**Interleukin-20 differentially regulates bone mesenchymal stem cell activities in RANKL-induced osteoclastogenesis through the OPG/RANKL/RANK axis and the NF-κB, MAPK and AKT signalling pathways**

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**Abstract**

The immune and skeletal systems share common mechanisms, and the crosstalk between the two has been termed osteoimmunology. Osteoimmunology mainly focuses on diseases between the immune and bone systems including bone loss diseases, and imbalances in osteoimmune regulation affect skeletal homeostasis between osteoclasts and osteoblasts. The immune mediator interleukin-20 (IL-20), a member of the IL-10 family, enhances inflammation, chemotaxis and angiogenesis in diseases related to bone loss. However, it is unclear how IL-20 regulates the balance between osteoclastogenesis and osteoblastogenesis; therefore, we explored the mechanisms by which IL-20 affects bone mesenchymal stem cells (BMSCs) in osteoclastogenesis in primary cells during differentiation, proliferation, apoptosis and signalling. We initially found that IL-20 differentially regulated preosteoclast proliferation and apoptosis; BMSC-conditioned medium (CM) significantly enhanced osteoclast formation and bone resorption, which was dose-dependently regulated by IL-20; IL-20 inhibited OPG expression and promoted M-CSF, RANKL and RANKL/OPG expression; and IL-20 differentially regulated the expression of osteoclast-specific gene and transcription factors through the OPG/RANKL/RANK axis and the NF-κB, MAPK and AKT pathways. Therefore, IL-20 differentially regulates BMSCs in osteoclastogenesis and exerts its function by activating the OPG/RANKL/RANK axis and the NF-κB, MAPK and AKT pathways, which make targeting IL-20 a promising direction for targeted regulation in diseases related to bone loss.

**1 | INTRODUCTION**

Osteoimmunology is an evolving and emerging concept in bone-immune crosstalk in health and disease. The functions and interactions of the ‘osteoimmune system’ are performed by both bone cells and immune cells in the bone marrow; both cell types grow and develop in the same micromilieu and share many cytokines and signalling pathways.¹-³

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However, imbalanced osteoimmunological regulation is not the only factor affecting immune responses in skeletal homeostasis between osteoclasts and osteoblasts, but molecules secreted from osteoclasts, osteoblasts and osteocytes differentially regulate the immune system, leading to diseases that affect bone loss, such as rheumatoid arthritis, osteoporosis and periodontitis.4–6

In the bone marrow microenvironment, bone mesenchymal stem cells (BMSCs), as a cell type with multilineage potential, can differentiate into osteoblasts and secrete many osteoclast-associated cytokines, such as M-CSF, RANKL and OPG.7,8 It has gradually become clear that M-CSF is essential in the differentiation, survival and apoptosis of pre-osteoclasts and that RANKL promotes the formation of mature osteoclasts and bone resorption pits by binding RANK; this interaction is blocked by OPG.9 Moreover, the binding of RANKL-RANK contributes to the activation of osteoclastogenic downstream signalling pathways, including the NF-κB, MAPK and AKT pathways,10 all of which subsequently promote the expression of osteoclast-specific genes such as tartrate-resistant acid phosphatase (TRAP), cathepsin K (CK), MT1-MMP and MMP9.11

As an area of interest in osteoimmunology, the cross-talk among cytokines during osteoclast differentiation is an increasingly explored area. Previous research has shown that inflammatory cytokines, such as TNF, IL-1, IL-6, IL-10, IL-17, IL-18, IL-21, IL-22, IL-23, IL-27 and IL-33, are mainly secreted by macrophages and T cells to mediate osteoclastogenesis.1,12–17 Unfortunately, all of the above-mentioned factors act together with OPG/RANKL/RANK axis and are incapable of completely curing bone loss diseases as targets in osteoclast, and most of them are regulated by the pleiotropic proinflammatory factor IL-20, a member of the IL-10 cytokine family. Remarkably, IL-20 is mainly secreted by activated macrophages and acts by binding to one of its heterodimeric receptors, IL-20RA/IL-20RB or IL-22RA1/IL-20RB and consequently enhancing inflammation, chemotaxis and angiogenesis in rheumatoid arthritis, osteoporosis, cancer-induced osteolysis and bone fracture.18 In addition, treatment with an anti-IL-20 monoclonal antibody completely blocks osteoclastogenesis and promotes osteoblastogenesis in several bone-related diseases, such as osteoporosis and bone fracture,19,20 which suggests that IL-20 might be a vital upstream mediator of the interaction between osteoblasts and osteoclasts.

However, it is unclear how IL-20 regulates the balance between osteoclastogenesis and osteoblastogenesis in diseases that can cause bone loss. Therefore, we explored the roles of IL-20 in the involvement of BMSCs during osteoclastogenesis with regard to differentiation, survival, apoptosis and signalling and found that targeting IL-20 is a promising approach for the directed regulation of bone production and loss.

2 MATERIALS AND METHODS

2.1 Cytokines, reagents and antibodies

Recombinant rat M-CSF and RANKL were purchased from PeproTech and R&D Systems, respectively. Recombinant rat IL-20 was purchased from Sino Biological Inc. Dulbecco’s modified Eagle’s medium (DMEM; high glucose), foetal bovine serum (FBS), penicillin and streptomycin were purchased from Invitrogen. Red blood cell (RBC) lysis buffer was purchased from CWBIO. Primary antibodies against NF-κB, phospho-NF-κB, p38, phospho-p38, ERK, phospho-ERK, JNK, phospho-JNK, AKT and phospho-AKT were obtained from Cell Signaling Technology Inc. Primary antibodies against RANKL and OPG were obtained from Abcam.

2.2 Animals and animal ethics

We purchased 4-week-old Sprague Dawley rats (n = 120) from the Animal Experimental Center of Sun Yat-sen University. According to the Institutional Animal Care and Use Committee (IACUC) of Sun Yat-sen University, we fed, anesthetized and killed the rats. The experiments were in accordance with the Animal Ethical and Welfare Committee of Sun Yat-sen University (SYSU-IACUC-2018-000099, Guangzhou, China).

2.3 Assessment of the involvement of BMSCs in osteoclastogenesis

Primary rat bone marrow-derived macrophages (BMMs) and BMSCs were harvested from 4-week-old Sprague Dawley rats. Briefly, RBCs were removed with RBC lysis buffer, and the bone marrow cells were cultured overnight in a cell culture flask at 37°C in a humidified 5% CO2 atmosphere. The BMMs obtained from the non-adherent bone marrow cells were plated in 24-well plates (4 × 106 cells/well) in osteoclast medium supplemented with M-CSF (20 ng/mL) for 2 days, which induced the cells to become preosteoclasts. To induce osteoclastogenesis, the osteoclast medium was replaced every 2 days with medium containing M-CSF (30 ng/mL) and RANKL (30 ng/mL). To explore the involvement of BMSCs in osteoclastogenesis, BMSCs were recovered from adherent cells at passages 2–4. Then, the BMSCs were incubated with various concentrations of IL-20 for 3 days, and the CM from BMSCs in the above groups was added to osteoclast medium containing M-CSF (30 ng/mL) and RANKL (30 ng/mL) at a ratio of 1:1.
2.4 | Cell viability assay

Cell viability and proliferation were assessed by using a Cell Counting Kit-8 assay (CCK-8; Dojindo). Briefly, pre-osteoclasts were cultured in 96-well plates with complete medium supplemented with varying concentrations of IL-20 (0 ng/mL–200 ng/mL). After 1, 3, 5 or 7 days, the absorbance at 450 nm in each well was determined with a microplate reader (Tecan SUNRISE microplate reader, Tecan, Switzerland).

2.5 | Flow cytometry evaluation of cell apoptosis

To assess apoptosis, we performed flow cytometry analysis with an Annexin V-APC/7-AAD apoptosis kit (MultiSciences Biotech). Briefly, we stained cells with APC-labelled Annexin V and 7-AAD to distinguish 4 cell populations according to the manufacturer's instructions: early apoptotic cells (APC+/7-AAD-), late apoptotic cells (APC+/7-AAD+), viable cells (APC-/7-AAD-) and secondary necrotic cells (APC-/7-AAD+). Subsequently, we immediately analysed the cells after staining with a CytoFLEX flow cytometer (Beckman).

2.6 | TRAP staining and a bone resorption pit assay

For in vitro TRAP staining, mature osteoclasts were fixed in 4% paraformaldehyde and stained with an Acid Phosphatase Leukocyte (TRAP) Kit (Sigma) according to the manufacturer's protocol. For a bone resorption pit assay, the bone resorption activity of mature osteoclasts was observed with a 24-well osteo assay surface multiple-well plate (Corning) coated with a thin inorganic 3-dimensional crystalline material. We counted the numbers of TRAP + multinucleated cells with at least 3 nuclei and bone resorption pits with an inverted fluorescence microscope (Zeiss).

2.7 | qRT-PCR analysis

Total mRNA was harvested from cells with a RNA-Quick Purification Kit (ES Science) and reverse transcribed into cDNA with PrimeScriptTM RT Master Mix (Perfect Real Time) (Takara). We purchased commercially synthesized primers from Takara, which are listed in Table 1. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed with SYBR® Premix Ex Taq™ (Tli RNaseH Plus) (Takara) using a MicroAmp Optical 96-Well or 394-Well Reaction Plate with Barcodes (Thermo Fisher Scientific) on a QuantStudio 5 or 7 Flex Real-Time PCR System (Applied Biosystems™). The relative RNA expression of target genes was normalized to that of GAPDH with the 2-ΔΔCT method.

2.8 | Immunochemistry

BMSCs were seeded on sterile glass coverslips in six-well plates and incubated with primary antibodies and secondary antibodies. The cells were subsequently visualized with an immunoreaction by using the chromogenic agent DAB. Finally, we captured images under an inverted fluorescence microscope (Zeiss).

2.9 | Western blotting analysis

After cells were treated as indicated, samples were on ice, washed and lysed in radioimmunoprecipitation assay buffer (Sigma). The cell lysates were collected, and the obtained supernatants were used for further experiments. After the concentration of total protein was measured and the samples were denatured, 50 µg of protein was loaded in each lane, separated using SDS-PAGE through an 8% gel and then transferred to an Immobilon®-P Transfer Membrane (Millipore). Subsequently, the membranes were blocked and incubated with primary antibodies and secondary antibodies. Immunoreactivity was visualized with the ImmobilonTM Western Chemiluminescent HRP substrate (Millipore).

2.10 | Statistical analysis

All results are expressed as the means ± standard deviations, and the values were obtained from at least three experiments. Statistical differences were evaluated with GraphPad Prism 7.04 software using Student's t test or one-way ANOVA with Tukey's post hoc analysis. Values of P < .05 were considered statistically significant.

3 | RESULTS

3.1 | IL-20 differentially regulated preosteoclast proliferation and apoptosis

To determine the effect of IL-20 on preosteoclast proliferation, we treated M-CSF-induced preosteoclasts with IL-20 and detected cell viability representing cell proliferation ratio by the CCK-8 assay at 1, 3, 5 and 7 days after initial
# Table 1

| Gene | Forward primer sequence (5'-3') | Reverse primer sequence (5'-3') |
|------|---------------------------------|-------------------------------|
| IL-20 | ACTGCAAACCTACAGGCGATACAA | AGAACCTCACTAGATGGCAGGAGA |
| IL-20RA | GGGTCTACGGAGTCCAAGTCA | AGCCTATAGCAGGGGAGTCTCAA |
| IL-20RB | AGCATTGATGGTAAACGCC | GAAAACAGAGACACACAGCCCTCC |
| IL-22RA | CCTACACGTGGCAGAGTGAAGA | AGAACCTCACTAGATGGCAGGAGA |
| M-CSF | TAGAAAGATCTTCAAGCTGG | CTTTGAAGATGGCAGGAGTCTCAA |
| RANKL | CTCATGAGGAAATCAAC | TTTCACTAGATGGCAGGAGTCTCAA |
| OPG | GACCAAGATGAATGCGGAGG | CGGTGTTCACAGAGGACAGG |
| Cathepsin K | CGGCTATAGCAGGGGAGTCTCAA | TTTGCCGTTGAGTGCAGCATA |
| TRAP | TGGCAATGCTCGGCAAC | AGCATCAGGAGTCCAGCATA |
| MT1-MMP | GAGAATCCAGGTGGGATAGCAC | TTTCTGGGTATCAGAGGACAGG |
| MMP9 | CATCAGGCTGGCCTAGATCA | GAGGGCTGTGGCAGGAGTCTCAA |
| NFATc1 | CAGTCTCACAGCGGGTCTACTA | TCAGCCTAGCCTAGCAGCATA |
| c-Fos | CGTCTTCTTGCCTACCATCAC | TTGCCTAGCCTAGCAGCATA |
| RANK | CAGGACAGGCGTCTGCAA | TGACTAGCCTAGCAGCATA |
| GAPDH | GGCACAGTCAAGGCTGAGAATG | ATGGTGAGGACACGCAGTA |

**Figure 1** IL-20 differentially regulated preosteoclast viability and apoptosis. Cell viability and apoptosis were detected in M-CSF-derived preosteoclasts by the CCK8 and flow cytometry assays, respectively. (A) Cell viability analysis of IL-20-treated M-CSF-induced preosteoclasts on days 1, 3, 5, and 7. Bars represent mean ± SEM from six independent experiments (n = 12). (B-G) Flow cytometry evaluation of cell apoptosis. After 1 day of IL-20 treatment, we examined viable cell percentages (B) or apoptotic cell percentages (C) using an Annexin V-APC/7-AAD apoptosis kit. FMO controls that were used to separate negative gates from positive gates included unstained preosteoclasts (D) and preosteoclasts which were stained with Annexin V-APC (E) or 7-AAD (F). (G) Preosteoclasts treated with IL-20 were stained with Annexin V-APC/7-AAD. Bars represent mean ± SEM from three independent experiments (n = 18). *P < .05 vs the 0 ng/mL IL-20 group, †P < .05 vs. the 200 ng/mL IL-20 group; ns, not significant.
FIGURE 2 IL-20 in BMSC CM differentially regulated osteoclast formation and bone resorption at different concentrations. (A-C) M-CSF-induced preosteoclasts were cultured in CM from BMSCs in the presence or absence of 30 ng/mL RANKL or different concentrations of IL-20. The control group represented M-CSF-induced preosteoclasts was cultured in 0 ng/mL IL-20 CM. (A) TRAP staining was performed to identify TRAP-positive osteoclasts. (B, C) The number and size of TRAP-positive osteoclasts with at least 3 nuclei were examined and measured. (D-H) M-CSF-induced preosteoclasts were cultured in CM from BMSCs treated with different concentrations of IL-20 in the presence of 30 ng/mL RANKL. (D) We performed TRAP staining to distinguish TRAP-positive osteoclasts. (E, F) The number and size of TRAP-positive osteoclasts were examined. The control group comprised BMMs induced with 30 ng/mL M-CSF and 30 ng/mL RANKL. (G) A bone resorption pit assay was performed to detect osteoclast function. (H) The area of bone resorption pits was quantified. Bars represent mean ± SEM from six independent experiments (n = 18). *P < .05 vs. the control group.
treatment. Compared with control group (0 ng/mL IL-20), 2 ng/mL IL-20 significantly promoted preosteoclast proliferation all the time, while 200 ng/mL IL-20 gradually inhibited preosteoclast proliferation over time (Figure 1A). To determine whether IL-20 regulated preosteoclast apoptosis, we further analysed apoptosis ratio in preosteoclasts treated with varying concentrations of IL-20 using an Annexin V-APC/7-AAD kit for flow cytometry. Compared with control group (0 ng/mL IL-20), treatment with gradient concentration of IL-20 slightly increased the proportion of viable cells and decreased the apoptotic cell population to varying degrees (Figure 1B-F). And it was worth noting that 2 ng/mL IL-20 notably decreased the apoptotic cell population compared to other groups. Based on the above results, it is reasonable to conclude that IL-20 had an effect on preosteoclast proliferation and apoptosis.

3.2 IL-20 dose-dependently regulated the roles of BMSCs in RANKL-mediated osteoclast formation and function

To explore whether RANKL or IL-20 was essential for BMSC CM-induced osteoclastogenesis, we treated M-CSF-induced preosteoclasts with or without RANKL and IL-20 in BMSC CM. After 3 days, we found BMSC CM significantly increased the number and size of TRAP-positive osteoclasts in combination with RANKL compared to control group.

FIGURE 3 IL-20 and its receptors were expressed in BMSCs, and IL-20 increased the expression of RANKL/OPG in BMSCs. The control group comprised BMSCs treated with 0 ng/mL IL-20. (A-D) The mRNA expression levels of IL-20 and its receptors in BMSCs were evaluated by qRT-PCR after 3 days of treatment with IL-20. *P < .05 vs. the 0 ng/mL IL-20 group. (E) The expression of IL-20 and its receptors in BMSCs was determined by immunohistochemical staining. (F-M) The mRNA and protein expression levels of M-CSF, RANKL and OPG were examined in BMSCs using qRT-PCR and Western blotting, respectively. Bars represent mean ± SEM from three independent experiments (n = 12). *P < .05 vs. the control group; ns, not significant.
Further analysis found that 2 ng/mL IL-20 enhanced this effect, whereas 200 ng/mL IL-20 completely inhibited the formation of TRAP-positive osteoclasts (Figure 2A-C). To further examine the roles of IL-20 in the involvement of BMSCs in osteoclast formation, we isolated BMMs and BMSCs from rat bone marrow. The BMSCs were cultured with varying concentrations of IL-20 for 3 days, and CM containing IL-20 was added to preosteoclast medium supplemented with M-CSF and RANKL. After 4 days, we observed that the number and size of TRAP-positive osteoclasts were dramatically increased after treatment with 0 ng/mL CM and 2 ng/mL IL-20 CM, whereas treatment with CM containing 200 ng/mL IL-20 distinctly reduced these parameters (Figure 2D-F). The trend in increased osteoclasts in CM exhibited the following trend: 2 ng/mL IL-20 CM > 0 ng/mL IL-20 CM > control > 200 ng/mL IL-20 CM. To further investigate whether IL-20 contributes to the osteoclast function of bone resorption, preosteoclasts were seeded in osteo assay surface plates with CM to conduct the bone resorption pit assay. A similar trend was observed: the area of the bone resorption pits was significantly enhanced in the groups treated with 0 ng/mL IL-20 CM and 2 ng/mL IL-20 CM compared with the control group, whereas no obvious pits were observed in the 200 ng/mL IL-20 CM group (Figure 2G,H). Therefore, the results of the TRAP staining and pit-formation assays suggested that IL-20 upregulated or downregulated the involvement of BMSCs in RANKL-mediated osteoclastogenesis and bone resorption in a dose-dependent manner.

3.3 IL-20 promoted feedback regulation of BMSC involvement in osteoclastogenesis through the OPG/RANKL/RANK axis

BMSCs are multipotent stromal cells that can produce many molecules with immunomodulatory effects and differentiate into osteoblasts that can secrete M-CSF, RANKL and OPG to regulate osteoclast differentiation. The qRT-PCR results revealed that stimulation with different concentrations of IL-20 downregulated IL-20 expression and upregulated IL-20RA/IL-20RB/IL-22RA1 expression in BMSCs (Figure 3A-D). In addition, we found by immunochemistry that IL-20 and its receptors were highly expressed in BMSCs (Figure 3E). To investigate whether BMSC expression of M-CSF, RANKL and OPG was modulated by IL-20, we cultured BMSCs with different concentrations of IL-20 for 3 days and then cultured preosteoclasts in the resulting CM. mRNA expression analysis showed that M-CSF and RANKL expression in BMSCs treated with IL-20 was remarkably enhanced (Figure 3F,G). By contrast, IL-20 slightly inhibited the OPG expression in BMSCs (Figure 3H). Further analysis showed that the RANKL/OPG ratio changed substantially based on the concentration of IL-20, and the trend was as follows: 2 ng/mL IL-20 > 200 ng/mL IL-20 > 0 ng/mL IL-20 (Figure 3I). In addition, similar trends in protein expression were observed for RANKL and OPG in the immunoblotting results (Figure 3J-L). The RANKL/OPG protein expression ratio was obviously higher in the 2 ng/mL IL-20 and 200 ng/mL IL-20 groups than in the 0 ng/mL IL-20 group (Figure 3M). Taken together, the data clearly indicated that...
M-CSF, RANKL and OPG expression in BMSCs was endogenously induced by IL-20 and its receptors, which supported the notion of regulatory feedback on osteoclast differentiation through the OPG/RANKL/RANK axis.

3.4 | IL-20 differentially regulated the expression of osteoclast-specific genes and transcription factors in a dose-dependent manner

CM from BMSCs was shown to contain numerous cytokines, such as M-CSF, RANKL and OPG, and these cytokines are theoretically capable of activating osteoclastogenic signalling pathways to regulate the transcriptional activation of osteoclast-specific marker genes. The qRT-PCR results showed that the mRNA expression of marker genes, including cathepsin K, TRAP, MT1-MMP, MMP-9, NFATc1, c-Fos and RANK, was remarkably increased in the 0 ng/mL IL-20 CM and 2 ng/mL IL-20 CM groups compared with the control groups, with the 2 ng/mL IL-20 CM group exhibiting higher levels than the 0 ng/mL IL-20 CM group. In contrast, 200 ng/mL IL-20 CM slightly inhibited the expression levels of these genes (Figure 4A-D). Further analysis revealed a similar trend in the mRNA expression of RANKL-specific membrane receptors and transcription factors, such as RANK, c-Fos and NFATc1 (Figure 4E-G). In conclusion, these findings further confirmed that IL-20 may act together with M-CSF/RANKL/OPG in upstream regulation of osteoclastogenesis and differentially regulate the expression of osteoclast-specific genes and transcription factors.

3.5 | IL-20 differentially regulated BMSC activities in RANKL-mediated osteoclastogenic downstream signal transduction

To explore a possible mechanism that IL-20 regulated bone mesenchymal stem cell activities in RANKL-induced osteoclastogenesis, we added CM from BMSCs treated with IL-20 to preosteoclasts and detected whether the signalling pathways for osteoclast differentiation were activated at the indicated times (0, 10, 20, 40 or 60 minutes). Western blotting confirmed that 2 ng/mL IL-20 CM activated RANKL-RANK signalling-mediated downstream signalling pathways in preosteoclasts, such as the NF-κB, p38, ERK, JNK and AKT pathways, while 0 ng/mL IL-20 CM inhibited the activation of NF-κB pathways and activated other pathways (Figure 5A). Moreover, 2 ng/mL IL-20 CM significantly enhanced the phosphorylation rates of NF-κB, p38 and JNK, while 0 ng/mL IL-20 CM notably enhanced the phosphorylation of ERK and AKT, which negatively regulated osteoclastogenesis (Figure 5B-F). In conclusion, these results indicated that IL-20 regulated the involvement of BMSCs in osteoclastogenesis by activating RANKL-mediated osteoclastogenic signalling pathways, including the NF-κB, MAPK and AKT signalling pathways.

4 | DISCUSSION

As a powerful proinflammatory, chemotactic and angiogenic cytokine of the IL-10 family, IL-20 has been found and widely studied in chronic inflammatory diseases, such as rheumatoid arthritis, psoriasis and atherosclerosis. Nowadays, researchers have reported that IL-20 was related to several bone-related diseases and may play a crucial part in regulating formation of osteoclast and osteoblast, such as osteoporosis, bone fracture, rheumatoid arthritis and cancer-induced osteolysis. Besides, recent studies have shown that the anti-IL-20 monoclonal antibody 7E prevents bone resorption in ovariectomized mice by completely blocking osteoclastogenesis and inducing osteoblastogenesis in osteoporosis and bone fracture. Researchers have made an initial breakthrough in the molecular mechanism of osteoclastogenesis with coculture systems comprising BMMs and BMSCs or T cells. More and more inflammatory and chemotactic mediators have been confirmed to launch the activation of BMSCs leading to bone remodelling, such as TNF-α, IL-1, IL-6, IL-17 and IL-22. In our study, we found that BMSC CM notably increased RANKL-induced osteoclastogenesis and bone resorption, which was upregulated or downregulated by IL-20 in a dose-dependent manner. Moreover, IL-20 may act on BMSC by binding to its receptor complexes and lead to expression changes of some osteoclast-associated molecules, such as M-CSF, RANKL and OPG. Further studies found that IL-20 may act together with RANKL/OPG in upstream regulation of osteoclastogenic downstream signalling pathways, which leads to gene expression of osteoclast-specific genes and transcriptional activators. These findings validated the possible regulatory mechanism of IL-20 regulating the involvement of BMSC activities in RANKL-induced osteoclastogenesis.

Recent evidence in vitro and in vivo has confirmed that some cytokines and hormones can act on osteoclast survival, osteoclastogenic signalling pathways and bone resorption, such as IL-1 and IL-10. In the past, it was commonly recognized that the proliferation and apoptosis of preosteoclasts were primarily regulated by osteoblast-secreted cytokines such as M-CSF, which is crucial for differentiation into mature osteoclasts. Moreover, recent studies have indicated that the new key molecule IL-20 is able to regulate the proliferation and apoptosis of epithelial cells and endothelial cells by activating related signalling pathways and proteins, including caspase 9, FGF2, VGEF, JAK/STAT3 and MAPK. These findings suggest that IL-20 may play
a significant role in osteoclast differentiation by regulating proliferation and apoptosis. In this study, we found that a low concentration of IL-20 promoted M-CSF-induced preosteoclast proliferation and inhibited apoptosis, whereas a high concentration of IL-20 inhibited preosteoclast proliferation over time which may be caused by cumulative cell cytotoxicity on preosteoclasts. Anyhow, we initially confirmed that IL-20 differentially regulated preosteoclast growth and apoptosis during osteoclastogenesis.

Regulation of osteoimmunology is vital for bone homeostasis, a process that depends on osteoblast-osteoclast communication, which is regulated by various molecules, cytokines and signalling pathways, such as the vital OPG/RANKL/RANK axis. Recent studies have shown that osteoimmunology mediators target IL-20 in MC3T3-E1 cells (mouse osteoblasts) and mature osteoclasts and that IL-20 acts as a vital regulator between osteoblastogenesis and osteoclastogenesis by activating the OPG/RANKL/RANK regulatory axis. In this study, we confirmed that IL-20 and its receptors were highly expressed in rat BMSCs and that IL-20 engages in auto-regulatory feedback. Subsequently, we found that IL-20 dose-dependently inhibited the expression of OPG and increased the expression of M-CSF, RANKL and RANKL/OPG, all of which are crucial for the activation of osteoclastogenic signalling pathways and the expression of osteoclast-specific genes and proteins. Overall, we concluded that IL-20 regulated the expression of M-CSF, RANKL and OPG by BMSCs to contribute to osteoclast differentiation, which was tested by evaluating gene and protein expression.

RANK-RANKL binding contributes to the activation of osteoclastogenic downstream signalling pathways and ultimately promotes the expression of osteoclast-specific genes. While exploring the molecular mechanism(s) underlying the differential regulation of osteoclastogenesis by IL-20, we found that IL-20 dose-dependently upregulated and downregulated the expression of osteoclast-specific genes and transcriptional activators to perform bone resorption function, such as RANK, CK, TRAP, MT1-MMP, MMP9, NFATc1 and c-Fos. In signal transduction, activation of the transcription factors NFATc1 and c-Fos is mainly regulated by several key signalling pathways including NF-κB, p38, ERK, JNK and AKT pathways, which are involved in
proliferation, apoptosis, differentiation and function in osteoclasts. In this case, NF-κB, p38 and JNK pathways act as positive regulation in osteoclastogenesis, whereas ERK and AKT pathways are involved in negative regulation. Remarkably, Western blot analysis showed that the NF-κB, p38, ERK, JNK and AKT pathways were activated to varying degrees within 60 min after 2 ng/mL IL-20 CM treatment. Besides, compared to 0 ng/mL IL-20 CM, 2 ng/mL IL-20 CM mainly activated three positive osteoclastogenic pathways including NF-κB, p38 and JNK, which was consistent with higher RANKL/OPG ratio in 2 ng/mL IL-20 CM. Therefore, we recognized that IL-20 may act together with RANKL/OPG in upstream regulation of osteoclastogenic downstream signalling pathways, including the NF-κB, MAPK (p38, ERK and JNK) and AKT pathways (Figure 6).

In summary, the most important finding in this study is that IL-20 may act together with M-CSF/RANKL/OPG of BMSC productions in upstream differential regulation of osteoclastogenesis in primary cells. In addition, we confirmed the possible molecular mechanism involving IL-20 together with BMSC productions in activating the OPG/RANKL/RANK axis and the NF-κB, MAPK and AKT pathways. Therefore, targeting IL-20 might be a promising approach for targeted regulation in diseases that present bone loss. And there are some deficiencies in our studies. Although we have conducted a preliminary experiment to confirm that IL-20 differentially regulated RANKL-induced osteoclastogenesis without BMSC (unpublished data), we could not rule out the possible effect of IL-20 left in CM on osteoclasts. However, it is still imperative that more researchers should be committed to deeper studies in order to clarify specific molecular mechanism involving IL-20 underpins osteoclastogenesis.

CONFLICT OF INTEREST
All authors declare that they have no conflicts of interest.
AUTHOR CONTRIBUTIONS
Y. Cao and C. Liu designed the experiments. B. Meng, D. Wu and Y. Cheng carried out the experiments. P. Huang, Y. Liu and L. Gan collected and analysed the data. B. Meng and D. Wu wrote and revised the manuscript.

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