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

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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 (\textit{CCR6}\textsuperscript{-/-}) 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 \textit{CCR6}\textsuperscript{-/-} 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. \textit{CCR6} deletion inhibited \textit{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 (\textit{Runx2}) and \textit{Collagen-1}. The ratio of osteoprotegerin (\textit{OPG}) / receptor activator of nuclear factor \kappa-B ligand (\textit{RANKL}) mRNA level in osteoblasts was decreased by \textit{CCR6} deficiency in the culture treated with 1,25(OH)\textsubscript{2}D\textsubscript{3}/PGE\textsubscript{2}, while there was no effect observed in the normal culture environment. The results provide novel insights, such as that \textit{CCR6} deletion suppresses osteoblast differentiation by downregulating the expression levels of the transcription factor \textit{Osterix}, and indirectly promotes osteoclast production by increasing transcription of \textit{RANKL}. This may be one of the mechanisms via which \textit{CCR6} deletion regulates bone metabolism.

Keywords

osteoporosis, C-C chemokine receptor type 6, osteoprotegerin, receptor activator of nuclear factor \kappa-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-\alpha, 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

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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 chemokines, 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 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 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 × 106 cells were seeded onto 10% FBS (Gibco) and phenol red-free α-MEM supplemented with 10 U/mL penicillin and 10 μg/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...

Table 1. Summary of oligonucleotide primers for CCR6+ mice genotyping

| Oligonucleotide | Sequence’ (5’-3’) |
|----------------|-----------------|
| Common forward primer | AAAACCCCATGGTGCTGCTGATGAG |
| Wild-type reverse primer | CCTAGAAGAGGTGGAACACTTCAC |
| Mutant reverse primer | GGTTGGATAGATAAAATGCCTGCTT |

The oligonucleotide sequences for genotyping were obtained from the Jackson Laboratory website: https://www.jax.org/search?q=+005793
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.). Gene expression was normalized to the level of housekeeping gene GAPDH and analyzed using the standard 2^ΔΔCT 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

| Gene          | Forward Primer       | Reverse Primer       |
|---------------|----------------------|----------------------|
| Osterix       | GCTCGTAGATTTCTATCCTCCTCACAGA | GACGGCTAGATGGAAACAC |
| Collagen-1    | TGACTGGAAGACGGAGGATA | GCCGAGTGGAAAGGGACAC |
| Runx2         | GACGGTCCACTTCCTGTG | GCCGAGTGGAAAGGGACAC |
| OPG           | CTTGCCTCCGTGACACTCTAT | CGCCTCTCCACACTCAC |
| RANKL         | CAAGATGGCTCTTTACACTTC | TTAGCTGCTGGTTTTAAGAC |
| GAPDH         | GGTGCTCCCTGCGACTTCA | GGTGCTCCAGGGTTTCTTA |

Runx2: runt-related transcription factor 2; OPG: osteoprotegerin; RANKL: nuclear factor-κ B ligand.
β-glycerol phosphate and 100 nM dexamethasone (differentiation medium). Each well contained 100 μL medium, and six replicate wells were analyzed for each group. After 12, 24, 48 and 96 h, 10 μL 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 μL 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 μL/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 ± 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 difference.
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 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)\(_2\)D\(_3\)/PGE\(_2\)

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)\(_2\)D\(_3\)/PGE\(_2\) to common culture medium, which was used to simulate the coculture 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...
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 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 ± SEM and representative of at least three experiments.

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 deposition, 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 deposition 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.
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