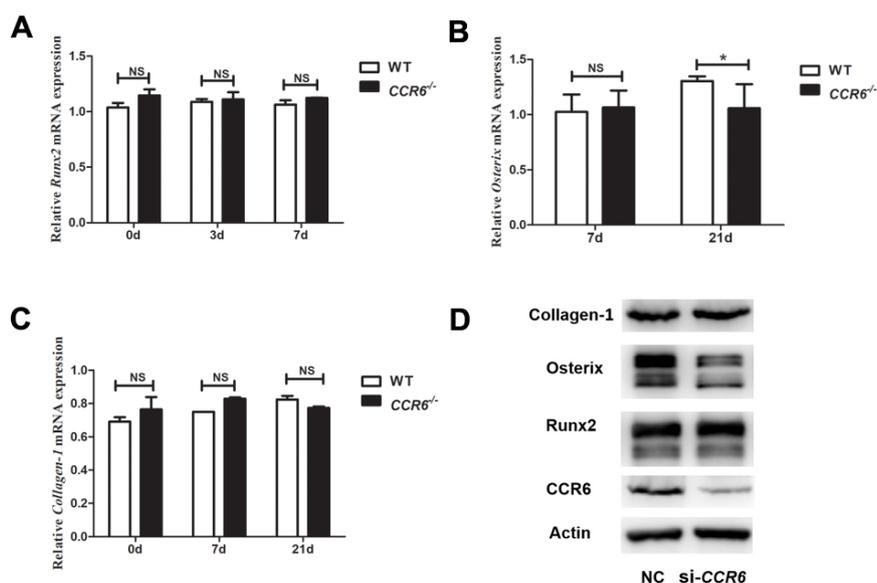


Figure 4. CCR6 affects mRNA expression levels of osteoblastogenesis-related genes. Primary osteoblasts from *CCR6*^{-/-} or WT mice were treated with osteogenic induction medium for 0, 3, 7 and 21 days. The mRNA expression levels of (A) *Runx2*, (B) *Osterix* and (C) *Collagen-1* were detected. (D) The protein expression levels of Runx2, Osterix and Collagen-1 were detected by transfection with si-NC or si-*CCR6* in MC3T3-E1 cells. * $p < 0.05$. All data are presented as the mean \pm SEM and representative of at least three experiments.

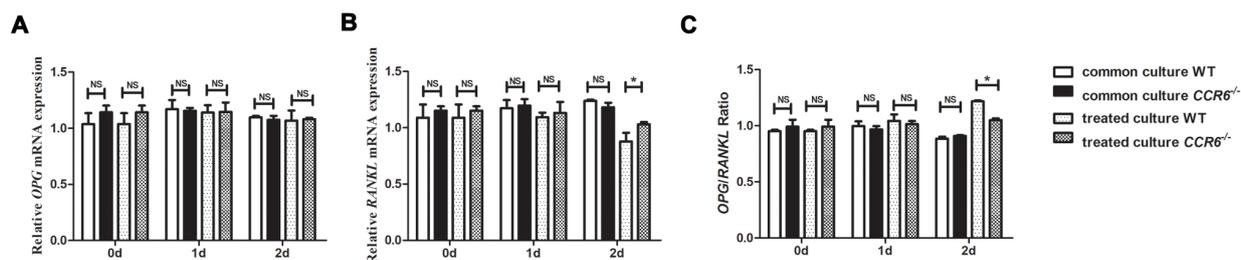


Figure 5. CCR6 deficiency decreases the OPG/RANKL level in osteoblasts treated with 1,25(OH)2D3/PGE2. Primary osteoblasts from *CCR6*^{-/-} or WT mice were cultured in growth medium or treated medium. (A) *CCR6* deficiency did not affect the mRNA expression levels of *OPG* in osteoblasts compared with WT controls in both the normal culture environment and the treated group. (B) *CCR6* deletion increased the mRNA expression levels of *RANKL* in osteoblasts compared with WT controls in the treated group. (C) The *OPG/RANKL* ratio was decreased in osteoblasts with *CCR6* deletion compared with WT control in the treated group, while no change was observed in the normal culture environment. * $p < 0.05$. All data are presented as the mean \pm SEM and representative of at least three experiments.

not significantly different from those in WT osteoblasts in both the normal culture environment and the treated environment (Figure 5A). Furthermore, *CCR6* deletion did not affect the mRNA expression levels of RANKL in osteoblasts in the normal culture environment, whereas it increased the mRNA expression levels of RANKL in osteoblasts in the treated group (Figure 5B). The ratio of OPG/RANKL was decreased in osteoblasts with *CCR6* deletion compared with WT control cells in the treated group (Figure 5C).

4. Discussion

Previous studies have demonstrated that osteoblasts and osteoclasts can secrete CCR6 and CCL20, and the CCL20/CCR6 signaling pathway is closely associated with bone metabolism (18). When the overall level of CCR6 in mice decreases, trabecular bone mass decreases, which is consistent with the reduced

number of osteoblasts. CCR6 and CCL20 are co-expressed in osteoblast progenitor cells. The expression levels of CCR6 and CCL20 are increased during the differentiation of osteoblasts, suggesting that the CCL20/CCR6 signaling pathway affects osteoblasts via autocrine and paracrine pathways (21,22). Whether CCR6 is involved in osteoblastogenesis and what role it serves remains to be elucidated. Therefore, the present study proposed the hypothesis that the decrease in CCR6 expression in osteoblasts suppresses osteoblast differentiation and regulates bone metabolism, which leads to osteoporosis.

The main cause of osteoporosis is the imbalance of bone formation induced by osteoblasts and bone resorption induced by osteoclasts. Osteoblasts are derived from mesenchymal stem cells (MSCs) and have the potential to differentiate into a variety of cells, such as chondrocytes, myoblasts or adipocytes. Osteoblast differentiation is divided into three stages:

cell proliferation, extracellular matrix formation and maturation, and mineralization. Each stage has a characteristic gene expression profile (23-25). Under suitable culture conditions, osteoblasts can secrete certain unique extracellular matrix proteins, including osteocalcin (OCN), ALP and a large amount of *Collagen-1* (26,27). The extracellular matrix does not mineralize at the beginning of deposition and is rich in *Collagen-1* (28). With the accumulation of calcium phosphate in the form of hydroxyapatite, the matrix mineralizes to form hard but light-weight sediments (both organic and inorganic), which are the main components of bone tissue (29,30). These osteoid calcium nodule deposits represent the end products of proliferation and differentiation of osteoblasts (31). In the present study, we isolated pre-osteoblasts from the calvarium of newborn *CCR6*^{-/-} mice and detected ALP activity, calcium deposit formation, and osteoblastogenesis related factor expression 0, 3, and 7 days after differentiation. The activity of ALP decreased in *CCR6*^{-/-} osteoblasts 7 days after differentiation compared to wild-type controls, combined with impaired calcium deposit formation that indicated inhibited mineralization of differentiated osteoblasts.

During different stages of osteoblast growth and development, sequential expression of different osteoblast-related genes has different effects on differentiation. Runx2 and Osterix are important transcription factors in osteoblast growth and differentiation. Runx2 is expressed in the early stage of differentiation, whereas Osterix is only expressed in the late stage of differentiation (32-34). Runx2 is essential for osteoblast differentiation in the process of chondrogenesis and intramembrane osteogenesis. Runx2 can directly stimulate the transcription of OCN, Collagen-1, osteopontin, collagenase 3 and suppression of Tumorigenicity 2 during the differentiation of bone marrow MSCs (BMSCs) into osteoblasts (35-37). Osterix is a downstream transcription factor of Runx2 in osteoblasts and is required for osteoblast differentiation (38). If Runx2 and Osterix expression is inhibited, this will affect the growth and differentiation of osteoblasts, which will lead to a differentiation disorder. Therefore, the present study isolated RNA from osteoblasts, which had been treated in different stages of differentiation and analyzed the expression levels of osteoblast differentiation-related genes using RT-qPCR. The results demonstrated that *CCR6* deletion did not affect the transcription levels of *Collagen-1* and *Runx2* in osteoblasts, whereas the transcription levels of *Osterix* were markedly lower in *CCR6*^{-/-} osteoblasts than in WT osteoblasts, indicating that *CCR6* deletion inhibited *Osterix* expression in the late stage of mineralization of osteoblasts *in vitro*.

CCR6 deletion can weaken the activity of osteoblasts and inhibit the mineralization of osteoblasts *in vitro*. The present study assessed the osteoblasts of the two

groups using an MTT assay, and the effect of *CCR6* deletion on osteoblast proliferation was observed. The results revealed that there was no significant difference in the proliferation activity between the two groups at the four time points (12, 24, 48 and 96 h). The results demonstrated that *CCR6* deletion did not affect the proliferation of osteoblasts. Therefore, it was concluded that *CCR6* deletion may directly affect the differentiation of osteoblasts but not proliferation.

Postmenopausal estrogen deficiency is associated with increased bone resorption and increased production of pro-inflammatory factors, such as RANKL. A mature osteoclast is a multinucleated giant cell, which is induced and differentiated by bone marrow hematopoietic stem cells stimulated by macrophage colony-stimulating factor and RANKL. RANKL serves an important role in osteoclast generation. OPG, also known as osteoclast inhibitory factor, is a growth factor receptor belonging to the tumor necrosis factor receptor family (39,40). RANKL expression in osteoblasts and BMSCs can promote the differentiation and activation of osteoclasts and inhibits the apoptosis of osteoclasts. In addition, osteoblasts and BMSCs secrete and express OPG, which competitively binds with RANKL, preventing binding between RANKL and RANK (41,42). The OPG/RANKL/RANK system is an important signaling pathway in osteoclast differentiation. Numerous hormones and immune factors affect bone metabolism *in vivo* by affecting the expression levels of OPG or RANKL (43,44). The present results demonstrated that *CCR6* deletion did not affect the transcription levels of OPG in osteoblasts in the simulated co-culture environment or in the common culture environment, but increased the transcription of *RANKL* in mice osteoblasts in the treated culture environment only. Therefore, it was speculated that the deletion of the *CCR6* could indirectly promote osteoclast formation by increasing the transcription levels of *RANKL*, while the bone mass and bone microarchitecture of *CCR6*^{-/-} and wild-type mice *in vivo* should be analyzed in further studies.

In conclusion, *CCR6* deletion weakened osteoblast activity and inhibited osteoblast mineralization *in vitro*, whereas it did not affect the proliferation of osteoblasts. This suggests that *CCR6* deletion may directly inhibit osteoblast differentiation by downregulating the expression levels of *Osterix*, a key transcription factor in osteoblast differentiation, and indirectly promote osteoclast production by increasing the transcription levels of *RANKL* in osteoblasts without affecting the transcription levels of *OPG*. However, the transcription levels of *Collagen-1* and *Runx2* were not significantly altered in *CCR6*^{-/-} osteoblasts. Therefore, it was speculated that *CCR6* deletion can alter the biological function of osteoblasts in osteogenesis in mice. The present study provides novel evidence to explain the mechanisms *via* which *CCR6* deletion regulates bone metabolism.

Funding: This work was supported by grants from the Science and technology innovation action plan of Shanghai Natural Science Foundation Project (No.20ZR1409100 to Ling Wang), the Shanghai Municipal Science and Technology Commission (No.18401902200 to Ling Wang), the Fund for Young Scientists of the Shanghai Municipal Health and Family Planning Commission (No. 20184Y0218 to Lisha Li), the National Natural Science Foundation of China (Nos. 31571196 and 30801502 to Ling Wang), the Shanghai Program for Support of Leading Disciplines-Integrated Chinese and Western Medicine (Nos. 20180101 and 20150407).

Conflict of Interest: The authors have no conflicts of interest to disclose.

References

- Drake MT, Clarke BL, Lewiecki EM. The pathophysiology and treatment of osteoporosis. *Clin Ther.* 2015; 37:1837-1850.
- Wu S, Zhao F, Zhao J, Li H, Chen J, Xia Y, Wang J, Zhao B, Zhao S, Li N. Dioscin improves postmenopausal osteoporosis through inducing bone formation and inhibiting apoptosis in ovariectomized rats. *Biosci Trends.* 2019; 13:394-401.
- Musumeci G, Loreto C, Clementi G, Fiore CE, Martinez G. An *in vivo* experimental study on osteopenia in diabetic rats. *Acta Histochem.* 2011; 113:619-625.
- Xu Y, Chu N, Qiu X, Gober HJ, Li D, Wang L. The interconnected role of chemokines and estrogen in bone metabolism. *Biosci Trends.* 2017; 10:433-444.
- Jia M, Dahlman-Wright K, Gustafsson JA. Estrogen receptor alpha and beta in health and disease. *Best Pract Res Clin Endocrinol Metab.* 2015; 29:557-568.
- Faienza MF, Ventura A, Marzano F, Cavallo L. Postmenopausal osteoporosis: the role of immune system cells. *Clin Dev Immunol.* 2013; 2013:575936.
- Charatcharoenwitthaya N, Khosla S, Atkinson EJ, McCready LK, Riggs BL. Effect of blockade of TNF-alpha and interleukin-1 action on bone resorption in early postmenopausal women. *J Bone Miner Res.* 2007; 22:724-729.
- Castrogiovanni P, Trovato FM, Szychlinska MA, Nsir H, Imbesi R, Musumeci G. The importance of physical activity in osteoporosis. From the molecular pathways to the clinical evidence. *Histol Histopathol.* 2016; 31:1183-1194.
- Andersson A, Stubelius A, Karlsson MN, Engdahl C, Erlandsson M, Grahne L, Lagerquist MK, Islander U. Estrogen Regulates T Helper 17 Phenotype and Localization in Experimental Autoimmune Arthritis. *Arthritis Res Ther.* 2015; 17:32.
- Yan M, Su J, Li Y. Rheumatoid arthritis-associated bone erosions: evolving insights and promising therapeutic strategies. *Biosci Trends.* 2020; 14:342-348.
- Yu Q, Lou XM, He Y. Preferential recruitment of Th17 cells to cervical cancer *via* CCR6-CCL20 pathway. *PLoS One.* 2015; 10:e0120855.
- Lee AYS, Korner H. The CCR6-CCL20 Axis in Humoral Immunity and T-B Cell Immunobiology. *Immunobiology.* 2019; 224:449-454.
- Singh TP, Zhang HH, Borek I, Wolf P, Hedrick MN, Singh SP, Kelsall BL, Clausen BE, Farber JM. Monocyte-derived inflammatory Langerhans cells and dermal dendritic cells mediate psoriasis-like inflammation. *Nat Commun.* 2016; 7:13581.
- Paulissen SM, van Hamburg JP, Dankers W, Lubberts E. The role and modulation of CCR6⁺ Th17 cell populations in rheumatoid arthritis. *Cytokine.* 2015; 74:43-53.
- Chen X, Wang Z, Duan N, Zhu G, Schwarz EM, Xie C. Osteoblast-osteoclast interactions. *Connect Tissue Res.* 2018; 59:99-107.
- Brylka LJ, Schinke T. Chemokines in physiological and pathological bone remodeling. *Front Immunol.* 2019; 10:2182.
- Tsao YT, Huang YJ, Wu HH, Liu YA, Liu YS, Lee OK. Osteocalcin mediates biomineralization during osteogenic maturation in human mesenchymal stromal cells. *Int J Mol Sci.* 2017; 18:159.
- Doucet M, Jayaraman S, Swenson E, Tusing B, Weber KL, Kominsky SL. CCL20/CCR6 signaling regulates bone mass accrual in mice. *J Bone Miner Res.* 2016; 31:1381-1390.
- Zhang N, Zhang Y, Lin J, Qiu X, Chen L, Pan X, Lu Y, Zhang J, Wang Y, Li D, Wang L. Low-density lipoprotein receptor deficiency impaired mice osteoblastogenesis *in vitro*. *Biosci Trends.* 2018; 11:658-666.
- Kim Y, Sato K, Asagiri M, Morita I, Soma K, Takayanagi H. Contribution of nuclear factor of activated T cells c1 to the transcriptional control of immunoreceptor osteoclast-associated receptor but not triggering receptor expressed by myeloid cells-2 during osteoclastogenesis. *J Biol Chem.* 2005; 280:32905-32913.
- Pathak JL, Bakker AD, Verschueren P, Lems WF, Luyten FP, Klein-Nulend J, Bravenboer N. CXCL8 and CCL20 enhance osteoclastogenesis *via* modulation of cytokine production by human primary osteoblasts. *Plos One.* 2015; 10:e0131041.
- Lisignoli G, Manferdini C, Codeluppi K, Piacentini A, Grassi F, Cattini L, Filardo G, Facchini A. CCL20/CCR6 chemokine/receptor expression in bone tissue from osteoarthritis and rheumatoid arthritis patients: different response of osteoblasts in the two groups. *J Cell Physiol.* 2009; 221:154-160.
- Watts NB, investigators G. Insights from the Global Longitudinal Study of Osteoporosis in Women (GLOW). *Nat Rev Endocrinol.* 2014; 10:412-422.
- Corina M, Vulpoi C, Branisteanu D. Relationship between bone mineral density, weight, and estrogen levels in pre and postmenopausal women. *Rev Med Chir Soc Med Nat Iasi.* 2012; 116:946-950.
- Twine NA, Chen L, Pang CN, Wilkins MR, Kassem M. Identification of differentiation-stage specific markers that define the *ex vivo* osteoblastic phenotype. *Bone.* 2014; 67:23-32.
- Sahin IO, Gokmenoglu C, Kara C. Effect of concentrated growth factor on osteoblast cell response. *J Stomatol Oral Maxillofac Surg.* 2018; 119:477481.
- Zeng R, Ma Y, Qiao X, Zhang J, Luo Y, Li S, Liu L, Xu L. The effect of His-tag and point mutation on the activity of irisin on MC3T3-E1 cells. *Biosci Trends.* 2018; 12:580-586.
- Costa DG, Ferraz EP, Abuna RPF, de Oliveira PT, Morra M, Beloti MM, Rosa AL. The effect of collagen coating on titanium with nanotopography on *in vitro* osteogenesis.

- J Biomed Mater Res A. 2017; 105:2783-2788.
29. Xiao W, Wang Y, Pacios S, Li S, Graves DT. Cellular and molecular aspects of bone remodeling. *Front Oral Biol.* 2016; 18:9-16.
 30. Wischmann J, Lenze F, Thiel A, Bookbinder S, Querido W, Schmidt O, Burgkart R, von Eisenhart-Rothe R, Richter GHS, Pleshko N, Mayer-Kuckuk P. Matrix mineralization controls gene expression in osteoblastic cells. *Exp Cell Res.* 2018; 372:25-34.
 31. Cai N, Li C, Wang F. Silencing of LncRNA-ANCR promotes the osteogenesis of osteoblast cells in postmenopausal osteoporosis *via* targeting EZH2 and RUNX2. *Yonsei Med J.* 2019; 60:751-759.
 32. Choi YH, Han Y, Jin SW, Lee GH, Kim GS, Lee DY, Chung YC, Lee KY, Jeong HG. Pseudoshikonin I enhances osteoblast differentiation by stimulating Runx2 and Osterix. *J Cell Biochem.* 2018; 119:748-757.
 33. Perez-Campo FM, Santurtun A, Garcia-Ibarbia C, Pascual MA, Valero C, Garces C, Sanudo C, Zarrabeitia MT, Riancho JA. Osterix and Runx2 are transcriptional regulators of sclerostin in human bone. *Calcif Tissue Int.* 2016; 99:302-309.
 34. Lee YJ, Park SY, Lee SJ, Boo YC, Choi JY, Kim JE. Ucmr, a direct transcriptional target of Runx2 and Osterix, promotes osteoblast differentiation and nodule formation. *Osteoarthritis Cartilage.* 2015; 23:1421-1431.
 35. Komori T. Runx2, an inducer of osteoblast and chondrocyte differentiation. *Histochem Cell Biol.* 2018; 149:313-323.
 36. Bruderer M, Richards RG, Alini M, Stoddart MJ. Role and regulation of Runx2 in osteogenesis. *Eur Cell Mater.* 2014; 28:269-286.
 37. Bonyadi Rad E, Musumeci G, Pichler K, Heidary M, Szychlinska MA, Castrogiovanni P, Marth E, Bohm C, Srinivasiah S, Kronke G, Weinberg A, Schafer U. Runx2 mediated induction of novel targets ST2 and Runx3 leads to cooperative regulation of hypertrophic differentiation in ATDC5 chondrocytes. *Sci Rep.* 2017; 7:17947.
 38. Qiao X, Nie Y, Ma Y, Chen Y, Cheng R, Yin W, Hu Y, Xu WM, Xu L. Irisin promotes osteoblast proliferation and differentiation *via* activating the MAP kinase signaling pathways. *Sci Rep.* 2016; 6:18732.
 39. Park K, Ju WC, Yeo JH, Kim JY, Seo HS, Uchida Y, Cho Y. Increased OPG/RANKL ratio in the conditioned medium of soybean-treated osteoblasts suppresses RANKL-induced osteoclast differentiation. *Int J Mol Med.* 2014; 33:178-184.
 40. Martin TJ, Sims NA. RANKL/OPG; Critical role in bone physiology. *Rev Endocr Metab Disord.* 2015; 16:131-139.
 41. Fan F, Shi P, Liu M, Chen H, Tu M, Lu W, Du M. Lactoferrin preserves bone homeostasis by regulating the RANKL/RANK/OPG pathway of osteoimmunology. *Food Funct.* 2018; 9:2653-2660.
 42. Varley I, Hughes DC, Greeves JP, Fraser WD, Sale C. SNPs in the vicinity of P2X7R, RANK/RANKL/OPG and Wnt signalling pathways and their association with bone phenotypes in academy footballers. *Bone.* 2018; 108:179-185.
 43. Weitzmann MN. Bone and the immune system. *Toxicol Pathol.* 2017; 45:911-924.
 44. Pichler K, Loreto C, Leonardi R, Reuber T, Weinberg AM, Musumeci G. RANKL is downregulated in bone cells by physical activity (treadmill and vibration stimulation training) in rat with glucocorticoid-induced osteoporosis. *Histol Histopathol.* 2013; 28:1185-1196.
- Received May 24, 2021; Revised June 23, 2021; Accepted July 3, 2021.
- [§]These authors contributed equally to this work.
- *Address correspondence to:
Ling Wang, Obstetrics and Gynecology Hospital of Fudan University, 419 Fangxie Road, Shanghai 200011, China.
E-mail: Dr.wangling@fudan.edu.cn
- Released online in J-STAGE as advance publication July 11, 2021.